Laboratory Guide for Identification of

PLANT PATHOGENIC BACTERIA

Third Edition

Edited by N. W. Schaad, j. B. **Jones, and W. Chun** For the Bacteriology Committee of The American Phytopathological Society

APSPRESS

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PREFACE **TO** THE **THIRD EDITION**

This laboratory guide is written to assist in the identification of plant pathogenic bacteria and not in their classification. Whereas characteristics useful for classification such as DNAIDNA homologies and sequencing of 165 rRNA can be quite difficult to determine, characteristics useful for identification at the genus and species level should be easily determined and few in number.

It has been over ten years since the second edition of the APS Laboratory Guide for Identification of Plant Pathogenic Bacteria was published. Nobel Laureate Kary Mullis had only recently (1984) discovered the polymerase chain reaction (PCR). Few plant pathologists knew what peR meant or realized that a new revolution in biological science was beginning. No other single technique has advanced disease diagnosis and pathogen identification so quickly. Because of this we have added a list of available PCR primers to most chapters and an appendix on Molecular Techniques.

Because of the newer taxonomy, many nomenclatural changes have been made in the third edition. Several species have been re-classified into new genera. Most notable changes have occurred to the pseudomonads. Members of *Pseudomonas* have been placed into four genera, *Pseudomonas, Acidovorax* (many of the non-fluorescent, oxidase positive ones), *Burkholderia,* and *Ralstonia* (non-fluorescent). Other new genera include *Pantoea, Rhizomonas, Xylophilus,* and *Liberobacter* (fastidious phloem-limited bacterium). These changes have made identification of most plant pathogenic bacteria with a few simple phenotypic tests much easier. Each chapter briefly describes the nomenclature and taxonomy being followed.

Like the second edition, the third edition is written to assist the researcher, student, and diagnostician in obtaining an accurate identification of the most common plant pathogenic bacteria. Most common organisms can be identified very quickly whereas the fastidious ones take considerably longer. Detailed methods are provided, including the source of all chemicals and reagents. Additional color photographs have been added to the third edition to assist in interpretations of results.

By following this manual, most every pathogen isolated from a diseased plant is easily identified to the species or pathovar level by a few simple phenotypic tests. However, if one has no pathology background or does not know the host of the unknown bacterium then a molecular, serological, or automated commercial technique can be helpful. We have added to most chapters a section on molecular, serological, and automated commercial techniques for rapid, presumptive identification. For additional details we have added an appendix on each of the above sections. Serological or peR-based techniques are especially useful for identification of fastidious organisms such as *Xyelta* or uncultivable organisms such as *Liberobacter.* Many new semiselective agar media have been developed since the last edition. These media are extremely helpful in presumptive identification because most are based on some metabolic uniqueness of the organism Whether working with molecular or phenotypic tests, known positive and negative control strains should always be included. Because cultures obtained from collections can be contaminated or incorrectly identified, one should always confirm the organism's purity, identity, and pathogenicity. Time must be taken to be absolutely sure of using a pure culture. This is especially important

when isolating directly from plants. Cultures should be cloned at least twice by streaking onto a nonselective medium in a manner to obtain well separated colonies and adequate time (4-5 days) provided for any nearby contaminating bacteria to grow. Since it is the identification of a pathogen that we are concerned with in this manual, confirming the pathogenicity of the known control and unknown target organism is of great importance.

I wish to express a special thanks to Gail Hoover our ARS Word Processing Specialist and friend for her energetic devotion and steadfastness in preparation of this manuscript and dealing with the many re-typing and electronic conversions and formatting she had to deal with. I want to thank Jeff Jones and Wes Chun for their helpful input and advice and support in co-editing this manual. I thank each of the authors for their suggestions in developing the contents for this edition and the timely preparation of their chapters. The excellent reviews provided by T. Burr, A Alvarez, and D. Cooksey are appreciated. A special thanks to my wife Yvette Berthier-Schaad for her suggestions and moral support and understanding during this endeavor.

Ft. Detrick, Maryland, April 2000 Norman Werth Schaad

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I. Initial Identification of Common Genera

N. W. Schaad

A. INTRODUCTION

Relatively few differential characters are required to identity the predominant genera of plant pathogenic bacteria. They can be conveniently divided into 1) those that are isolated easily on standard bacteriological media (Table 1) and those that are not (Table 2).

Table 1. Characters used to differentiate genera of plant pathogenic prokaryotes that grow on standard media.

Character														
Gram positive											+	+	+	+
Grows anaerobically	+	+										+	+	
Grows aerobically	+	+	+	+	+	+	+	+	+	+			+	+
Colonies yellow or orange on YDC, or NBY		+a					+b	+c			+<1			
Colonies mucoid on YDC at 30°C			+		+		+		+	+		ND	ND	
Fluorescent pigment on KB				+										
Diffusible non-fluorescent pigments on KB						+								
Urease	-e		+		+	V		+	ND			ND	ND	ND
Oxidase			+		+	+r			+				V	+
Grows at 40°C		V	+			~						+	+	
More than four peritrichous flagella	+	+										V	V	
Growth on DIM agar									+					
Spores formed												+	+	
Aerial mycelium														+

+,80% or more strains positive after five days; V, between 21-79% of strains positive; -,80% or more strains negative; ND, not determined.

• - Colonies of *Pan toea citrea* and some strains of *P. agglomerans* are generally white.

b - Colonies of *X campestris* pathovars *manihotis* and *mangiferaeindicae* are white.

c _ *Xylophilus* grows very slowly on these media, but somewhat better on Difco nutrient agar,

d - Colonies of *Clavibacter michiganensis* subsp. *sepedonicus* are generally white.

e - *Erwinia nigrifluens is positive.*

r" *Burkholderia andropogodis* is oxidase negative.

g- *Burkholdena andropogodis* and *B. glumae* pv. *agricola* are negative.

Genera within the first group are relatively easy to differentiate from one another on the basis of the characters presented in Table 1 and the simplified flow chart presented in Fig. 1 (see p.6). The Gram reaction (see 1, p. 7) is related to structural and chemical properties of the cell wall, and this characteristic serves as a rapid and very basic step in the initial identification of unlmown plant pathogenic bacteria. It is important when doing the Gram reaction to use rapidly growing, freshly prepared cells (see l a, p. 7). The anaerobic test is a key test for identification of the genera *Erwinia*, *Pantoea*, and *Clostridium.* Also, the presence of spores is a key test for differentiation of Gram-positive bacteria. Colony morphology and pigmentation, growth at 400C, and the oxidase reaction are key tests for differentiation of *Acidovorax, Burkholderia, Ralstonia,* and *Pseudomonas.* Always be sure to include known cultures as positive and negative controls for each of the tests. When identifying an unknown bacterium, it is absolutely essential that you use a pure culture of the organism being identified. It should be cloned at least twice by streaking onto a nonselective agar medium. Be sure that the selected colony is well separated and is allowed to grow for 4-5 days to be sure other colonies do not appear.

More rapid commercial automated techniques (see Appendix C) such as fatty acid analysis, Biolog, and ELISA kits are useful for presumptive identification of a large number of bacteria isolated from soil or plant leaves, however, they should not be relied upon for final identification. Details for molecular and serological-based techniques are presented in Appendix A and B, respectively.

Members of the second group (Table 2) are relatively new to plant pathologists and are initially identified on the basis of 1) growth on S-medium (see h, p, 5),2) growth on PW (see g, p. 5) and other complex media (see a), p. 204-205), 3) presence or absence of a cell wall (see p. 12, p. 13), and 4) helical morphology (see 1 p. 293). If no growth occurs on any of the three agar media, it is advisable to prepare samples for the electron microscope, and use serological (see p. 310) and/or PCR-based techniques (see p. 303).

Table 2. Characters used to differentiate fastidious and non-culturable plant pathogenic bacteria.

Character	*Rhizomonas*	*Xyella fastidiosa*	*Spiro-plasma*	*Phyto-plasma-like*	*Phloem-limited'*
Growth on S-medium	+				
Growth on PW agar		+			
Possess cell wall	+	+			+
Growth on serum agar			+		
Helical morphology			+		

[a] Includes proposed *Liberobacter* spp. and rickettsia-like (papaya bunchy top) bacterium (see p. 275).

B. ISOLATION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

1. Plant Material

When isolating an unknown plant pathogenic bacterium it is a good practice to use several different common agar media. Most *Erwinia, Xanthomonas, Acidovorax, Burkholdera, Ralstonia,* and *Agrobacterium* spp. grow well and produce characteristic colonies on nutrient broth-yeast extract agar (NBY) (15) or yeast extract-dextrosecalcium carbonate (YDC) (16) agar. Without experience it is advisable to streak cultures of known strains for comparing the colony characteristics. A few species or pathovars do not grow well on YDC. For example, *P. stewartii, X oryzae, X albilineans,* and *X fragariae* grow poorly on YDC but quite well on NBY. *Xylophilus ampelinus* grows poorly on YDC or NBY but much better on nutrient agar. Most pseudomonads and *Clavibacter* spp. grow well and are very characteristic on King et al's medium B (KB) (6) and NBY agar, respectively. If *Bacillus* or *Clostridium* is suspected, casein agar plus glucose or PI medium (see a, p. 268) should be included. One plate should be incubated in an anaerobic chamber (see 2, p. 262). If *Streptomyces* is suspected, include water agar (10).

For isolation, remove a small amount of tissue from the advanced portion of the lesion using a sterile scalpel. The tissue should be washed with sterile water or surface sterilized for 3 min. in a 1: 10 dilution of a household bleach (5.25% active sodium hypochlorite). After rinsing in sterile water, the tissue should be chopped up with a sterile scalpel in a droplet of water in a plastic Petri dish. If the tissue is too difficult to cut, a sterile mortar and pestle can be used. After sitting for 2 or 3 minutes, the macerate is streaked onto agar media with a platinum wire loop. If a particular nomenspecies is suspected to be the causal agent, follow the isolation techniques suggested for that particular organism. Streaks should be made in four right angle directions, flaming the loop after each directional streak. A single colony (well spaced from other colonies) should be restreaked on one of the media to be sure the bacterium is pure. Ifno colonies result from streaking onto a common medium, determine if the causal organism is anaerobic *(Clostridium* spp.), a fastidious organism (such as *Xylella fastidiosa,* or *Rhizomonas),* or a non-culturable phloem-limited bacterium such as Huanglongbin (citrus greening) bacterium *(Liberobacter).* For the latter organisms PCR techniques should be used (see F, p. 278).

2. Recipes for common and semiselective media

a. Nutrient agar (NA) and nutrient broth (N'B)*

	perL
Beef extract (D ifc 0)	3.0 g
Peptone	5.0 g
Agar	15.0 g

Nutrient agar (NA) or nutrient broth may be purchased in dehydrated form from Difco.

*Do not add agar if nutrient broth is desired.

b. Yeast extract-dextrose-$CaCO_J$ ([YDC] [16])

	perL
Yeast extract	10.0 g
Dextrose (glucose)	20.0 g
Calcium carbonate, USP light powder	20.0 g
Agar	15.0 g

To obtain an even milky white medium, the CaCD) must be of the finely ground form, otherwise it will precipitate to the bottom. Autoclave at 10 PSI for 1 h or autoclave dextrose in 100 ml of water separately. The autoclaved medium should be cooled to 50[a]C in a waterbath and CaCO) suspended by swirling before pouring the plates. For tubes, a vortex mixer should be used to mix thoroughly the CaCO) after removing tubes from the waterbath.

c. Nutrient-broth yeast extract agar (NBY) (IS)

	per L 8.0 g 2.0 g 2.0 g
Nutrient broth (Difco)	0.5 g
Yeast extract	2.5 g
$KJIP0_4$	15.0 g
$KH,^P04$	
Glucose	
Agar	

After autoclaving, add 1.0 rnl of a sterile solution of 1M $MgS0_4'7H_20$.

d. King et at's medium B agar [(KB (6»)

	per L
Proteose peptone #3 (Difco)	20.0 g
K_2HP0_4	1.5 g
$MgS0_4'7H_20$	1,5g
Glycerol	15.0 ml
Agar	15.0 g

e. Casein agar plus glucose

	perL
Casein acid hydrolysate	10.0 g
Yeast extract	5.0 g
Glucose	5.0 g
K_2HP0_4	4.0 g
Agar	17.0 g

f. Water agar

	perL
Agar	20 g

g. PW agar (3)

	perL
Soytone	4.0 g
Tryptone Hemin	1.0g
chloride	10.0 mg*
Phenol red (0.2% aqueous)	10.0 mI
$MgS0_4'7H_20$	0.4 g 1.2
K_2HP0_4	g LOg
KH_2HP0_4	4.0 g
Lsglutamine	15.0 g
Agar	30.0 mI**
Bovine serum albumin V	

* Or add 10 mI of a 0.1 % solution in 0.05 n NaOH

* Add 20% filter sterilized (0.2 urn filter) stock solution after autoclaving

*

h. S-medium (14)

Rhizomonas is isolated from soil and plant tissue using S-medium.

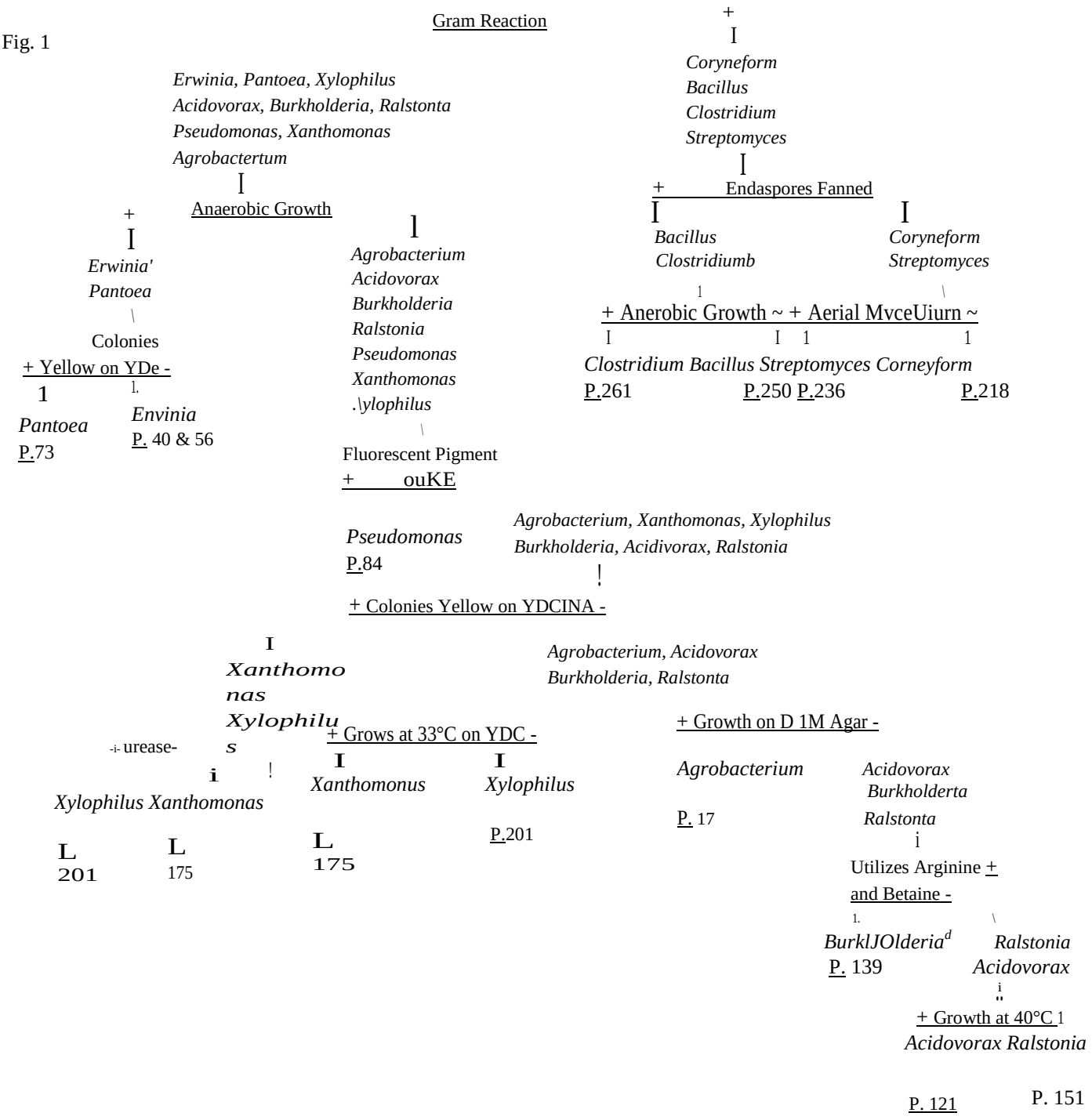
	per L
Casein acid hydrolysate	5.0 g
Glucose	2.5 g
$K_2 HP0_4 03H_20$	1.3 g
$KN0_3$	0.5 g
$MgS0_4 \cdot 7H_20$	0.5 g
Ca(N03)2e4H20	60.0 mg
Streptomycin	1.0 ml*
sulfate Agar, Noble	11.0 g

*Streptomycin sulfate (aqueous. filter-sterilized, 15 mg/ml stock solution) is added to the medium after autoclaving and has cooled to about 50°C.

C. DIFFERENTIA nON OF COMMONLY ISOLATED GENERA

The following flow chart (Fig. 1) should be used as a quick guide for identifying a particular species or pathovar:

Fig. 1



a Colonies *ofPal1!oea atrea* and some strains of *P. aggLomiirans* are generally white. b *Closmdia* cells with ~'Pores are swollen, whereas *Baer/Ii* are not.

c Colonies of *Xanthomonas cassavae* and twa pathovafS of *X campestris* are white .

• *81lrkhoiderta andropogonis* is negative tor *arginIne* and *betaine.* However, it is oxidasenegativc, whereas *Ralstoma andAcidovorax* are positive.

D. DIAGNOSTIC MEDIA AND TESTS

1. Gram reaction

This test is essential for differentiating plant pathogenic bacteria into two broad groups: Gram-positive and Gram-negative. The simple KOH technique can be used as a rapid test for presumptive identification (13). However. the Gram-stain is *very* important when identifYing a newly described bacterium and should not be omitted.

a. KOH test (13):

Mix loopful of bacterial with 2 drops of3% KOH. Gram-negative bacteria will become gummy upon mixing with a loop while Gram-positive bacteria will not. If questionable results are obtained the Gram stain should be used.

b. Gram stain (1, 4, 11, A. Vidaver, Personal communication)

Gram-positive bacteria retain the primary dye, giving a purple to blue-black appearance. Gram-negative bacteria take up the color of the counter stain. Only a few bacteria cannot be readily grouped into either category.

The major points in successful staining are as follows:

a. Use reagents less than one year old. Particular attention is needed for the iodine solution. If the solution is not put in a brown bottle or kept in the dark, it will decolorize and become ineffective. The reagents may also break down.
b. Use freshly grown bacteria, ideally a liquid overnight culture in exponential phase. Older stationary phase cells may give a Gram-variable reaction.
c. Use a known positive and known negative for controls.
d. The bacterial smears must show some well-separated groups of bacteria. Clumps are to be avoided.

Staining procedure:

a. On a clean slide, dry a thinly spread bacterial film in air, without heat. Then lightly flame the underside of the slide twice to fix the bacteria to the slide.
b. Flood the smear with crystal violet solution for 1 minute.
c. Wash in tap water a few second. Drain off excess water, and lightly blot dry on a paper towel.
d. Flood the smear with iodine solution for 1 minute
e. Wash in tap water a few seconds; blot dry.
f Decolorize with solvent, e.g. ethyl alcohol, until the solvent flows colorlessly from the slide (about 30 seconds). Blot dry. (If decolorizer is used longer, the Grampositive bacteria may lose color.)
g. Rinse in tap water for about 2 seconds.

h. Counterstain for about 10 seconds with safranin solution.

1. Wash briefly in tap water. Blot dry and examine.

Results:

Gram-positive bacteria stain purple to blue-black; Gram-negative bacteria stain red (Plate 1, Fig 1).

Solutions:

a. Hucker's ammonium oxalate crystal violet

Solution A	
Crystal violet	2.0 g
Ethyl alcohol (95%)	20.0 ml
Solution B	
Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Mix solutions A and B. Store 24 hours before use. Filter through paper into storage bottle.

b. Gram's modification ofLugol's solution

Iodine Potassium iodide	~ LO g
Distilled water	2.0 g 300.0 ml

Allow iodine solution to dissolve several hours or overnight in a dark place. Alternately, grind the dry iodine and KI in a mortar, adding water slowly. Continue grinding until I and KI are in a solution. Rinse the solutions with remaining water into a dark bottle.

c. Decolorizers

1. Ethyl alcohol, 95%, slowest agent
2. Acetone: fastest agent
3. Acetone-alcohol: intermediate (95% ethyl alcohol, 100 ml; acetone, 100 ml.).

With practice, any of the three decolorizing agents will yield good results.

d. Counterstain

Stock solution	
Safranin 0	2.5 g
Ethyl alcohol, 95%	100.0 m1
Working solution	
Stock solution	10.0 ml
Distilled water	90.0 m1

2. Spore determination (4) (also, see c, p. 11)

Suspend colony of bacteria growing on agar medium in a drop of water on a slide and air dry. Flood slide with 5.0% (w/v) aqueous malachite green and stain for 10 minutes. Wash thoroughly under running water and dry briefly. Counter stain by flooding slide with 0.5% (w/v) aqueous safranin for 15 seconds. Rinse thoroughly with water and blot dry. Observe cells at 40x. Bacterial cells stain red and spores green.

3. Anaerobic growth (Hugh and Leifson [5])

	perL2
Peptone	.0 g
NaCl	5.0 g
KH_2P0_4	0.3 g
Agar Bromthymol	3.0 g
blue	3.0ml
(1 % aqueous solution)	

Dissolve ingredients and adjust the pH to 7.1. Add 5 m1 of basal medium to 13 em diameter test tubes and sterilize at 121°C for 20 min. Prepare a 10% aqueous solution of glucose and sterilize by filtration. Add 0.5 m1 of the sterile glucose aseptically to each tube of basal medium. Inoculate two tubes with each strain of the organism to be tested. Cover one tube with a layer of sterile melted Vaseline or paraffin to a depth of about 5mm. A color change from blue to yellow in both tubes is recorded as positive for anaerobic growth (fermentation).

4. Growth on DIM agar (12).

	perL
Cellobiose	5.0
NH_4CI NaH_2P0_4	g
K_2HP0_4	1.0
$MgS0_4$&$7H_2O$	g
Malachite green	1.0
Agar	g 1.0
	g
	3.0
The medium is adjusted to pH 7.0 before autoclaving.	g
	10.0 mg
	15.0 g

Useful for differentiating *Agrobacterium* from *Acidovorax, Burkholderia,* and *Ralstonia.*

5. Growth at 33 or 40°C

Grow culture overnight in 5 to 10 ml of liquid NBY in a 25 cm diameter test tube on a slanted rack on a rotary shaker at 25 - *2r* C. Remove 50 ,ul and add to a new tube of NBY and incubate at 40°C on a rotary shaker. Record growth after 15 and 24 h

6. Urease (see e, p, 47)

7. Oxidase test (7)

For inoculum use 24-hour-old cultures grown on NGA (false negatives may result if greater than 0.25% glucose is used). Rub a small loopful of the inoculum on a filter paper impregnated with 1 % (w/v) aqueous tetramethyl-p-phenylenediamine dihydrochloride solution* (freshly made). The strain is rated oxidase-positive if a purple color develops within 10 seconds (Plate 1, Fig. 2), delayed positive if coloration develops within 10-60 seconds; and negative if no color develops after 60 seconds. A platinum or plastic loop or a toothpick is recommended since traces of iron can catalyze the oxidation of the phenylenediamine compound.

* Commercial oxidase strips are available from Difco; some investigators prefer dimethyl to tetramethyL

8. Colony coloration and consistency on YDC agar. Streak onto agar plate and incubate at 30°C.

9. Fluorescent and diffusible non-fluorescent pigments on KB. Streak onto agar plate and incubate at 25°C. After 48 h, observe for non-fluorescent pigment under normal light and in dark with long wave length uv light (366 nm) for fluorescent pigment.

10. Flagella

a. Silver impregnation (2)

Preparation of bacteria and precautions:

1) Grow bacteria on agar plates of a standard medium such as YDe at 27^0 C for 24-48 h.
2) Use a known peritrichous species such as *E. carotovora* or a polar species such as *P. marginalis* as a control.
3) Prepare a faintly cloudy suspension by carefully adding 1.0 ml of distilled water to butt of slant.
4) Do not mechanically agitate slant.
5) Place a loopful of distilled water on an alcohol-cleaned slide.
6) A loopful of bacterial suspension is placed just touching the distilled water.
7) Allow slides to air dry.

Staining procedure:

1) Cover the smear with reagent A for 2 to 4 mins then rinse in distilled water.
2) Add reagent B (pH 10.0) for about 30 sees and immediately wash in distilled water.
3) Air dry and examine under oil immersion.

Results:
Bacteria and flagella are stained dark-brown to black on a light to golden background.

Reagents:

A. Tannic acid solution

Tannic acid	5.0 g
Ferric chloride Formalin	1.5g
(15%) Sodium	2.0 m1
hydroxide (1%)	1.0 m1

Bring to 100 m1 with distilled water.

B. Ammoniated silver nitrate solution

Silver nitrate (2%)	100.0 ml

Remove and save 10 m1 of silver nitrate. Then add ammonium hydroxide to remaining 90 ml until a heavy precipitate is formed. Continue adding ammonium hydroxide until precipitate is dissolved. From the 10 ml of silver nitrate saved, back titrate until a slight clouding appears and persists. Adjust pH to 10.0 with ammonium hydroxide and silver nitrate. Use Within 4 h of Preparation.

b. Electron microscopy (G. Gaard, University of Wisconsin, Madison, Wisconsin, personal communication.)

Prepare a test tube agar slant culture of the test organism on an optimal growth medium. Culture should be sampled when it is in the logarithmic growth phase (15-72 h). However, it is also helpful to incubate the culture at a temperature 2 JOe below the optimum for growth.

Prepare the culture for viewing by the following procedure:

1) Rinse the base of the agar slant about 5 times with double distilled water and discard water. This is to remove the cells that accumulate at the base of the slant.
2) Gently rinse bacteria from surface growth of the agar slant with about 1 ml of double distilled water.
3) Prepare a dilution series using a suspension of about 10^9 cells as the initial concentration. Mix dilutions very gently to avoid loss of flagella. Dilute

10. this suspension by l/2, *114.* 1/16, and 1/32. then put a smail droplet of each on different grids. For optimal viewing, the final concentration on the grid should be 5 cells/grid hole on 200 mesh grids. Use of a spreading agent (detergent) is normally not necessary.

-l) Shadow or negative stain.

a) To shadow, freeze dry grids at _lO° C in a desiccator overnight and shadow with carbon-platinum at an angie of 8 ° .

b) To stain. place grid on a drop of 1 % aqueous uranyl acetate tor ~.: min or 2% potassium phosphotungstate pH 6.5 for 30 sees. Remove stain by touching the edge of the grid to filter paper.

5) Observe under an electron microscope (Fig 2).

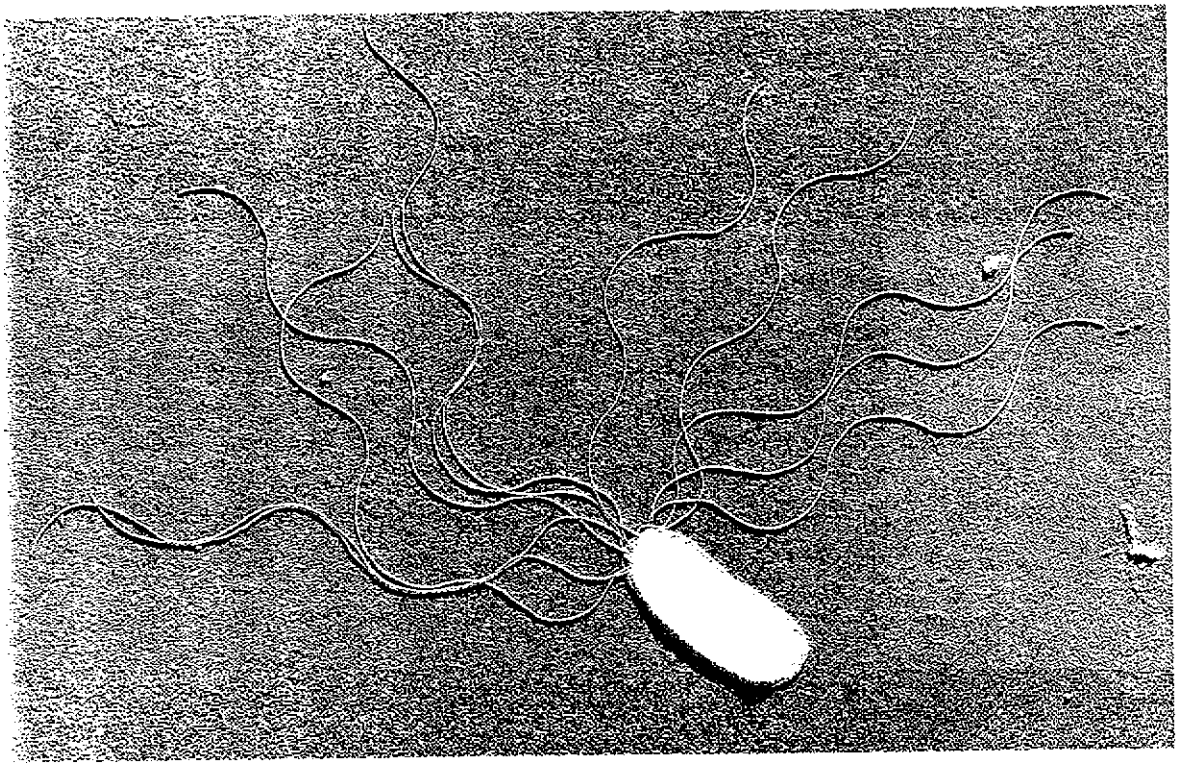


Fig. 1. Polar flagella of *Pseudomonas marginalis* shadowed with carbon-platinum and observed by an electron microscope. {Photograph courtesy ofG. Gaard, Univ. Wis.)

11. Serum agar ([Liao and ellen medium i l8D

	ner L
PPLO broth base	1,5 g
Sucrose	12.0 g
Horse serum	202 g
Phenol red	2.5 mg
Agar	i.5 g

12. Cell **wall determination**

This test is needed to identify a microorganism such as a mycoplasma or spiroplasma and should be performed by an experienced electron microscopist. The following method is that of J. Worley, USDAIARS Beltsville Agricultural Research Center, Beltsville, MD 20705 (personal communication):

Selection of material is critical since some organisms are at low concentrations or limited to certain tissues.

a. Remove pieces of tissue 1 to 2 mnr' using a razor blade and place immediately into freshly prepared 3% glutaraldehyde in O.IM phosphate buffer (pH 7.0).

b. After infiltration in a vacuum desiccator for several hours (depending upon tissue), replace glutaraldehyde with fresh solution and continue fixation under vacuum for about 4 hr at room temperature.

c. Remove glutaraldehyde and then wash tissue briefly three times in fresh phosphate buffer.

d. Place tissue pieces in 2% osmium tetraoxide in the same buffer for 2-4 hr at room temperature or overnight a 4°C.

e. Rinse three times in fresh buffer.

f Dehydrate tissue in ethanol-propylene oxide using single steps (15 - 30 min) of 20%, 50%, 70%, and 95% ethanol, two steps of absolute alcohol, one step in absolute alcohol-propylene oxide (1: 1), and finally twice in propylene oxide.

g. Embed in Araldite resin (9).

Araldite 502	13.0 ml
Dodecanylsuccinic anhydride	10.0 ml
DMP-30	0.4 ml

Stir mixture well before use.

Embed in propylene oxide-resin mixture (2: 1) for 1 hr, then in propylene oxide-resin mixture (1:2) for 4 hr covered and overnight uncovered. Finally, embed in resin mixture for 4 hr.

h. Remove tissue from resin, blot dry, and transfer to aluminum weighing dish containing fresh resin.

1. Arrange tissue in resin and cure at 35°C for 8-12 hr, 45°C for 8-12 hr, and 60°C for 48 hr.

l Saw tissue from block and mount on plastic cylinders. Trim and section transversely at a setting to obtain silver-to-gold (about 100 urn) and purple-togreen (about 300 run) sections.

k. Spread the compressed ribbons by passing a cotton swab saturated with chloroform over the ribbons.

1. Mount on Formvar - coated copper grids.

m. Stain for 10 mins in saturated aqueous uranyl acetate, wash thoroughly, stain for 4-5 mins in lead citrate, wash thoroughly, then dry and view in electron microscope (Fig.3).

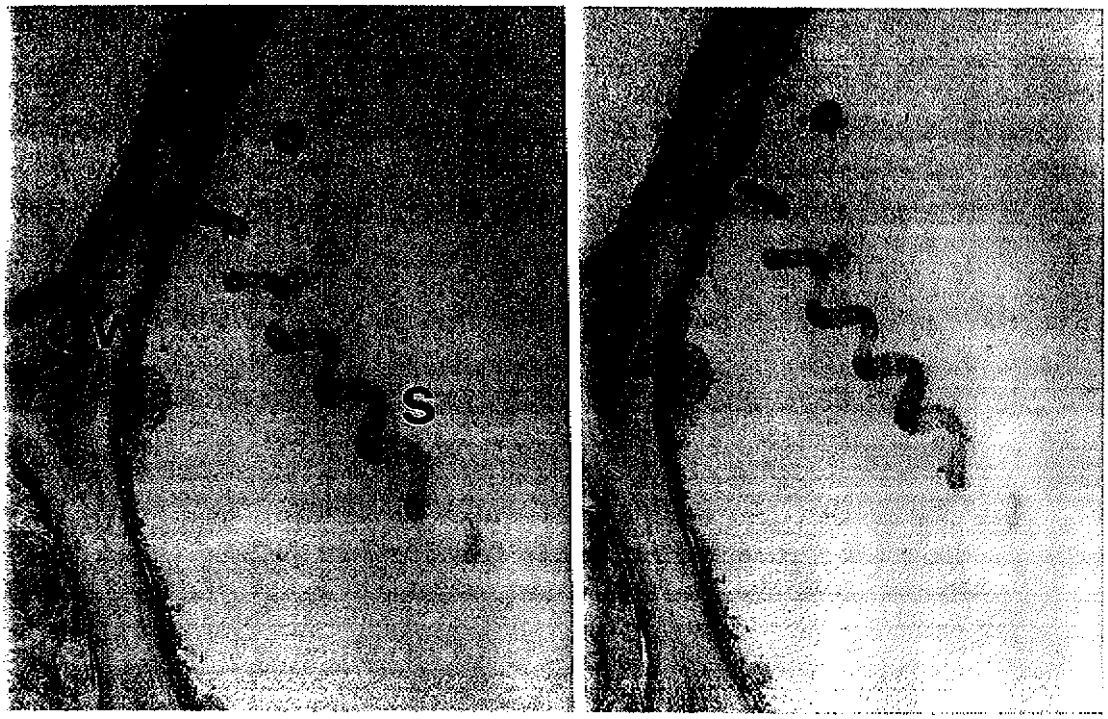


Fig. 3. Stereo pair of electron micrographs of a thick ultramicrotome section (cut at about 0.3 *IJ.)* of phloem tissue from a com stunt-infected com plant. S = helically coiled cell of com stunt spiroplasma. CW- plant cell wall. (From Davis and Worley [1973], *Phytopathology 63:403-408).*

E. LITERATURE CITED

1. Annette, E., H. A Bazows, W. 1. Hausller, and H. 1. Shadomy. 1985. Manual of Clinical Microbiology. American Society for Microbiology, Washington, D.C. Fourth Edition.

2. Blenden, D. C. and H. S. Goldberg. 1965. Silver impregnation stain for leptospira and flagella. 1. Bacteriol. 89: 899-900.

3. Davis, M. 1., W. 1. French, and N. W. Schaad. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curro Microbial. 6:309-314.

4. Gerhardt, P. (Ed.). 1981. Manual of Methods of General Bacteriology. Am. Soc. MicrobioL Washington, D.C.

5. Hugh, R. and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J. Bacteriol. 66:24~26.

6. *King,* E. 0., M. K. Ward and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.

7. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature. 178:703.

8. Liao, C. H. and T. A. Chen. 1977. Culture of com stunt spiroplasma in a simple medium. Phytopathology 67:802-807.

9. Luft. J. H. 1961. Improvements in epoxy resin embedding methods. 1. Biophys. Biochem, Cytol. 9:409-414.

10. Menzies, 1. D. and C. E. Dade. 1959. A selective indicator medium for isolating *Streptomyces scabies* from potato tubers or soil. Phytopathology 49:457-458.

11. Meynell, G. G. and E. Meynell. 1970. Theory and practice in experimental bacteriology. 2nd ed. Cambridge University Press, London.

12. Perry, K. L. and C. I. Kado. 1982. Characteristics ofTi plasmids from broad-host-range and ecologically specific biotype 2 and 3 strains for *Agrobacterium tumefaciens. 1.* Bacteriol. 151:343-350.

13. Suslow,T. V., M. N. Schroth, and M. Isaka. 1982. Application ofa rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology 72:917-918.

14. van Bruggen, A. H. C., R. G. Grogan, C. P. Bogdanoff, and C. M. Waters. 1988. Corky root oflettuce in California caused by a Gram-negative bacterium. Phytopathology 78: 1139-1145.

15. Vidaver, A. K. 1967. Synthetic and complex media for rapid detection of fluorescence of phytopathogenic pseudomonads: Effect of the carbon source. Appl. Microbiol. 15: 15231524.

16. Wilson, E. E., F. M. Zeitoun and D. L. Fredrickson. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. Phytopathology 57:618-621.

F. Chemicals List

Chemicals	Source
Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.	
Agar	Sigma, Difco, BBL
Ammonium oxalate	Fisher
Arginine	
Araldite 502	Ladd Research Inc., Burlington, VT

Beef extract	Difco
Betaine	
Bovine serum albumin fraction V	
Bromthymol blue - sodium salt	Matheson Coleman Bell
Calcium carbonate, precipitated light powder	Mallinekrodt or 1. T. Baker
$Ca(N°3)2-^{4H}20$	
Casein acid hydrolysate	Difco
Cellobiose	
Crystal violet, certified	Difco
Dl\1P-30	Ladd Research Inc.
Dodecanylsuccinic anhydride	Ladd Research Inc.
Ferric chloride	
Formalin (15%)	Mallinckrodt or Fisher
Glucose	
Glutaraldehyde	
Glycerol	
Hemin chloride - bovine	Grand Island Biological
Horse serum	
Iodine	Mallinckrodt or Fisher
K_2HP0_4	
K_2HP0_4' 3 H_20	Mallinckrodt or Fisher
$KH_2{}^P04$	
KH_2PO_{4-} anhydrous	
KOH	
$KN0_3$	Fisher
Malachite green	J. T. Baker
$MgS0_4'7H_20$	Difeo
Nutrient agar or broth	
Osmium tetraoxide	Difeo
Peptone	
Phenol red	J. T. Baker
Potassium iodide	
Potassium phosphotungstate	Difco
PPLO broth base	Difco
Proteose peptone #3	Ladd Research
Propylene oxide	Fisher
Safranin 0, certified	
Silver nitrate (2%)	Difco
Soytone	
Streptomycin sulfate	
Tannic acid	
Tetramethyl-p-phenylenediamine dihydrochloride	Difco
Tryptone	
Uranyl acetate	Difco, BBL
Yeast extract	

D. GRAM-NEGA TIVE BACTERIA

A. *Agrobacterium*

L. W. Moore, H. Bouzar, and T. Burr

1. INTRODUCTION

Agrobacterium spp. are commonly known as soil-borne bacteria that infect dicotylendonous plants from over 90 different plant families (7, 13), including economically important fruit and nut crops, grapes, ornamental and landscape plants (e.g. rose, euonymous, dahlia, chrysanthemum). Upon infection of plants, some strains of *Agrobacterium* incite abnormal cell proliferation (hyperplasia) which result in tumor formation in the case of the crown gall disease and excessive adventitious roots in the hairy root disease. Financial losses from the disease have been estimated in the millions of dollars per year (24), occurring primarily at nurseries, where diseased plants are discarded (30). Crown gall can also stunt mature plants by causing inferior development ofthe root system and/or disruption of vascular flow in the stem. A new cultivar of walnut has been especially susceptible in California where crown gall stunts and sometimes kills the tree, and production is almost always affected. Almond trees in California with crown gall are more susceptible to blow-over during wind storms and the wood rotting fungi.

Pathogenic strains of *Agrobacterium* share a common feature; they contain at least one large plasmid, the tumor- or root-inducing (Ti and Ri, respectively) plasmid (44). Virulence is determined by different regions of the plasmid including the transferred DNA (T-DNA) and the virulence *(vir)* genes. The virulence genes mediate transfer ofT-DNA into infected plant cells, where it integrates into the plant DNA (11). Rhizogenic (root-inducing) agrobacteria T -DNA contains *rol* (root locus) genes that render plant cells more sensitive to endogenous auxin (40). Although Ti and Ri plasmids vary considerably between strains, they all carry similar *vir* genes (20).

The genus *Agrobacterium* belongs to the family *Rhizobiaceae* (27) which has been included in the alpha-2 subclass of *Proteobacteria* on the basis of ribosomal characteristics (45). Members of this family are aerobic and Gram-negative. The cells are normally rod-shaped (0.61.0 um by 1.5-3.0 urn), occur singly or in pairs, without endospores, and are motile by one to six peritrichous flagella. Considerable extracellular polysaccharide slime is usually produced during growth on carbohydrate-containing media.

The classification of gall- and root-inciting agrobacteria is in constant evolution. Until recently, the species nomenclature was based primarily on plasmid-borne phytopathogenic characters and included *Agrobacterium tumefaciens,* causal agent of crown gall; *A. rhizogenes*, causal agent of hairy root disease; and *A. rubi*, causal agent of cane gall on *Rubus* (27). All nonpathogens were lumped into *A. radiobacter*. However, there was no correlation between this nomenclature based on pathogenicity characters and the taxonomic structure based on

phenotypical and genotypical traits of the genus (27). Analysis of chromosome-encoded characters (37), chromosomal DN A (11) and comparison of electrophoretic protein patterns (26) showed that most members of the genus *Agrobacterium* grouped into at least three taxonomic groups not corresponding to pathogenicity. Not including the five marine species recently described as a distinct subdivision within the genus *Agrobacterium* (37), the genus is composed of at least four distinct and separate taxa (4, 18,38). DNA-DNA homology studies showed that these taxa corresponded to the genospecies: *Agrobacterium tumefaciens* (formerly biovar 1) (5), *A. rhizogenes* (formerly biovar 2) (38), *A. vitis* (foonerly biovar 3) (32) and *A. rubi.* Although this proposed speciation scheme appears to be a good solution to objectively differentiate the agrobacteria, it has been difficult for scientists to accept because oflong historical usage of the previous names (Table 1). We recommend that the new speciation scheme be accepted. The phylogenetic relationships of members of the genus *Agrobacterium* have been determined by ribosomal RNA sequencing (rrs) (38, 45). Two major lineages were distinguishable: one includes *A. rhizog*^{enes} along with most *Rhizobium* species; the second includes *A. tumefaciens, A. vitis, A. rubi* and a distinct strain, NCPPB 1650, along with *Rhizobium galegae.* Thus, biochemical traits permit the characterization of species in the genus *Agrobacterium* which in turn can be identified by rrs sequencing. Because of the molecular tools now available for classification and phylogenetic studies, it is predictable that in the future new species will be identified [e.g., distinct strains have been isolated from *Ficus* (6) and wild blackberry (10)). The genetic diversity within *A. vitis* has been recently studied. In addition to Ti plasmid groups, chromosomal characterizations of *A. vitis* strains were done by fingerprinting the region between the 168 and 23S rRNA genes. A high correlation was found between chromosomal type and the type ofTi plasmid carried by strains (33).

Table 1. Comparison of aid and new nomenclature for species of *Agrobactertum*

New taxonoro *	Old taxonom
A. ttlmefaciens	*A. tumefaciens* biovar 1 *A. radiobacter* biovar I *A. rhizogenes* biovar 1
J. *rhizogenes*	*A. tumefaciens* biovar 2 *A. radiobacter* biovar 2 *A. rhizogenes* biovar 2 *A. tumefaciens* biovar 3 *A. radiobacter* biovar 3
A. vi tis	
A.robi	*A. robi*

*Individual strains in the species may be tumorigenic, rhizogenic, or nonpathogenic

2. ISOLATION TECHNIQUES USING SEMISELECTIVE MEDIA

a. Plant Material

Agrobacterium strains can be isolated from aerial and subterranean galls, vascular fluids and from symptomless plants. Isolation of the pathogen is made most easily from white to cream-colored gall tissue of young, actively growing galls. Wash the gall surface

in running water and remove darkened necrotic areas of tissue, or surface sterilize for 1020 minutes in 1 % sodium hypochlorite solution (or 20% household bleach) prior to isolating. Rinse the tissues with several changes of sterile water before proceeding. Remove and combine two to three small subsamples from different locations within the gall. Cut the subsamples into small pieces and place in a culture tube with sterile distilled water or buffer, vortex and let stand for 30 minutes or longer to allow the bacteria to diffuse into the liquid. Streak a loopful of the tissue suspension onto agar media (Table 2) and incubate at 25-27°C. Some agrobacteria grow very slowly (e.g. wild Blackberry types), and colony morphology and pigmentation varies. Some strains isolated from pecan produced a diffusible blue pigment while some isolated from grape produced a reddish, diffusible pigment when cultured (Bouzar, unpublished). Because bacteria other than *Agrobacterium* can grow on the selective media, it is helpful to plate known strains on the same media to aid in identification. Both pathogenic and non-pathogenic agrobacteria are commonly isolated from galls. Warning: many colonies look morphologically correct on selective media following initial plating, but may contain other bacteria, hence the colonies should be cloned for purity before performing additional characterization. To clone for purity, individual colonies from selective media are suspended uniformly in sterile distilled water or buffer and streaked onto PDA+$CaC0_3$ (Table 2). Single colonies are then again selected, suspended uniformly in buffer or sterile distilled water and streaked. Repeat this process until all the colonies on the plate appear homogeneous. On PDA+$CaC0_3$, *Agrobacterium* strains grow well, and their colonies are distinguishable from other species. They are convex, glistening, circular with an entire edge, and white to tannishcream in color.

Selective media for *Agrobacterium* include (Table 2); medium lA (8) for *A. tumefaciens* (Plate 1, Fig. 3), medium 2£ (8) for *A. rhizogenes* (Plate 1, Fig. 4) and Roy and Sasser's (36) medium for *A. vitis* (Plate 1, Fig. 5). Because more than one strain of *Agrobacterium* can cohabit the same tumor, it may be necessary to use all three media when attempting to determine which species are present.

b. Soil and Water

Use of selective media is especially helpful for isolation of *Agrobacterium* from soil and water. Even on selective media a few other bacterial species can grow. For a qualitative determination of the presence of *Agrobacterium,* streak a loopful ofthe sample suspension onto the selective medium. For quantitative determinations, prepare l C-fold serial dilutions and spread 0.1 ml of each suspension over the entire surface ofthe selective medium with an L-shaped glass rod. Sensitivity of the selective media is limited to about 10^4 colony forming units (CFU)/g soil. Incubate plates at 25-27°C. Utilization of the pour plate method with selective media allows higher concentrations of soil suspensions to be assayed, thus increasing the sensitivity of detection, especially when combined with immunofluorescent colony detection/isolation (Moore and Van Vuurde, manuscript in review). Prepare pour plates by adding 0.1 to 0.5 ml ofa particular dilution series to an empty, sterile Petri dish followed by 12 ml molten (cooled to 50°C), selective

Table 2. Selective media for isolation of the major species of *Agrobacterium*

SELECTIVE MEDIUM	MAJOR SPECIES ISOLATED	COMMENTS
Patel (34)	*A. tumefaciens* andA. *rhizogenes*	Discriminated poorly against the normal soil microflora; however, all agrobacteria tested by Brisbane and Kerr grew (8).
Schroth et al. (41)	*A. tumefaciens*	Excluded 99% of other species growing on nonselective media; efficiency of recovery was 38%. Reducing antibiotics increased efficiency of recovery; some *A. rhizogenes* grew when ammoniacal nitrogen was used; use of this selective medium as a liquid enrichment medium allowed detection of nonpathogenic agrobacteria in 28 soils (41). Omission of streptomycin helped improve recovery of someA. *tumefaciens* strains (8).
Clark (12)	*A. tumefaciens*	*A. rhizogenes* also grew, but poorly; *A. vitis* was recovered as well. The manganese sulphate concentration had to be reduced by half to allow growth of *Agrobacterium* strains in Australia (8).
DIM (35)	*A. tumefaciens andA. rhizogenes*	*A. vitis* also grew on this medium (Moore, LW., unpublished). (see 4, p. 9)
New-Kerr (31)	*A. rhizogenes*	Nitrate was reported to prevent growth of *A. rhizogenes* and *Rhizobium trifolii*. However, Anderson (1) found that 26/27 *Rhizobium* andA. *rhizogenes* strains grew in the presence of nitrate.
lA 2E and 3DG (8)	*A. tumefaciens, A. rhizogenes* and *A. vitis*	Each of these media was reported as highly selective and efficient (99%) for the respective species.
Roy-Sasser (36)	*A. VIlis*	Selective for *A. vitis* strains and effective when isolating from soil. Colony morphology is very distinct. (Burr, T., unpublished and Moore, L. W., unpublished).

medium from a culture tube. Swirl the contents of the dish to thoroughly mix the aliquot of diluted suspension with the molten agar. Allow to solidify and then incubate the poured plate upside down. Colonies buried in the agar layer appear lenticular in shape. Compare colony morpholohogies in the dilution series to a similarly prepared pour plate containing a known culture of *Agrobacterium.*

c. Vascular Sap

The presence of endophytic agrobacteria has been reported in grape (28) and chrysanthemum (19). *A. vitis* is a common inhabitant of the vascular tissues of grape and can be isolated from extracted xylem fluids. In the spring, one can collect the exuded sap and spread it to selective medium. Alternatively, a pressure chamber has been constructed which allows cane sections to be processed throughout the year (43). Similar procedures can be used to isolate *Agrobacterium* from other woody plants. Canes or branch sections are cut into 15-cm segments, the ends dipped in 95% ethanol and flamed. Flamed segments are inserted through holes in rubber stoppers and the stopper and stem section are fitted into the lid of the sap-extraction apparatus with the basal part of the cutting positioned in 10 m1 of sterile water contained in a conical tube, and the lid is secured tightly. Pressure is applied by pumping air into the chamber to force water through the vascular tissues. Extruded sap is collected at the apical end, and 0.1 m1 is spread to selective media. Colonies from the selective medium are purified as described under 2.a, p. 18.

d. Recipes fOI" semiselective media

1) Medium 1A for *A. tumefaciens* (formerly by. 1) (8)

	perL
L (-) arabitol	3,04 g
NH_4N0_3	0.16 g
KH_ZP0_4	0.54 g
K_ZHP0_4	1.04 g
Sodium taurocholate	0.29 g
$MgS0_4@7H_20$	0.25 g
Agar	15.00 g 2 ml
Crystal violet, 0.1% *(w/v)* aqueous	

Autoclave, cool to about 50°C, then add filter-sterilized cycloheximide (1.0 ml of 2% solution) and N~Se03 (6.6 m1 of 1 % solution).

2) Medium 2E for *A. rhizogenes* (formerly by. 2) (8)

	perL0
NH_4N0_3	.16 g
Erythritol	3.05 g
$KH_2{}^P04$	0.54 g
$KlIPO_4$ $MgS0_4{}^07H_20$	1.04 g
Sodium taurocholate	0.25 g
Yeast extract, 1% *(w/v)* aqueous	0.29 g
Malachite green, 0.1 % *(w/v)* aqueous	1 m1
Agar	5 m1
	15.00 g

Autoclave, cool to 50°C, then add filter-sterilized cycloheximide *(1.0 ml* of2% solution) and N~Se03 (6.6 *ml* of 1 % aqueous).

3) Roy and Sasser medium for *A. vitis* (formerly bv. 3) (36)

	per L
MgSO~.7H20	*0.2* g
KH_2^P04 *KlIP0*$_4$	*0.7* g
Adonitol Yeast	0.9 g
extract NaCI	4.0 g
H_3B0_3	*0.14* g
Agar	*0.2* g
Chlorothalonil (Bravo *500),* 4% *(w/v)* aqueous	1.0 g
	15.0 g 0.5 ml

Adjust to pH 7.2, autoclave, cool to 50°C and add aseptically the following (after dissolving separately in a few *ml* of distilled water and filter sterilizing):

Trimethoprim (with 1 drop HCl to the distilled water)	20 mg
Triphenyltetrazolium chloride	80mg
D-cycloserine	20 mg

Colonies of *A. vitis* are countable after four days of incubation at 27°C. They usually have dark red centers with white edges, but some strains fail to develop the dark red centers. In all cases, comparison to a known strain plated at the same time is recommended.

3. DIFFERENTIA nON OF COMMONLY ISOLA TED SPECIES

The following diagnostic tests (Table 3) separate strains of *Agrobacterium* into the three most common species. Because genetic mutation and recombination likely occurs among *Agrobacterium* strains in nature, we expect to isolate some strains with phenotypic traits that are intermediate between the designated boundaries of the different species; however, their frequency of occurrence seems to be low. A simplified, rapid procedure to putatively classify the three main species of *Agrobacterium* is presented in Table 4, and the authors request scientists who do comparative studies of the two procedures to inform them of their results.

These diagnostic tests do not differentiate between pathogens and non-pathogens. Inoculation of susceptible plants is necessary to determine pathogenicity, but beware that some strains have a limited host range (1). DNA probes using gene sequences from a Ti plasmid provide a quick presumptive prediction of pathogenicity, but suspect colonies should still be inoculated to plants to confirm pathogenicity.

Table 3. Major biochemical characteristics differentiating species of the genus *Agrobacterium*

Diagnostic test	*A. tumefaciens*	*A. rhlzogenes*	*A. vitis*
3-Ketolactose production	+		V
Growth in 2% NaCl	+		+
Growth at 35°C	+	V	V
Action on litmus milk	ALK	AC	ALK
Acid from:			
Erythritol		+	
Melezitose	+		
Alkali from:			
Malonic acid		+	+
L-tartaric acid		+	+
Mucic acid		+	
Ferric ammonium citrate	+		
Oxidase reaction	+	V	V
Citrate utilization	V	+	+

+, 80% or more strains positive; V, between 21-79% of strains positive; ", 80% or more strains negative; ALK, alkaline; AC, acid.

Note: We typically streak each isolate onto each selective medium as an additional test. Some strains grow on more than one selective medium, but they grow more slowly than on the medium designed specifically for another species (L. Moore, unpublished).

Table 4. Simplified method for putative classification of *Agrobacterium* species (6)

Diagnostic T est	*A. tumefaciens*	*A. rhizogenes*	*A. vitis*
3-ketolactose production	+		
Acid-clearing on PDA plus $CaC0_3$		+	
Motility at pH 7.0	+	+	
Pectolytic at pH 4.5			+

+,80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative.

4. DIAGNOSTIC MEDIA AND TESTS

The following general procedures apply to all of the following recipes:

Use distilled or deionized water to prepare the media. Liquid test media are seeded with 0.1 m1 of a 48 h old culture. The culture is grown for 40-48 h on NGA or PDA slants that were streaked from working cultures (working cultures are grown on either PDA or YDC slants and kept at 4°C). The bacterial suspension is prepared by scraping bacterial cells from the slant, washing once in 0.85% NaCl solution and diluting lOO-fold to provide lO:5_{to} 10^6CFU per test medium.
Diagnostic test responses are recorded after 14 days incubation at 27°C, except for tests performed in liquid medium or on slant media which are incubated an additional week.

a. Acid clearing on PDA plus $CaC0_3$

Prepare from Difco dehydrated PDA as recommended by manufacturer and supplement with 0.5% $CaC0_3$.

b. 3-ketolactose test (3)

Smear bacterial inoculum over about a 1.0 cm diameter spot on medium containing 1 % o<-lactose, 0.1 % yeast extract, and 2% agar. Four to six strains can be applied to the same plate. Incubate the plate at 27 C for 2 days. Then, flood the agar surface with a shallow layer of Benedict's reagent and leave at room temperature. If3ketolactose is present, a yellow ring of Cu_20 becomes visible around the cell mass in about one hour (Plate 1, Fig. 6).

Benedict's reagent: Dissolve 17.3 g of sodium citrate and lag of anhydrous sodium carbonate in 60 ml of distilled water with heating (filter the resulting solution if a precipitate forms). Dissolve 1.73 g of cupric sulfate in 15 ml distilled water. Slowly add the cupric sulfate solution into a large beaker containing the sodium citratesodium carbonate solution, while stirring constantly. Dilute to one liter.

c. Ferric ammonium citrate broth test (16).

	per L
Ferric ammonium citrate	10.0 g
$MgS0_4 \cdot 7H_20$	0.5 g
K_2HP0_4	0.5 g
$CaC1_2$	0.2 g

Adjust to pH 7.0 before autoclaving. Inoculate the culture tubes containing the broth and incubate in a stationary position. *A. tumefaciens* strains produce a reddish brown pellicle at the surface of the medium (Plate 1, Fig. 7). Auxotrophs may require media supplements of O. 1 % L(-) glutamic acid or 0.001 % yeast extract to grow.

d. Growth in 2% sodium chloride (23).

Inoculate NGA slants containing 2% *(w/v)* sodium chloride and check for growth.

e. Acid production from erythritol and D(+) melezitose (15).

	perL
$NH_4H_zP0_4$	1.0 g
KCI	0.2 g
Yeast extract	1. a g
Bromthymol blue, 1% *(w/v)* in 50% ethanol	3.0 ml
Mg_ZS0_4 -$7H_zO$	0.2 g
Agar	1.5 g

Adjust pH to 7.1 with IN NaOH before adding agar.
After autoclaving, add 1 part of filter-sterilized 10% *(w/v)* erythritol, melezitose or sucrose solution to 9 parts sterile and cooled basal medium; then dispense about 4 ml of medium to sterile plugged tubes. Development of a yellow color in the medium indicates production of acid from the oxidation of erythritol, melezitose or sucrose.

f. Oxidase test (see 7, p, 10)

g. Citrate utilization (42)

Cultures are inoculated to sodium citrate agar slants of the following composition:

	perL
Sodium citrate (anhydrous)	2.0 g
$MgS0_4$ -m.o	0.2 g
NH_4 H_zP0_4	1.0 g
K_zHP0_4	1.0 g
NaCl	5.0 g
Agar	20.0 g
Bromthymol blue, 1% *(w/v)* in 50% ethanol	15.0 ml

Dissolve the salt." add Bromthymol blue and adjust the medium to pH 6.8. Add the agar, and heat the suspension to melt the agar. Dispense the medium in test-tubes, autoclave and slant the tubes to cool. After 24-48 hr, the inoculated medium turns to a deep Prussian blue if citrate has been utilized (Plate 1, Fig. 8).

h. Action on litmus milk.

Usc Difco dehydrated and prepare as recommended by manufacturer. Adjust pH to 7.0 by addition of IN NaOH prior to sterilizing. For preparation from scratch see e, p. 190. Acid production turns the milk red whereas alkaline turns the milk blue (Plate 1, Fig. 9).

1. Alkali from malonic acid (25).

	per L
(NH4)2S04	2.0 g
KH_2P0_4	0.4 g
K_2HP0_4	0.6 g
NaCl	2.0 g
Yeast extract	0.1 g
Malonic acid, sodium salt	3.0 g
Bromthymol blue, 1% (w/v) in 50% ethanol	2.5 ml

Adjust to pH 7.0, dispense 3-4 ml of medium in test tube before autoclaving. After inoculation, incubate at 2rC for about two weeks. The medium will turn blue when alkali is produced (Plate I, Fig. 10).

j. Alkali from mucic acid and L-tartaric acid (25).

	per L
$NaNH_4P0_4$	0.5 g
NaH_2P0_4	0.17 g
xci	0.2 g
Bromthymol blue, 1 % *(w/v)* in 50% Ethanol	2.5 ml

Adjust to pH 7.0 and dispense 4.5 ml basal medium to each test-tube before autoclaving. After autoclaving, add aseptically 0.5 ml of a filter-sterilized I % solution of either L-tartaric acid or mucic acid previously neutralized with NaOH. After inoculation, incubate at 27°C for about two weeks. The medium will turn blue when alkali is produced.

k, Motility at pH 7.0. (see d, p. 46)

I. Pectolytic at pH 4.5 (see 3, medium A, p, 87)

5. PA THOGENICITY TESTS

Agrobaeterium pathogenicity is controlled primarily by genes on tumor-inducing (Ti) plasrnids, Individual strains of *Agrobacterium* typically infect a narrow range of hosts (1). No host was infected by more than 81 % of the pathogenic strains in the study by Anderson and Moore (1) nor was the host range of a strain predictably determined by the plant from which the pathogen was isolated.

a. Preparation of inoculum

Prepare an inoculum containing 10^6 to 10^7 CPU/rol (see 5, p. 226) grown in liquid medium 523 (21).

1) Liquid medium 523 **(21)**

	perL
Sucrose	10.0 g
Casein (acid hydrolysate)	8.0 g
Yeast extract	4.0 g
K_2HP0_4	2.0 g
$MgS0_4$-$7H_20$	0.3 g*

*Dissolve separately in 50 ml distilled water and add prior to autoclaving.

b. **Inoculation**

Although the economics oftime and space must be considered when testing the pathogenicity of an unknown strain, the following is recommended:

1) Inoculate two or more plants of each host species per strain.

2) Tomato, datura, sunflower and bryophyllum are infected by a large number of *Agrobacterium* strains. Using combinations of these species, pathogenicity of a strain was identified with 78-94% certainty (1).

3) Some strains infect only the host species from which they are isolated and therefore must be tested on that host.

4) Always include controls which consist of i) plants that are wounded but not inoculated, ii) plants inoculated with a known pathogenic strain of *Agrobacterium* and, iii) plants inoculated with a known nonpathogenic *Agrobacterium* strain.

Temperatures above 32°C inhibit pathogenicity (2).

Wounding is required for infection and can be done with a scalpel or razor blade or by making multiple punctures with a thin, sharp needle. Young and actively growing plants are recommended. Inoculum can be applied (before or after wounding) as a spray, with a transfer loop, hypodermic syringe, micropipette, sterile wooden toothpick, or cotton swab.

Allow tumors to develop for at least 3 weeks on herbaceous plants (Plate 2, Fig. 3A) and for at least five weeks on woody plants. Incubation periods of 4-6 months were required for tumor development on inoculated incense cedar (Moore, L. W., unpublished), and roses may require 18 months. If tissue proliferation at the wound of datura plants is relatively small after 4 weeks, they are recorded as negative because *Datura* plants often form callus in response to wounding (17). Callus on datura usually ceases enlargement after 2-3 weeks of growth, whereas agrobacteria-induced tumors grow.

In the laboratory, disks of carrot can be used to test tor pathogenicity of *A. tumefaciens* and *A. rhizogenes.* We prefer the carrot root assay (29) (Plate 2, Fig. IB) to test for pathogenicity ofrhizogenic agrobacteria strains. For best results, avoid carrots held in prolonged storage. After peeling and washing with 95% ethanol, rinse the tissue with 70% ethanol and surface sterilize in a freshly prepared 1: 10 dilution of household bleach for 15 minutes. Rinse serially in three changes of sterile water and aseptically cut 5 rnm-thick slices perpendicular to the axis of the carrot. Place the slices on moistened sterile filter-paper in petri-dishes and inoculate with a bacterial suspension. Observe tumor formation or root proliferation after 3 weeks. The abaxial side of the carot slice appears more susceptible to infection.

DNA probes with homology to key gene sequences on the Ti plasmid offer a fast method of predicting the pathogenicity of a strain isolated from different hosts and/or environments (9).

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTO:MA TED TECHNIQUES

a. Molecular techniques.

Various peR-primer sets have been used to identify pathogenic *Agrobacterium.* Sawada et al. (39) reported a universal primer set (Table 5) based on sequences from the virC operon. These primers detected 75 of 77 pathogenic strains, which represented the major three *Agrobacterium* species. Haas et al. (14) designed peR primers (Table 5) to identify a wide variety of pathogenic *Agrobacterium* strains from a broad strain collection containing pathogenic and nonpathogenic strains of *A. tumefaciens, A. rhizogenes, A. vitis.* They chose oligonucleotides specific for the endonuclease-encoding portion of the virD2 gene as potential universal primers capable of identifying all pathogenic strains of *Agrobacterium.* The primers based on *virD2* identified pathogenic agrobacteria from all three virulent species tested, whereas the *ipt* primers identified only tumor-inciting *Agrobacterium* strains.

Recently, an immunocapture cultivation (with selective medium 3DG) followed by peR (primer set derived from a *6b* gene) was developed (22). This procedure proved effective for identification of *A. vitis* in tumors as well as extracts from systemically colonized symptomless plants. See Appendix A for details.

Table 5. Universal primer sets for identification of pathogenic *Agrobacterium* strains

Primers	Citation
Primer A: 5'-ATG CCC GAT CGA GCT CAA GT-3' Primer C': 5'~TCG TCT GGC TGA CTT TCG TCA TAA-3' Primer E': 5'-CCT GAC CCA AAC ATC TCG GeT occ CA-3' These oligonucleotides were used in two different pairs to produce PCR products of338 base pairs (bp) (A-E') and 224 bp (A-C).	Haas et al. (14)
Primer VCF: 50-ATCATTTGTAGCGACT-30 Primer VCR: 50-AGCTCAAACCTGCTTC-30 These primer sets produced a product of730 bp.	Sawada et al. (39)

a. Serology. A species-specific monoclonal antibody was developed for identification of *A. vitas* strains (9). See Appendix B for details.

b. Fatty Acid Analysis. Bouzar et al. (4) reported that the MIDI (MIDI, Microbial 10, Inc., Newark, DE) GC system for analysis of fatty acids in *A. tumefaciens, A. rhizogenes, A. rubi and A. vitis* provided a qualitative and/or quantitative measure of differentiation between members of these four species. Fatty acid analysis revealed that cis-vaccenic acid accounted for more than 60% of the fatty acid content of agrobacteria. Another fatty acid shared by agrobacteria was palmitic acid, and amounts of these two fatty acids differed among species.

   The presence or absence of an oncogenic plasmid did not seem to impact the classifciation. Cluster analysis of fatty acid profiles showed a closer relationship between *A. vitis* and *A. tumefaciens,* even though these two species had fewer acids in common than did *A. vitis and A. rhizogenes.*

c. Carbon source utilizations. Bouzar et al.(4) tested 59 *Agrobacterium* strains using the Biolog system (Biolog, Inc., Hayward CA) and reported that metabolic fingerprints generated from utilization patterns of the different Biolog carbon sources allowed them to distinguish between *A. tumefaciens, A. rhizogenes, A. rubi* and *A. vitis.* This system subsequently proved useful in describing what is likely a new species of *Agrobacterium* isolated from aerial galls on weeping fig trees (6). Results from Biolog analysis agreed with results from classical diagnostics. As with the MIDI system, careful attention should be given to standardization of temperature, growth-medium and age of culture. One should be aware that natural strains will be isolated from time to time with carbon-utilization patterns that differ from those in the database. For details see Appendix C.

7. CUL TURE PRESERVATION

Agrobacterium strains can be maintained on PDA (supplemented with 0.5% $CaC0_3$) or MGY for about 6 months at 4°C. Long-term preservation can be achieved when cultures are lyophilized or stored at or below -70°C. For storage at -70°C, grow the cells on slants of NGA or MGY for 48 h. Wash the growth from the agar surface with a sterile 30% (v/v) glycerol solution (glycerol serves as a cryoprotectant). Dispense 1.0 ml of the cell suspension containing at least 10^8 CFU/rnl into sterile 1.8 ml-cryotubes *(NS* Nunc, Kamstrup, DK-4000 Roskilde, Denmark) and store the cryotubes in a freezer at -70°C. Long-term preservation can also be achieved by freezing *Agrobacterium* cultures at minus 179-196°C in liquid nitrogen (30). An economical and simple alternative for long-term storage is the use of sterile-water blanks. Two loopsful of fresh bacterial growth (agar medium) are placed at the bottom of test-tubes containing sterile distilled water. To minimize evaporation, use screw caps with Teflon liners and wrap Parafilm (American Can Co., Greenwich, CT) around the tube cap or place the tubes in a plastic bag. These cultures should be kept at 4°C.

8. LITERA TURE CITED

1. Anderson, A. R. and L. W. Moore. 1979. Host specificity in the genus *Agrobacterium.* Phytopathology 69:320-323.

2. Banta. L., M. 1. Bohne, and S. D. Lovejoy. 1998. Stability of the *Agrobacterium tumefaciens* VirB 10 protein is modulated by growth temperature and periplasmic osmoadaption. 1. Bacterial. 180:6597-6506.

3. Bernaerts, M. 1. and J. DeLey. 1963. A biochemical test for crown gall bacteria. Nature 197:406-407.

4. Bouzar, H., 1. B. Jones and N. C. Hodge. 1993. Differential characterization of *Agrobacterium* species using carbon-source utilization patterns and fatty acid profiles. Phytopathology 83:733-739.

5. Bouzar, H. 1994. Request for a judicial opinion concerning the Type Species of *Agrobacterium.* Int. J. Syst. Bacterial. 44: 373-374 (Letter to the Editor).

6. Bouzar, H., W. S. Chilton, X. Nesme, Y. Dessaux, V. Vaudequin, A. Petit, J. B. Jones and N. C. Hodge. 1995. A new *Agrobacterium* strain isolated from aerial tumors on *Ficus benjamina* L. AppL Environ. Microbiol. 61:65-73.

7. Bradbury, J. F. 1986. Guide to Plant Pathogenic Bacteria. Ferry Lane, Kew, Surrey, England. C.A.B International. 332 pp.

8. Brisbane, P. G. and A. Kerr. 1983. Selective media for three biovars of *Agrobacterium.* 1. Appl. Bacteriol. 54:425-431.

9. Burr, T. 1., 1. L. Norelli, B. H. Katz and A. L. Bishop. 1990. Use ofTi plasmid DNA probes for determining tumorigenicity of *Agrobacterium* strains. Appl, Env. Microbiol. 56: 1782-1785.

10. Canfield, M. L. and L. W. Moore. 1989. Unusual characteristics of *Agrobacterium tumefaciens* isolated from wild blackberry. Phytopathology 79: 1181. (Abstr.).

11. Chilton, M. D., R. K. Saiki, N. Yadav, M. P. Gordon, and F. Quetier. 1980. T-DNA from *Agrobacterium* is in the nuclear DNA fraction of crown gall tumor cells. Proc. Natl. Acad. Sci. USA 77:4060-4064.

12. Clark, A. G. 1969. A selective medium for the isolation of *Agrobacterium* species. 1. Appl. Bacterial. 32:348-351.

13. DeCleene, M. and 1. Deley. 1976. The host range of crown gall. Bot. Rev. 42:389466.

14. Haas, 1. H, L. W. Moore, W. Ream, and S. Manulis. 1995. Universal primers for detection of pathogenic *Agrobacterium* strains. Appl. Environ. Microbial. 6: 2879-2884.

15. Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum.* 1. Appl. Bacteriol. 27:265-277.

16. Hendrickson, A A, I. L. Baldwin, and A 1. Riker. 1934. Studies on certain physiological characters of *Phytomonas tumefaciens, Phytomonas rhizogenes* and *Bacillus radiobacter.* II. 1. Bacteriol. 28:597-618.

17. Hildebrand, D. C, 1. P. Thompson, and M. N. Schroth. 1966. Bacterial enhancement of self-limiting outgrowth formation on *Datura.* Phytopathology 56:365-366.

18. Holmes, B. and P. Roberts. 1981. The classification, identification and nomenclature of agrobacteria. J. AppL Bacteriol. 50:443-467.

19. Jones, 1. B. and B. C Raju. 1988. Systemic movement of *Agrobacterium tumefaciens* in symptomless stem tissue of *Chrysanthemum morifolium.* Plant Dis. 72:51-54.

20. Jouanin, L. 1984. Restriction map of an agropine-type Ri plasmid and its homologies with Ti plasmids. Plasmid. 12:91-102.

21. Kado, E. Land M. G. Heskett. 1970. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas,* and *Xanthomonas.* Phytopathology 60:969-976.

22. Kauffman, M., H. H. Kassemeyere, and L. Otten. 1996. Isolation of *Agrobacterium vitis* from grapevine propagating material by means ofPCR after immunocapture cultivation. Vitis, 35: 151-153.

23. Keane, P. 1., A. Kerr, and P. B. New. 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23:585-595.

24. Kennedy, B. W. 1980. Estimates of U.S. crop losses to procaryote plant pathogens. Plant Dis. 647:674-676.

25. Kerr, A. and C. G. Panagopoulos. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopath. Z. 90: 172-179.

26. Kersters, K. and 1. De Ley. 1975. Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. J. Gen. MicrobioL 87:333-342.

27. Kersters, K. and J. D. Ley. 1984. Genus III. *Agrobacterium* Conn 1942. Pages 244-254 in: N. R. Krieg and 1. G. Holt, ed., Bergey's Manual of Systematic Bacteriology, vol. 1. Williams & Wilkins Co., Baltimore,

28. Lehoczky.T. 1968. Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine after natural infection. Phytopath. Z. 63:239-246.

29. Lippincott, 1. A. and B. B. Lippincott. 1969. Tumor-initiation ability and nutrition in the genus *Agrobacterium.* J. Gen. Microbiol. 59:57-75.

30. Moore, L. W. and D. A. Cooksey. 1981. Biology of *Agrobacterium tumefaciens:* plant interactions. Pages 15-46 in: K. L. Giles and A. G. Atherly, eds., Biology of the Rhizobiaceae. Academic Press, New York.

31. New, P. B. and A. Kerr. 1971. A selective medium for *Agrobacterium radiobacter* Biotype 2. 1. Appl. Bacteriol. 34:233-23.

32. Ophel, K. and A. Kerr. 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. Int. J. Syst. BacterioL 40:236-241.

33. Otten, L., P. D. Ruffray, A. E. Momol, M. T. Momol, and T. 1. Burr. 1996. Phylogenetic relationships between *Agrobacterium vitis* isolates and their Ti plasmids.

Mol. Plant-Mirobe Interact. 9: 782-786.

34. Patel, M. K. 1926. An improved method of isolating *Pseudomonas tumefaciens.* (Smith and Towsend). Phytopathology 16:577.

35. Perry, K. L. and C. I. Kado. 1982, Characteristics ofTi plasmids from broad-hostrange and ecologically specific biotype 2 and 3 strains for *Agrobacterium tumefaciens.* 1. Bacteriol. 151:343-350.

36. Roy, M. and M. Sasser. 1983. A medium selective for *Agrobacterium tumefaciens* biotype 3. Phytopathology 73:810 (Abstr.).

37. Ruger, H. 1. and M. G. Hofle. 1992. Marine star-shaped aggregate-forming bacteria: *Agrobacterium atlanticum* sp. nov.; *Agrobacterium meteori* sp. nov.; *Agrobacterium ferrugineum* sp. nov., nom. rev.; *Agrobacterium gelantinovorum* sp. nov., nom. rev.; and *Agrobacterium stellulatum* sp. nov., nom. rev. Int. 1. Syst. Bacteriol. 42: 133143.

38. Sawada, H., H. Icki, H. Oyaizu, and S. Matumoto. 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes.* Int. 1. Syst. Bacteriol. 43:694-702.

39. Sawada, H., H. Ieki, and L Matsuda. 1995. rcn detection ofTi and Ri plasmdids from phytopathogenic *Agrobacterium* stains. Appl. Environ. Microbiol. 61: 828-831.

40. Schmulling, T., 1. Schell, and A. Spena. 1988. Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO 1. 7:2621-2629.

41. Schroth, M. N., 1. P. Thompson, and D. C. Hildebrand. 1965. Isolation of *Agrobacterium tumefaciens-A. radiobacter* group from soil. Phytopathology 55:645647.

42. Simmons, 1. S. 1926. A culture medium for differentiating organisms of typhoidcolon aerogenes groups and for islolation of certain fungi. 1. Infect. Dis. 39:209-214.

43. Tarbah, F. A and R. N. Goodman. 1986. Rapid detection of *Agrobacterium tumefaciens* in grapevine propagating material and the basis for an efficient indexing system. Plant Dis. 70:566-568.

44. Van Larebeke, N., C. Genetello, 1. Schell, R. A. Schil-eroort, A. K. Hermans, M. P. Hernalsteens, and M. Van Montagu. 1975. Acquisition of tumor-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature 255:742-743.

45. Willems, A and M. D. Collins. 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. Int. 1. Syst. Bacteriol. 43:305-13.

9. Chemicals List

Chemicals	Source

Source: Unless stated otherwise, all the chemicals in this list were obtained from Sigma Chemical Co., St. Louis, Mo.

Acetic acid, glacial
Adonitol agar
Anunonium nitrate
Anunonium phosphate
Ammonium sulfate
L(-) Arabitol
d-Biotin
Boric acid
Bromcresol purple
Bromthymol blue
Calcium carbonate
Calcium chloride Caseine,
acid hydrolysate
Chloroform
Chlorothalonil (Bravo 500) SDS Biotech Co., Painesville, OH
Crystal violet
Cupric sulfate
Cycloheximide
D-Cycloserine
EDT A (Ethylenediamine tetraacetic acid), disodium salt
Erythritol
Ethanol (ethyl alcohol)
Ethidium bromide
Ferric ammonium citrate
FerrousmnmoniumsuWme
Ferrous sulfate
Ferrous sulfate
Gelrite, Kelco Merck & Co., Inc., San Diego, CA
D-Glucose
L(-) Glutamic acid
Glycerol
Hydrochloric acid
L-Lactose
Lauryl sulfate, sodium salt Biorad, Richmond, CA

II. GRAM-NEGA TIVE BACTERIA

B. *Erwinia* and *Pantoea*

S. H. De Boer, D. L. COpUl\ and A. L. Jones

1. INTRODUCTION

The taxonomic position, nomenclature, and interrelationships of the members of the genus *Erwinia* have been the subject of diverse proposals. The broadest classification is that of Dye (5, 6, 7, 8), which separates *Erwinia* into the pectolytic soft rot *"carotovora"* group, the yellow pigmented *"herbicola"* group, the white nonpectolytic wilt-causing *"amylovora"* group, and the "atypical" *Erwinia.* These have turned out to be valid clusterings based on DNA-DNA homology studies and 16S sequence homology (15, 17), but do not completely agree with purely phenetic groupings (23). Controversy remains on whether or not the differences are great enough to constitute new genera. On the basis of 165 rRNA sequence homologies, Hauben et al. (15) recommended that these four groups be split into the genera *Pectobacterium* emend, *Pantoea*
gen. nov., *Erwinia* emend., and *Brenneria* gen. nov., respectively. In this manual, these groups are recognized as valid divisions of the species within the traditional *Erwinia* genus but, except for *Pantoea,* the recommended generic designations will not be adopted at this time. Further taxonomic studies on the relationship of the plant associated enterobacteria-like bacteria to those associated with humans and animals may require further changes in nomenclature.

The following species and subspecies are currently included in the pectolytic soft rot or *"carotovora"* group (p. 56): *Erwinia carotovora* subsp. *carotovora, E. carotovora* subsp. *atroseptica, E. carotovora* subsp. *betavasculorum* (22), *E. carotovora* subsp. *wasabiae* (14), *E. carotovora* subsp. *odorifera* (13), *E. chrysanthemi, E. cypripedii, E. rhapontici* (19) and *E. cacticida* (1). Sub specific division of *E. chrysanthemi* has been suggested. Although the heterogeneity of the species is now generally recognized, satisfactory descriptions of subspecies or pathovars have not yet been designated (2,3,4, 9, 10,22).

The "herbicola" group of yellow pigmented strains which consists of epiphytic as well as plant pathogenic bacteria., has now been classified as *Pan toea* (p. 73) together with some species of the genus *Enterobacter* (12, 16, 18). Initially, Ewing and Fife (11) recommended, on the basis of DNA-DNA homologies, that *Enterobacter agglomerans, E. herbicola* and *E. milletiae* be combined into the species *Enterobacter agglomerans* comb. nov. Subsequently Gavini et al. (12) proposed that *E. agglomerans* (including the type species of *E. herbicola* and *E. milletiae)* be transferred to a new genus *Pan toea,* which would consist of two species, *P. agglomerans* and *P. dispersa.* Later, Mergaert et al. (18) placed *E. ananas* and *E. stewartii* into *Pan toea* and deemed *E. uredovora* to be synonymous with *P. ananas.* Subsequently, three new 2, 5 diketo-D« gluconate producing bacteria isolated from fruit and soil were described as new *Pantoea* species:
P. citrea , P. punctata and *P. terrea* (16). The present genus *Pantoea* includes the phytopathogens *P. stewartii, P. ananas, P. citrea* and *P. agglomerans* pvs. *milletiae, gypsophilae* and *betae.*

The species in the "amylovora" (p. 40) group will be considered together with the atypical erwinias and will include *E. amylovora, E. persicina, E. pyrifoliae, E. mallotivora, E. psidii,* and *E. tracheiphila* along with *E. alni, E. nigrifluens, E. paradisiaca, E. quercina, E. rubrifaciens, and E. salicis* (24). The latter group comprises the species included in the proposed genus *Brenneria.* It should be noted that G+C ratios do not entirely support the division of the *Erwinia* species according to the presence or absence of pectolytic abilities, since they suggest a closer similarity between *E. amylovora* and *E. chrysanthemi* than between *E. amylovora* and the other species of the *"amylovora"* group (21) . Nevertheless, division of the *Erwinia* genus on the basis of phenotypic characteristics is useful for grouping together those species that require similar protocols for their manipulation within the laboratory.

Due to the heterogeneity of the genus, a general description of *Erwinia* is necessarily limited. As other members ofthe family Enterobacteriaceae, the erwinia occur as straight rods (0.5-1.0 X 1-3 zzm) singly, in pairs, or sometimes in short chains, are Gram negative and are motile by peritrichous flagella. They are facultatively anaerobic, chemoorganotrophic, and grow optimally at 23-30°C. **All** species are oxidase negative but catalase positive; nitrates are not reduced by most species. All species catabolize glucose and various other carbohydrates with the production of acid but usually without gas formation.

II. LITERA TURE CITED

1. Alcorn, S. M., T. V. Orum, A. G. Steigerwalt, 1. L. M. Foster, J. C. Fogleman, and D. J. Brenner. 1991. Taxonomy and pathogenicity of *Erwinia cacticida* subsp. nov. Int. 1. Syst. Bacteriol. 41:197-212.

2. Dickey, R. S. 1979. *Erwinia chrysanthemi:* A comparative study of phenotypic properties of strains from several hosts and other *Erwinia* species. Phytopathology 69:324-329.

3. Dickey, R. S. 1981. *Erwinia chrysanthemi:* reaction of eight plant species to strains from several hosts and to strains of other *Erwinia* species. Phytopathology 71: 23 -29.

4. Dickey, R. S., and 1. I. Victoria. 1980. Taxonomy and emended description of strains of *Erwinia* isolated from *Musa paradisiaca* Linnaeus. Int. 1. Syst. Bacteriol. 30: 129-134.

5. Dye, D. W. 1968. A taxonomic study ofthe genus *Erwinia.* I. The *amylovora* group. N. Z. 1. Sci. 11:590-607.

6. Dye, D. W. 1969. A taxonomic study of the genus *Erwinia.* II. The *carotovora* group. N. Z. 1. Sci.12:81-97.

7. Dye, D. W. 1969. A taxonomic study ofthe genus *Erwinia.* III. The *herbicola* group. N. Z. 1. Sci. 12:223-236

8. Dye, D. W. 1969. A taxonomic study of the genus *Erwinia.* IV. The 'atypical' erwinias, N. Z. J. Sci. 12:833-839

9. Dye, D. W. 1981. A numerical taxonomic study of the genus *Erwinia.* N. Z. 1. Agric. Res. 24:223-229.

10. Dye, D. W., J. F. Bradbury, M. Goto, A. C. Hayward, R. A. Lelliott, and M. N. Schroth. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list ofpathovar names and pathotype strains. Rev. Plant Pathol. 59: 153-168.

11. Ewing, W. H, and M. A. Fife. 1972. *Enterobacter agglomerans* (Beyerinck) comb. nov. (the *herbicola-lathyri* bacteria). Int. J. Syst. Bacteriol. 22:4-11.

12. Gavini, F., J. Mergaert, A. Beji, C. Mielcarek, D. Izard, K. Kersters, and J. De Ley. 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pan toea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. Int. 1. Syst. Bacteriol, 39:337-345.

13. Gallois, A., R. Samson, E. Agaron, and P. A. D. Grimont. 1992. *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory *(Chichorium intybus* L.). Int. J. Syst. Bacterial. 42:582-588.

14. Goto, M., and K. Matsumoto. 1987. *Erwinia carotovora* subsp. *wasabiae* subsp. nov. isolated from diseased rhizomes and fibrous roots of Japanese horseradish *(Eutrema wasabi* Maxim). Int. 1. Syst. Bacteriol. 37: 130-13 5.

15. Hauben, L., E. R. B. Moore, L. Vauterin, M. Steenackers, J. Mergaert, L. Verdonck, and J. Swings. 1998. Phylogenetic position of phytopathogens within the Enterobacteriaceae. System. Appl. Microbial. 21:384-397.

16. Kageyama., B., M. Nakae, S. Yagi, and T. Sonoyama. 1992. *Pantoea punctata* sp. nov., *Pantoea citrea* sp. nov., and *Pantoea terre a* sp. nov. isolated from fruit and soil samples. Int. 1. Syst. Bacterial. 42:203-210.

17. Kwon, S. W., S. J, Go, H. W. Kang, 1. C. Ryu, and 1. K. Jo. 1997. Phylogenetic analysis of *Erwinia* species based on 16S rRNA gene sequences. Int. 1. Syst. Bacteriol. 47: 10611067.

18. Mergaert, 1., L. Verdonck, and K. Kersters. 1993. Transfer of *Erwinia ananas* (synonym, *Erwinia uredovora)* and *Erwinia stewartii* to the genus *Pantoea* emend. as *Pantoea ananas* (Serrano 1928) comb. nov. and *Pantoea stewartii* (Smith 1898) comb. nov., respectively, and description of *Pan toea stewartii* subsp. *indologenes.* Int. 1. Syst. BacterioL 43: 162-173.

19. Sellwood, 1., and R A. Lelliott. 1978. Internal browning of hyacinth caused by *Erwinia rhapontici.* Plant Pathol. 27: 120-124.

20. Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (Eds.). 1980. Approved list of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.

21. Starr, M.P. and M. Mandel. 1969. DNA base composition and taxonomy of phytopathogenic and other enterobacteria. 1. Gen. Microbiol. 56: 113-123.

22. Thomson, S. v., D. C. Hildebrand, and M. N. Schroth. 1981. Identification and nutritional differentiation ofthe *Erwinia* sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi.* Phytopathology 71: 1037-1042.

23. Verdonck, L., 1. Mergaert, C. Ruckaert, 1. Swings, K. Kersters, and 1. De Ley. 1987. Genus *Erwinia:* Numerical analysis of phenotypic features. Int. 1. Syst. Bacteriol. 37A:4-18.

24. Young, J. M., Y. Takikawa, L. Gardan, and D. E. Stead. 1992. Changing concepts in the taxonomy of plant pathogenic bacteria. Annu. Rev. Phytopathol. 30:67-105.

II. GRAM-NEGA TIVE BACTERIA

B-l. *Erwinia amylovora* Group

A. L. Jones and K. Geider

1. INTRODUCTION

The non-soft rot group in the genus *Erwinia* corresponds to the "amylovora" group as proposed by Dye (6) and to a number of plant pathogenic species described in the last 10 to 15 years (10, 11, 15, 21, 30). The species of *Erwinia* included in this chapter include *E. alni* * (30), *E. amylovora, E. mallotivora, E. nigrifluens", E. paradisicina* (11), *E. persicinus* (10), *E. psidii (21), E. pyrifoliae* (15), *E. quercina*, E. rubrifacinens"; E. salicis*,* and *E. tracheiphila* .

Although most species in this group are found on different hosts, they occur primarily on trees causing various blights, cankers, leaf spots, wilts, and rots. *E. amylovora,* the fire blight pathogen, is not only the type species of the genus but it also the most important economically.

2. ISOLA TION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

Isolation of non-soft rot *Erwinia* species is often not difficult when above ground portions of plant material are visibly infected. The exception is *E. paradisiaca,* a pathogen on the roots of banana. Bacteria occur in high titer in newly infected tissues; titers decline and secondary microorganisms increase as lesions age. Small pieces of newly invaded water-soaked tissues from the leading edge of lesions on leaves, shoots, spurs, and fruits can be plated directly on suitable culture media. Alternatively, infected tissue is comminuted in sterile water and a small amount of suspension streaked or dilution plated on suitable culture media. After 3-4 days, bacteria consistently associated with infected tissues should be streaked 011 fresh media. Bacterial ooze can be streaked directly on the isolation medium. Some *Erwinia* exist as an epiphytic bacterium on flowers. Epiphytic bacteria are detected by washing blossoms in water and plating serial dilutions on culture media.

Either a standard medium or a semiselective medium can be used for the recovery of *Erwinia-like* bacteria. Standard media such as Luria-Bertani (LB) agar, King et al.'s medium B (KB), or nutrient agar are normally used when pathogen populations are expected to be high in relation to populations of secondary microorganisms. These media are normally supplemented with 50 to 100 ug/ml cycloheximide to inhibit fungal contaminants. Coalescing of colonies can be a problem when plating on KB or onto media with sucrose. Streaking is done in a pattern to obtain isolated colonies. Dilution of samples in water should be sufficient to obtain approximately 100 colonies on a standard agar plate. After incubation at 28°C for 2-3 days, individual colonies can be transferred to fresh plates. With experience, an investigator generally can distinguish colonies of a pathogen from miscellaneous saprophytic bacteria provided the numbers of contaminating bacteria are relatively few.

Semiselective media are used for the recovery of *Erwinia* from specimens that potentially harbor a significant mixture of attending saprophytic bacteria. Some media contain chemicals that inhibit the growth of certain bacterial species (selective), and some *Erwinia* species may form colonies on the media that can be differentiated from colonies of other bacteria by their characteristic color and colony formation.

a. Recipes for differential media:

1) Luria-Bertaini (LB) agar

	perL
Tryptone	10.0 g
NaCl	5.0 g
Yeast extract	5.0 g
Agar	15.0 g

2) Nutrient agar (NA)

	perL
Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

3) King et at's medium B (KB) (see d, p. 4)

4) Yeast extract - dextrose - $CaC0_3$ agar medium (YDC) (see b, p. 4)

This medium can be used for general isolation from plant tissues and is recommended for examination of pigmentation (Table 1).

A water soluble pink pigment is present around colonies of *E. rubrifaciens* on YDC medium (32).

b. Recipes for semiselective media:

1) CG medium (5)

	perL
Sucrose	160.0 g
Nutrient agar	2.0 g
Crystal violet	0.8 ml *
Cycloheximide	20.0 ml **
Distilled water	380.0 ml

* O. 1 % in absolute alcohol

* 0.1 % in 70% alcohol

*

Colonies of *E. amylovora* are distinguished by formation of small craters or depressions on the surface of the shiny, opaque colonies. Some strains may not produce craters, or the craters only exist for a short period of time.

2) CCT medium (14)

	perL
Sucrose	100.0 g
Sorbitol	10.0 g
Crystal violet	2.0 ml *
Nutrient agar	23.0 g
Tergitol anionic 7	30.0 ml **
Distilled water to	l.OL
After autoclaving add:	
Thallium nitrate (1% *w/v)* C	2.0 ml
yelo heximide	50.0 mg

* 0.1 % solution in absolute ethanol 1

** % aqueous solution

After 3 days, *E. amylovora* colonies are translucent, smooth, large (4-7 nun), tight blue with entire margins (Plate 2, Fig. 2). Blue striations radiating from the center of colonies are seen when viewed from the underside. Fluorescent *Pseudomonas syringae* pathovars are detected under UV light.

3) YIS medium (20)

	perL
Agar	20.0 g
Mannitol L-asparagine	10.0 g
Sodium taurocholate	3.0 g
$KzlIP0_4$	2.5 g
Nicotinic acid	2.0 g
$.\1gS0_4$	0.5 g
Nitrilotriacetic acid (NT A)	0.2 g
Sodium heptadecyl sulfate	0.5 g *
Bromthymol blue	0.1 ml **
Neutral red	4.0 mg ***
	10.0 mg ****

* Add 10 ml of2% aqueous solution neutralized with 0.73 g of potassium hydroxide per gram of NT A.

* Note: The Tergitol 7 is optional and can be eliminated, if not available. As 9

** ml of a 0.5% aqueous solution.

**** As 2.5 ml of a 0.5% solution.

Enterobacteriaceae produce red-orange colonies (Plate 2, Fig. 3); pseudomonads and other bacteria are distinguished by the bluish color oftheir colonies. Sorbitol is substituted for mannitol when isolating *E. amylovora. Pantoea agglomerans* (herbicola strains) cannot be distinguished from *E. amylovora* because of relatively similar colony morphology and COIOL

Erwinia quericina (13) also grows on MS medium and produces colonies of distinct color and morphology (28).

4) MM2Cu (3)

	perL
L-asparagine	4.0 g
$KzfIP0_4$	2.0 g
$MgS0_4$	0.2 g
NaCI	3.0 g
Nicotinic	0.2g
acid	0.2 g
Thiamine hydrochloride	10.0 g
Sorbitol	0.5 g
Cupric sulfate (2 mM)	15.0 g
Agar	

Erwinia amylovora forms yellow, highly mucoid colonies after 4 days at 28°C (Plate 2, Fig. 4); some strains are less mucoid. *Erwinia pyrifoltae* is also mucoid, but slightly yellow on the agar. Many other species will not grow on this medium. l\.1M2Cu medium should not be used for primary isolation and about 25 colonies are transferred to a MM2Cu agar plate for screening.

5) Modified EMU medium (27)

	perL
Glycerol	10.0 ml
NH_4S0_4	5.0 g
K_2HP0_4	2.0 g
Eosin Y	0.4 g
Methylene blue	65.0 mg
Agar	15.0 g

After autoclaving aseptically add the following antibiotics:

Cyclohexamide*	2.5 ml
Novobiocin	40.0 mg
Neomycin sulfate (680 ug/mg)	40.0 mg

~Use a stock solution (100 pg/ml of 70% ethanol)

Useful for isolating *Erwinia rubrifaciens* from soil. The bacterium forms visible colonies after 4 days at 27°C. After 6 days colonies are 1-2 mm in diameter, circular, with entire margins and a convex surface.

3. DIFFERENTIA TION OF COMMONLY ISOLATED SPECIES

Most species in the non-soft rot *Erwinia-group* are found on different hosts. Therefore, the identification of *Erwinia-like* bacteria from plant material is relatively easy provided unknown strains are tested in parallel with control strains of known identity from the same or closely related host plants (29).

The genus *Erwinia,* in the Enterobacteriaceae, was proposed by Winslow et aL (33) for Gram-negative plant-associated bacteria with the following characteristics: facultative anaerobes, peritrichous flagella, rod shaped, and acid produced from fructose, glucose, galactose, and sucrose. All species exhibit motility. Identification to species is based on the tests listed in Table 1.

4. DIAGNOSTIC MEDIA AND TESTS

a. Hypersensitive reaction (see e, p. 96)

Approximately 10^9 colony forming units (CFU)/ml water are injected into the intercellular space of a leaf of tobacco cv. "Burley" with a 25 gauge needle and syringe (for high bacterial densities, false positive reactions may occur). Complete collapse of the tissue after 24 h is recorded as positive. Since plant pathogenic bacteria elicit the hypersensitive response but saprophytic bacteria do not, a positive reaction indicates that the suspect bacterium should be identified further using other tests listed in this section.

Table 1. Major diagnostic tests differentiating species in the non-soft rot group of *Erwinia.* [a]

Test												
Tobacco hypersensitivity	[b]	+	+		ND	ND		+				ND
Pectate degradation"					+	+			+	+	+	
Growth factors required"		+	+		ND	ND		ND	+			+
Pink pigment on YDC					ND	+		ND		+		
Growth at 36°C	+D			+	NO	+						
Growth at 39°C					+							
H_2S from cysteine	+			+	+	+	+		+	+	+	+
Urease	+D			+								
Indole test					+							
Nitrate reduction					ND	+						
Gelatin liquification		+										
Acid production from";												
Salicin	+			+	+	+	+	NO	+		+	
Kvmethyl glucoside	ND			ND			+		+	+		
Melibiose				+	+	ND	ND	NO	ND	ND	NO	ND
Inositol				+	ND	ND	+	ND	ND		+	ND
L-arabinoise	+	v		+	+	+	+	+		+		ND

[a] Data taken from Hao et al. (10), Hauben ct al. (11), Neto et al. (21), Schroth and Hildebrand (28), Surico et al. (30).

[b] +,80% or more strains positive; +D, 80% or more strains delayed positive; -, 80% or more strains negative; ND, not determined; v, variable.

[c] Tests were made on Paton's media (22). Pitting after 3 days represents a positive test.

[d] All positive species required yeast extract to grow in basal media with glucose. E. *amylovora* requires nicotinic good growth in some minimal media.

[e] After 7 days growth at 2rC in unshaken aqueous solution of 1 % organic compound and 1 % peptone with purple as indicator.

b. Nitrate reduction

Nitrate broth

	per L
$KN0_3$	l.0 g
Peptone	5.0 g
Yeast extract	4.0 g
Oxoid ion agar No.2	3.0 g
pH 7.0-7.2	

1) Seed 4 m1 broth with inoculum and grow 24-48 hr.

2) Examine for foam, which indicates gas production.

3) Test cultures on various days. Add 1 m1 of a 0.6% (v/v) solution of N,Ndimethy1-1 naphthylamine (or 0.5% solution alpha naphthylamine) and 1 ml of a 0.8% (v/v) solution of sulfanilic acid. both in 5 N acetic acid (l part glacial acetic acid to 2.5 parts water), to each tube. A distinct pink or red color in the broth indicates the presence of nitrite and is considered positive. If within 1 hr a pink or red color does not appear, add zinc dust to the tube. The zinc dust will react with the nitrate, producing a pink color if present. This will confirm a negative test. However, if the tube does not tum pink then complete denitrification has occurred and the test is considered positive. Also test a sterile control tube kept under the same conditions to guard against errors due to absorption of nitrous acid from the air. CAUTION: The naphthy1amine reagents are carcinogenic - handle with care.

c. Gelatin liquefaction (6)

Add 120 g of gelatin (bacteriological grade) to 1 L water and adjust the pH to 7.0. Add 10 m1 to each tube, autoclave, and cool (do not slant) immediately. Inoculate by stabbing a loop with inoculum into the center of the medium, then incubate the tubes at 20-22°C or at room temperature. Observe whether rapid or slow liquefaction occurs. Also observe shape produced when the gelatin begins to liquefy.

d. Motility *tests*

Motility agar is prepared in tubes without slanting. Dissolve tryptone 10 g, sodium chloride 5 g, and agar 5 g in 1 L water and adjust pH to 6.8 - 7.0. Inoculate the sterile semi-solid medium by stabbing the center of the medium as above for gelatin. Incubate and observe after 8, 24, and 48 hr. Motility is shown by a diffused zone of growth spreading from the line of inoculation. Diffuse growth may extend throughout the entire medium or only from one or two points.

e. Urease production (most useful for *E. nigrijluens)*

Modified yeast salts (YS) broth

	per 800 ml
$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCI	5.0 g
Yeast extract	1.0 g
Cresol red	16.0 mg

After autoc1aving add:

Urea (stock solution)	200 m1 *

* Add 20.0 g to 180 m1 water and filter sterilize.

Disperse 5.0 m1 into sterile 25 em test tubes. These are inoculated and placed on an incubator shaker at approximately 28°C. Control tubes containing the basal medium without urea should also be included. An increase in alkalinity indicated by a magenta red color (pH approx. 9.0) is evidence of urease activity.

f. Acid production from carbohydrates

This should be observed from agar slants of medium C of Dye (6) [NH4HZP04, 0.5 g; KJ-IPO_4, 0.5 g; $MgSO_4G7H_zO$, 0.2 g; NaCI, 5 g; yeast extract (Difco), 1 g; agar, 12 g; add water, 1 L; bromcresol purple, 0.7 ml of 1.5% alcohol solution; pH 6.8; and heat sterilized. Add carbon source, 0.5% (v/v) aseptically from filter-sterilized, stock solutions. Because of the difficulty of dissolving and filter sterilizing some carbon sources, substances such as salicin should be prepared by dissolving in a large volume of water added to medium C, and sterilizing in steam on three successive days. Cultures should be examined for acid production after 2,4,6,21, and 28 days. A yellow color indicates production of acid.

g. Growth at 36^0 C

Observe growth in tubes ofliquid 523 medium (see 1), p. 27) or YS broth.

h. H_2S from cysteine

Cultures are shaker incubated in YS broth + cysteine hydrochloride, O. 1 gIL, or YS broth + peptone, 0.5 gIL for 3,6, and 14 days (6). Strips offilter paper moistened with a 10% solution of neutral lead acetate are held in place over the medium to maintain the lower end of the paper about 5 mm above the surface of the liquid medium. The

presence of H_2S is indicated by a blackening of the paper.

i. Peetate degradation

1) Hildebrand's medium (12).

A basal polypectate medium is adjusted to three pH levels. Ingredients for the basal medium are added in the following order: 1,000 ml distilled water (heated to near boiling); 1 ml bromthymol blue (1.5% alcoholic solution); 6 ml 10% $CaCl_z$- H_20 (freshly prepared) and 22 g of sodium polypectate. This mixture is stirred with a mechanical stirrer while being kept warm by a hot plate or other means. After the sodium polypectate is dissolved, add 100 ml of sterile 4% agar solution to each medium (A, B, C) and adjust the pH as follows:

Medium A. Adjust to pH 4.5-4.7 with 1 N Hel and autoclave.
Medium B. Adjust to pH 6.9-7.1 with 1 N Hel and autoclave.
Medium C. Autoclave and then adjust to pH 8.3-8.5 with sterile 1 N NaOH.

Petri dishes must be poured before the temperature falls below 70 ° C. The plates are stored at room temperature for several days until the surface of the gel is dry. Masses of bacteria (2-4 mm in diameter) are spotted using a sterile toothpick or transfer loop (four samples/plate) on the gel surface and the plates incubated for 1-6 days at 28°C before the occurrence of pitting is ascertained.

2). Paton's medium (22).

A 2% pectate gel is poured onto a calcium agar base (13). The agar base is composed of 1 L tap water plus 1 g K_ZHP0_4 or 1 g (NH4)2S04 or NH4-^{C1} added after 5 g $Cael_z$- H_20. The calcium agar base is poured into a Petri dish, allowed to solidify and an equal amount of the pectate solution poured on top.

j) Pigment on YDC (see b, p. 4).

k) Indole production (see i, p, 61).

5. PATHOGENICITY TESTS

All of the non-soft-rot *Erwinia* pathogens may be inoculated by injuring tissue, then applying inoculum by atomizing or injecting inoculum with a needle and syringe (28). A range of inoculum concentrations (10^4 - 109 CFU/ml) should be tested. Grow bacteria as described (see 5, p. 226). Care should be taken in evaluating results obtained with high inoculum concentrations *(109--10 CFU/ml);* high concentrations can result in false positives. Sterile water and known

pathogenic strains of the suspect pathogen or *are* included as controls. Re-isolations should be done to confirm the identity of the bacterium.

Erwinia amylovora may be inoculated with a needle by wounding succulent growing tissue laden with the test strain or by atomizing pomaceous flowers with a suspension of bacteria (23). Pathogenicity tests may also be done by inoculating slices of young, green pear or apple fruit (31) or by inoculating germinating apple or pear seedlings (25). The inoculated tissues are incubated in a moist chamber at 28 a C. Flowers appear water-soaked often with ooze droplets on the surface, then whole flowers tum brown to black, collapsing in 4 to 6 days. Tender shoots will produce typical fire blight symptoms, ooze, wilting, necrosis, and hooking at the tip, in 5 to 7 days. A positive test on fruit is evident by a browning around the wound site and oozing of bacteria in about 5 days. Seedlings inoculated with *E. amylovora* exhibit wilting, discolored and necrotic tissue, and copious amounts of ooze after 3 days. Other pathogens such as *Pseudomonas syringae* will produce necrotic symptoms on seedlings but not the discoloration and ooze production associated with *E. amylovora. Erwinia pyrifoliae* barely forms symptoms on apple plantlets and should be assayed on Nashi pear seedlings (24).

Erwinia quercina may be inoculated with a needle carrying the pathogen by wounding young acorns attached to the tree. An alternate, perhaps more effective, test is made by inoculating tender, young shoots of *Quercus agrifolia* Nee (California field oak, California live oak, coast live oak) or Q. *wislizenii* A. DC (interior live oak) (13) with a needle carrying the bacterium. Within 4 to 5 days, shoots wilt and a frothy ooze occurs at the point of inoculation.

The other species may be inoculated by using similar techniques. With *E. nigrifluens,* best results occur when the bacterium is injected into walnut bark with a needle or syringe during spring or summer. *E. rubrifaciens* is inoculated by inserting a carpet-needle tangentially along the cambium of a susceptible walnut host such as cv. Hartley and injecting 0.1 to 0.2 ml of inoculum containing 10^6 CFU/ml (27).

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular techniques

Although some highly specific molecular tests are available for the identification of several well-characterized species of *Erwinia,* classical biochemical tests as described in *Bergey's Manual* are still the standard in microbiology for identifying *Erwinia* spp.

Various DNA hybridization and polymerase chain reaction (Pf'R) techniques are available for the identification of *E. amylovora* but not the other non-soft rot *Erwinia* species (Table 4). Hybridization techniques based on DNA from pEA29 (7, 19) or the chromosome (9) can be used to identify *E. amylovora* colonies. The most widely used, fast, specific, and sensitive PCR method to identify colonies of *E. amylovora* and to detect *E. amylovora* in extracts from symptomatic and asymptomatic plants is based on the

amplification of a *PstI* fragment from universal plasmid pEA29 (4, 19). Use of nested PCR (19) can increase sensitivity of detection.

Several PCR primers based on chromosomal DNA can be used to supplement those based on plasmid pEA29 DNA. These include PCR assays based on primers derived from a 187 bp *HindIII* fragment from *E. amylovora* genomic DNA (8), from the chromosomal *ams* region (2), from the 235 rRNA gene (18), and from the 16S rRNA gene followed by restriction analysis (2). PCR methods involving chromosomal DNA may be slightly less sensitive than those involving pEA29. Pulsed-field gel electrophoresis of genomic DNA digested with various restriction enzymes may be useful, also (34).

Table 4. PCR primers for *Erwinia* spp.

S ecificitv	Primer designation	Sequence	Size	Reference
E. amylovora pEA29	A B	(5'-CGGTTTTIAACGCTGGG-3') (5'-GGGCAAA TACTCGGA TI -3')	1,000a	4
	AJ75 *AJ76*	(5'-CGTATICACGGCTICGCAGAT-3) (5'_ACCCGCCAGGATAGTCGCATA-3')	107	19
chromosomal *ams* region	AMSbL AMSbR	(S'.GCTACCAGCAGGGRGAG B3') (5'-TCATCACGATGGTGTAG B3')	1,635	2
	AJ24S[b] *AJ246*	(5'-AGCTGGCGGGCACTICACT - 3') (5'-CCCCGCACCGTICAGTTIT -3')	519	A. Jones, unpublished
chromosomal DNA		(5' -CCTGCATAAA TCACCGCTGACAGCTCAA TGB3') (5'.GCTACCACTGATCGCTCGAATCAAA TCGCCB3')	187	8
chromosomal 23S rDNA gene	EaF EaR	(5'_GCGCAGTAAAGGGTGACAGCCCCGTACACAAAAAGGCAT-3') (5'_CCCTAGCCGAAACAGTGCTCTACCCCCGG- 3')	565	18
E. pyrifoliae chromosomal *cps*	CPSIL CPS2Rc	(5' -CGCGGAAGTGGTGAGAA-3') (5' .GAACAGATGTGCCGAGTA-3')	1,200	Kim & Geider unpublished
16S rDNAIITS	Ep16A EpIG2c	(5' -AGATGCGGAAGTGCTICG-3') (5' -ACCGTIAAGGTGGAA TC-3')	700	
E. tracheiphila 16S rDNA	ETl E12	(5'-TGAGTICCCGACCAAAT-3') (5'-GGGAGGAAGGGACGCTG-3')	706	

[a] The size of the amplified fragment may differ slightly from strain to strain due to the number of 8-bp repeat sequences within the fragment.

[b] Can be combined in the same reaction with primers AJ75/ AJ76 to yield the two expected amplification products. These primers cross react with *E. pyrifoliae*

DNA sequence analysis of the 165 rDNA and the adjacent intergenic transcribed spacer (ITS) region can be used to confirm the identity of a plant pathogenic *Erwinia*

species (11, 15, 16). See Appendix A and C for details.

b. Serological techniques

Serological tests are sometimes used for the identification of *E. amylovora* in areas where the bacterium does not occur and reference strains cannot be maintained. Antisera prepared in response to a valid reference strain may be used in agglutination tests, doublediffusion tests, enzyme-linked immunosorbent assays, and immunofluorescence tests (29). Considerable care needs to be taken when utilizing this approach because polyclonal antibodies often cross-react with other bacteria (26) and monoclonal antibodies may be too specific to identify all strains of *E. amylovora* (17). See Appendix B for details.

c. Commercial automated techniques

1) Carbon source utilizations

An identification system consisting of a 96-well microtiter plate (Biolog GN Microplate, Biolog, Inc., Hayward, CA) tests for the ability of a bacterium to utilize 95 different carbon sources in the presence of an indicator dye. A disadvantage of this identification technique is that metabolic profiles are not available for all *Erwinia* species. See Appendix C for description and details.

7. CULTURE PRESERVATION

Cultures of most *Erwinia* species will remain viable for about 2 years on nutrient agar or LB agar slants covered with sterile mineral oil and stored at 5 -10°C. They can be stored at room temperature by stabbing heart infusion agar and covering with mineral oil (personal communication, N. W. Schaad). For long-term storage at -80°C, mix overnight cultures in LB with an equal amount of sterile LB containing 20% glycerol (final glycerol concentration 10%). Preservation can also be done by lyophilization.

8. LITEM TUBE CITED

1. Burton, B., U. Melcher, T. Zitter, S. Pair, J. Fletcher, and F. Mitchell. 1999. Polymerase chain reaction for detection of *Erwinia tracheiphila* in cucurbits. Phytopathology 89: S 10 . (Abstr.).

2. Bereswill, S., P. Bugert, L Bruchmuller, and K. Geider. 1995. Identification of the fire blight pathogen, *Erwinia amylovora,* by PCR assays with chromosomal DNA. App. Environ. Microbiol. 58:3522-3526.

3. Bereswill, S., S. Jock, P. Bellemann, and K. Geider. 1998. Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. Plant Dis. 82: 158-164.

4. BereswiU, S., A Pahl, P. Bellemann, W. Zeller, and K Geider, 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. App. Environ. Microbiol. 58:3522-3526.

5. Crosse, 1. E. and R. N. Goodman. 1973. A selective medium for and a definitive colony characteristic of *Erwinia amylovora.* Phytopathology 63: 1425-1426.

6. Dye, D. W. 1968. A taxonomic study ofthe genus *Erwinia.* 1. the *»amytovora'* group. New Zealand J. Sci. 11:590-607.

7. Falkenstein, H., P. BeUemann, S. Walter, W. Zener, and K Geider. 1988, Identification of *Erwinia amylovora,* the fire blight pathogen, by colony hybridization with DNA from plasmid pEA29. App. Environ. Microbiol. 54:2798-2802.

8. Guilford, P. J., R. K. Taylor, R. G. Clark, C. N. Hale, and R. L. S. Forster. 1996. PCRbased techniques for the detection of *Erwinia amylovora.* Acta Horticulturae 411 : 53 - 5^{6}.

9. Hale, C. N., R. G. Clark, andD. Harte. 1993. *ADNAapproachtoErwiniaamylovora* detection in large scale apple testing and in epidemiological studies. Acta Horticulturae 338:59-66.

10. Hao, M. v., D. J. Brenner, A G. Steigerwalt, Y. Kosako, and K. Komagata. 1990. *Erwinia persicinus,* a new species isolated from plants. Int. J. Syst. Bacteriol. 40~379-383.

11. Hauben, L., E. R. B. Moore, L. Vauterin, M. Steenackers, J. Mergaert, L. Verdonck, and J. Swings. 1998. Phylogenetic position of phytop atho gens within the *Enterobacteriaceae.* Syst. Appl. Microbiol. 21:384-397.

12. Hildebrand, D. C. 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens . Phytopathology 61: 1430-1436.

13. Hildebrand, D. C. and M. N. Schroth. 1967. A new species of *Erwinia* causing the drippy nut disease oflive oaks. Phytopathology 57:250-253.

14. Ishimaru, C. and E. J. Klos. 1984. New medium for detection of *Erwinia amylovora* and its use in epidemiological studies. Phytopathology 74: 1342-1345.

15. Kim, W.-S., L. Gardan, S.-L. Rhim, and K. Geider. 1999. *Erwiniapyrijoliae* sp. nov., a novel pathogen that affects Asian pear trees *tPyrus pyrif*^{olia} Nakai). Int. J. Syst. Bacteriol49:899-906.

16. Kwon, S.-W., 5.-J. Go, R-W. Kang, J.-c. Ryu, and l-K. Jo. 1997. Phylogenetic

analysis of *Erwinia* species based on 16S rRNA gene sequences. Int. 1. Syst. Bacteriol. 47: 1061-1067.

17. Lin, C. P., T. A. Chen, 1. M. Wells, and T. van der Zwet. 1987. Identification and detection of *Erwinia amylovora* with monoclonal antibodies. Phytopathology 77:376380.

18. Maes, M., P. Garbeva, and C. Crepel. 1997. Identification and sensitive endophytic detection of the fire blight pathogen *Erwinia amylovora* with 23S ribosomal DNA sequences and the polymerase chain reaction. Plant Pathology 54: 1139-1149.

19. McManus, P. S. and A. L. Jones. 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. Phytopathology 85:618-623.

20. Miller, T. D. and M. D. Schroth. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology. 62: 1175-1182.

21. Neto, J. R., C. F. Robbs, and T. Yamashiro. 1987. A bacterial disease of Guava *(Psidium guajava)* caused by *Erwinia psidii* sp. nov. Fitopatol. Bras. 12:345-350.

22. Paton, A. M. 1958. Pectin-decomposing strains of *Pseudomonas.* Nature 181:61-62.

23. Pusey, P. L. 1997. Crab apple blossoms as a model for research on biological control of fire blight Phytopathology 87: 1096-1102.

24. Rhim, S.-L., B. Volksch L. Gardan, J.-P. Paulin, C. Langlotz, W.-S. Kim, and K. Geider. *1999. Erwinia pyrifoliae,* an *Erwinia* species, different from *Erwinia amylovora,* causes a necrotic disease of Asian pear trees. Plant Pathol. 48:514-520.

25. Ritchie, D. F. and E. 1. KIos. 1974. A laboratory method of testing pathogenicity of suspected *Erwinia amylovora* isolates. Plant Dis. Rep 58: 181-183.

26. Roberts, P. 1980. Problems encountered during immunofluorescence diagnosis offire blight. Plant Path. 29:93-97.

27. Schaad, N. W. and E. E. Wilson. 1970. Survival of *Erwinia rubrifaciens* in soil. Phytopath. 60:557-558.

28. Schroth, M. N. and D. C. Hildebrand. 1994. *Erwinia amylovora* or non-soft rot group. Pages 37- 43 in: N. W. Schaad, ed., Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd Edition. American Phytopathological Society, St. Paul, MN.

29. Slade, M. B. and A. I. Tiffin. 1984. Biochemical and serological characterization of

Erwinia. Methods in Microbiology 15 :228-293.

30. Surico, G., L. Mugnai, R. Pastorelli, L. Giovannetti, and D. E. Stead. 1996. *Erwinia alni,* a new species causing bark cankers of alder *(Alnus* Miller) species. Int. 1. Syst. Bacteriol. 46:720-726.

3 L Vanneste,1. L., J. Yu, G. E. Harper, and 1. H. Perry. 1996. Plugs of immature pear fruit to assess the virulence of *Erwinia amylovora* and to study in the laboratory the interaction between biological control agents and the fire blight pathogen. Acta Horticulturae. 411:303-305.

32. Wilson, E. E., F. M. Zeitoun, and D. L. Fredrickson. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. Phytopathology 57:618-621.

33. Winslow, E. E. A., 1. Broadhurst, R. E. Buchanan, C. Krumwiede, n., L. A. Rogers, and G. H. Smith. 1920. The families and genera of the bacteria. Final report of the Committee of the Society of American Bacteriologists on characterization and classification of bacterial types. 1. Bacteriol, 5: 191-229.

34. Zhang, Y. and K. Geider, 1997. Differentiation of *Erwinia amylovora* strains by pulsedfield gel electrophoresis. Appl. Environ. Microbiol. 63:4421-4426.

9. CHEMICAL LIST

Chemical	**Source**
Unless stated otherwise, all chemicals in this list can be obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.	
Acetic acid	
Agar	
L-arabinose	Difco
L-asparagine	
Beef extract	Difco
Bromcresol purple	
Brornthyrnol blue	
Cobalt chloride	
Crystal violet	
Cresol red	
Cupric sulfate	
Cycloheximide L-cysteine hydrochloride	
Eosin Y	
Ethanol	

Gelatin	Difco
Glycerol	
Inositol	
HCI	
K_2HP0_4	
$KN0_3$	
KOH	
Mannitol	
$MgS0_4$	
$MgS0_4117H_20$	
Melbiose	
Methylene blue	
0< Methyl glucoside	
Mineral oil	
NH_4CI	
$NH_4H_2{}^P04$	
$(NH_4)2^S{}_{04}$	
NaCl	
NaOH	
Neomycin sulfate	
Neutral red	
Nicotinic acid	
Nitrilotriacetic acid N,N-dimethyl-l-naphthylamine	
Novobiocin	
Nutrient agar	
Nutrient broth	Difco
Peptone Salicin	Difco
Sodium heptadecyl sulfate (Terigitol 7)	
Sodium polypectate	Source presently unknown
Sodium taurocholate	Dr. M. Burger, Eton Ridge Road, Madison, WI. USA
Sorbitol	
Sucrose	
Tergitol anion 7 (sodium heptadecyl sulfate)	Union Carbide
Thallium nitrate	K & K Rare Chemicals
Thiamine hydrochloride	
Tryptone	
Urea	Difco
Yeast extract	Difco

II. GRAM-NEGATIVE BACTERIA

B~2 *Erwinia* Soft Rot Group

S. H. De Boer and A. Kelman

1. INTRODUCTION

The proposal presented 54 years ago by Waldee (58) that the soft-rot *Erwinia* species be placed in a separate genus, *Pectobacterium,* has not been generally accepted. Other similar recommendations to place the pectolytic *Erwinia* into a separate genus have also lacked support (13, 32,35). However, the soft rot or *"carotovora"* group comprises those species which incite soft rot diseases of plants and hence form a logical group from a phytopathological perspective.

In this manual, the following designations are used for the *"carotovora"* group: *Erwinia carotovora* subsp. *carotovora, E. carotovora* subsp. *atroseptica, E. carotovora* subsp. *betavasculorum, E. carotovora* subsp. *wasabiae* (19), *E. carotovora* subsp. *odorifera* (17), *E. chrysanthemi, E. cypripedii, E. rhapontici* (13, 14,48,50, 55) and *E. cacticida* (1). Only the general characteristics for *E. chrysanthemi* are included in this manual, although pathovars have been designated (13, 14) and described in several references (8, 9, 10,55). Even thoughE. *cypripedii* and *E. rhapontici* are non-pectolytic, Dye (12) considered them to be related to the pectolytic species and until further studies show otherwise, they are treated as members of this group.

Phenotypic characteristics of species in the genus *Erwinia* are presented by Starr (53) and Starr and Chatterjee (54), and more specifically for *E. chrysanthemi* by Dickey (8, 9). Ecology of the soft rot *Erwinia* was reviewed by Perombelon and Kelman (41) and by Stanghellini (52). The status of research on the potato blackleg disease is summarized in the Report on the International Conference on potato Blackleg Disease (21). Details of methods generally applicable for identification of bacteria in the family, *Enterobacteriaceae* are given in Gerhardt (18). Procedures for specific identification of soft-rot bacteria were outlined by Cother and Sivasitharnpararn (3) and a manual on methods for the detection and quantification of *E. carotovora* subsp. *atroseptica* on potatoes was published by Perombelon and van der Wolf (42).

2. ISOLATION TECHNIQUES USING SEMlSELECTIVE MEDIA

Standard procedures for isolating phytopathogenic bacteria from plant and soil samples can be used for the pectolytic *Erwinia* (31). Detailed procedures for isolation of soft rot bacteria are found in Kelman and Dickey (30). Isolation from decayed plant tissue and soil is facilitated by the use of selective and enrichment media (2, 4,28, 34, 37, 39, 40, 44, 56).

To isolate *E. chrysanthemi* and the subspecies of *E. carotovora,* homogenize a small piece of tissue from the periphery of the decay lesion in sterile water, and streak or spread

appropriate dilutions on a selective medium. The selective medium and temperature of incubation may affect the efficiency of recovery and growth of specific strains (39). Perhaps one of the most effective and easily prepared selective media is crystal violet pectate medium (CVP, 4). Isolation plates should be incubated at 23-27°C. On CVP the soft-rot *Erwinia* species form iridescent, cross-hatched, translucent colonies in deep, cup-like depressions or pits, whereas pectolytic *Pseudomonas* spp. form shallow pits (Plate 2, Fig. 5). Colonies are most clearly observed under a low powered dissecting microscope illuminated from below with oblique illumination. Pure cultures can often be obtained by streaking directly from the deep pits in CVP onto CPG medium (29).

To isolate pectolytic bacteria from environmental samples, prior enrichment of the sample in a liquid enrichment medium greatly enhances isolation of low populations of the bacteria. Samples are incubated in enrichment medium (34) for 3-5 days at *23-2rC* before they are streaked onto CVP.

Selective media for isolating the other members of this group, *E. cacticida, E. cypripedii,* and *E. rhapontici* have not been developed. However, these bacteria can be isolated on non-selective media such as nutrient agar or CPG medium.

a. CVP medium (4)

	per 500 ml water
1 NNaOH	4.5 ml
10% $CaCl_z'2H_zO$	3.0ml
NaNO}	1.0
Agar	g
Sodium polypectate	1.5g
SDS	10.0 g 0.5 ml
0.075% crystal violet	1.0
	ml

Pour 300 ml of boiling distilled water into a preheated Waring Blender jar and then add the NaOH, $CaCl_z'2H_zO$, $NaN0_3$, and agar. Blend at high speed for 15 sec. while slowly adding the sodium polypectate (some sources of sodium pectate do not work for this medium). Blend for another 15 sec while adding another 200 ml of boiling distilled water. Pour medium in a 2 litre flask and add the SDS and crystal violet.

Swirl contents, cap flask with aluminum foil and autoclave for 25 min at 120°C. Pour plates while medium is hot and ventilate plates while cooling to prevent condensation.

b. CPG medium (29)

	perL
Casamino acids	1.0 g
Peptone Glucose	10.0 g
Agar	10.0 g
	18.0 g

c. Polypectate enrichment medium (34)

	per L
Sodium polypectate	1.5 g
10% (NH~)2S0~	10.0 ml
10% $KJIP0_4$	10.0 ml
5% MgSO~'7H20	5.0 ml

3. DIFFERENTIA nON OF COMMONLY ISOLATED SPECIES AND SUBSPECIES

The soft rot *Erwinia*, like other members of the *Enterobacteriaceae* are facultatively anaerobic, peritrichously flagellated, Gram-negative rods. All strains of the five species considered in this chapter are catalase positive, oxidase negative, ferment glucose, reduce nitrate, produce p-galactosidase and H_2S, and utilize L-arabinose, D-galactose, D-glucose, glycerol, D-mannose, D-ribose, and sucrose, but do not produce urease, or acid from adonitol. Most strains utilize L-rhamnose and D-mannitol but not dextrin. Holt et al. (23) have noted in Bergey's Manual that a meaningful comparison of *Erwinia* with other genera of *Enterobacteriaceae* is still needed because erwiniae have not been fully tested with the complete range of standard tests used for other enterobacteria.

For the pectolytic *Erwinia* presumptive identification can be made simply on the basis of pectolytic activity and colony characteristics on CVP medium observed with oblique illumination. For CPG medium, addition of 0.005% 2,3,5 triphenyl tetrazolium chloride to the medium enhances the differences in colony types and makes presumptive identification easier (30). Further identification to genus can be made on the basis of Gram negative stain reaction, rod shape, facultative anaerobic metabolism, and peritrichous flagellation. F or the nonpectolytic species, *E. cypripedii* and *E. rhapontici*, further tests to establish species identity is required to ensure correct genus designation. Hyman et al. (25) suggest that pectolytic bacteria can be confirmed as *Erwinia* on the basis of being facultatively anaerobic, catalase positive and oxidase negative.

All the pectolytic *Erwinia* species and subspecies can be differentiated from one another on the basis ofa limited number of tests (Table 1). Although there are several different ways in which many of these tests can be completed, only one procedure is described for each test. The methods have been selected as the most convenient and simplest for testing a number of isolates at one time. It is essential that positive and negative control strains be included in each test.

Table 1. Biochemical and physiological tests that differentiate pectolytic species and subspecies of the *Erwinia* soft rot group"

Test	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ect
Growth at *3r* C	-I-		-I-	-I-		-I-	-I-
Reducing sugars from sucrose		-I-	-I-	+			
Phosphatase activity	b					+	V
Sensitivity to erythromycin				ND		+	
Indole production						+	
Acid produced from :							
sorbitol				-I-	ND		
melibiose	+	+		+		+	
citrate	+	+		+	+	+	ND
raffinose	+	-I-		ND		+	
arabitol				+	ND		ND
lactose	+	+	+	+	-I-	-I-	
Utilization of keto-methyl glucoside		-I-	+	-I-			

+,80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

з Ecc = *E. carotovora* subsp. *carotovora*
Eca = *E. carotovora* subsp. *atroseptica*
Ecb = *E. carotovora* subsp. *betavasculorum*
Eco = *E. carotovora* subsp. *odorifera*
Ecw = *E. carotovora* subsp. *wasabiae*
Ech = *E. chrysanthemi*
Eet = *E. cacticida*

b Some strains of *E. carotovora* subsp. *carotovora* may be weakly positive

4. DIAGNOSTIC MEDIA AND TESTS

u. Fermentation of glucose

The Hugh and Leifson (24) O-F medium is used to differentiate fermentative from oxidative metabolism of carbohydrates (see 3, p. 9)

b. Flagellar stain

The procedure ofRyu (45) or the silver stain (see 10 a, p. 10) is very effective in staining flagella of *Erwinia carotovora.*

c. Catalase test

Mix a loopful of a 48-h-old culture grown on nutrient agar with a drop of freshly prepared 3% H_20_2 on a glass slide. The formation of gas bubbles indicates a positive reaction.

d. Oxidase test (see 7, p. 10)

Use a platinum loop to transfer bacteria from a 48-h-old culture grown on nutrient agar onto filter paper impregnated with a drop of 1 % aqueous tetramethyl-p-phenylenediamine dihydrochloride solution. Development of a purple color within 10 sec indicates a positive reaction.

e. Growth at 37°C

Use CPG or nutrient agar medium at 15 ml per plastic Petri dish. Stab inoculate isolates from a dense aqueous suspension in a grid pattern. Place plates in a plastic bag to prevent drying out of the medium, and incubate at *3rc* for three days. Observe inoculation point for bacterial growth. The presence of a bacterial colony at the point of inoculation is considered a positive test.

f. Reducing substances from sucrose

Dissolve 10 g Bacto-peptone and 17 g agar in 800 ml distilled water, autoclave, and add 200 ml of a filter sterilized sucrose solution (40 g sucrose in 200 ml distilled water) and pour into Petri dishes. Stab incubate isolates from a dense aqueous suspension in a grid pattern and incubate for two days at 23 - *27°C.* Then flood each plate with 10 ml Benedict's solution and incubate 30 - 45 min at 60°C. A positive test is an orange zone around the colony against a blue background.

To prepare Benedict's solution add 35 g sodium citrate and 20 g N~C03'H20 to 160 ml distilled water and heat to dissolve. Separately dissolve 3.5 g of $CuS0_4'5H_20$ in 40 ml distilled water. Combine and mix the two solutions. The mixture is stable for several months at room temperature.

g. Phosphatase activity (23)

Prepare nutrient agar and when partially cooled after autoclaving add a filter-

sterilized solution of phenolphthalein diphosphate sodium salt to give a final concentration of 0.05% *(w/v),* mix, and pour plates. Stab inoculate isolates from a dense aqueous suspension in a grid pattern and incubate for 48 h at 23-27°C. Then place a drop (ca. 0.1 ml) of concentrated ammonia in the lid of the Petri dish and invert the medium over it. Colonies that have phosphatase activity turn a bright pink color almost immediately. Use glass Petri dishes for this assay because ammonia softens plastic dishes.

h. Sensitivity to erythromycin

Seed a «lawn" of bacteria on nutrient agar plates by pouring 6 ml of molten nutrient agar cooled to 50 $_{o}$C and inoculated with 1 00 ~l of a dense aqueous suspension of the test bacterium over the base layer of medium in the plate. After the medium has solidified place a commercially prepared disk impregnated with 15 !lg of erythromycin per disk on the surface ofthe medium. Incubate at 23-2rC for 48 h. A zone of inhibition around the disk is recorded as a positive test for sensitivity.

I. Indole production (36)

Prepare medium containing 109 tryptone, 1 g L-tryptophan, and 15 g agar per liter. Stab inoculate isolates from a dense aqueous suspension in a grid pattern and incubate for 48 hat 23-27°C. When ready for testing, pipet two or three drops of pdimethylamino-cinnamaldehyde (1 g in 100 ml of 10% hydrochloric acid) on filter paper in a Petri dish. Place a loopful of bacteria from a colony on the treated filter paper. A blue-green coloration of the treated area within 10 sec is a positive test. A negative reaction is denoted by a colorless or light pink coloration of the treated area.

j. Acid from carbohydrates (20)

Prepare 1% peptone broth and use bromcresol purple (10-30 mgIL) as an indicator. Add the carbohydrate to be tested to give a final concentration of 1 %, adjust pH to 7.0, filter sterilize, and dispense into sterile test tubes. Inoculate tubes with a loopful of cells from a 48-h-old culture and incubate at 23-27°C for 48 h. A color change to yellow indicates acid production (Plate 2, Fig. 6).

k, Utilization of keto-methyl glucoside (methyl-v-d-glueeside) (42)

	perL
KH_2P0_4	2.0 g
K_2HP0_4	7.0 g
NH_4C1	1.0 g
10% $MgS0_4'7H_20$	2.0 ml
Casamino acid	1.0 g
Agar	15.0 g

After autoclaving cool to 50 °C and immediately add 50 ml of filter sterilized 20% keto-methyl glucoside, and 2 ml of filter sterilized 1 % tetrazolium chloride, and pour into Petri dishes. Stab inoculate isolates from a dense aqueous suspension in a grid pattern and incubate for 48 hat *23-2rc. E. carotovora* subsp. *atroseptica* grows well and produces colonies with red centers (Plate 2, Fig. 7). *E. carotovora* subsp. *carotovora* grows poorly and colonies are white although a little red color may develop at the point where the inoculum was stabbed into the medium.

5. PA THOGENICITY TESTS

3. Tissue maceration

The ability of a bacterium to macerate plant tissue confirms its pectolytic nature and provides an indication of pathogenicity. Maceration ability, however, does not prove pathogenicity of the bacterium in a natural environment. False positive results may be due to naturally occurring endophytic or epiphytic microorganisms associated with inoculated tissue.

Various plant tissues such as potato tubers (Plate 3, Fig. 1), peppers or celery stalks can be used to test maceration ability. Disinfect the surface of the tissue by immersing in a 10% household bleach (5.25% sodium hypochlorite), solution for 10 min and air-dry. Repeat bleach treatment or alcohol flame. Cut tissue into convenient size pieces, place in a Petri dish on moist sterile filter paper, and inoculate with 0.1 - 1 ml of the bacterial suspension (ca. 10^6 CFU/ml) from a 24-h-old-culture. Incubate at 20-27°C for 48 h and probe the tissue surrounding the inoculation site with a spatula or needle to determine whether decay and tissue maceration has occurred.

b. Pathogenicity tests

The most precise method for determination of pathogenicity involves the use of infectivity titrations (15). Prepare bacteria as described (see 5, p. 226). Inoculate host plants using a range ofseriall0-fold dilutions from 10^2 - 10^8 *CFU/ml* of the test bacterium using a micropipette tip or syringe (33). At least 8 to 10 plants per inoculum level should be used for each of four to five dilutions. Seal the injection point after inoculation by application of a small amount of petroleum jelly over the puncture. Infection of inoculated plants will be enhanced if they are maintained at a high relative humidity or even placed under an intermittent misting system.

F or additional details on other inoculation procedures, reference should be made to studies on host range of *E. chrysanthemi* (9) and tests with *E. rhapontici (48).*

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular techniques

1) Polymerase Chain Reaction

Three sets of specific primers exist for the identification of *E. carotovora* subsp. *atroseptica* and two sets for *E. chrysanthemi* (Table 2). Primers, ECAlr and ECAlf for *E. carotovora* subsp. *atroseptica* were constructed by De Boer and Ward (7), primers ERWFOR and ATROREV were designed by Smid et aI. (51) and primers, Y45 and Y46 by Frechon et al. (16). Smid et al. (51) also designed a primer set for *E. chrysanthemi*, ERWFOR and CHRREV while another set of primers for *E. chrysanthemi,* ADEI and ADE2 was designed by Nassar et al. (38). See Appendix A for details.

Table 2. Sequence of oligonucleotide primer pairs currently in use in PCR analysis of *Erwinia* species.

Target	Name	Primer Sequence	Product size	Ref
Eca	ERWFOR	5'-ACGCATGAAA TCGGCCATGC-3'	389	51
	ATROREV	5'-A TCGA TA *TTTGA* nGTC~3'		
Eca	Y45	5'-TCACCGGACGCCGAACTGTGGCGT-3'	438	16
	Y46	5'-TCGCCAACGTICAGCAGAACAAGT-3'		
Eca	ECAlf	5'-CGGCA TCA T AAAAACACG-3'	690	7
	ECA2r	5'-GCACACTTCATCCAGCGA-3'		
Ech	ADEI	5'-GATCAGAAAGCCCGCAGCCAGAT-3'	420	38
	ADE2	5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3'		
Ech	ERWFOR	S'-ACGCA TGAAA TCGGCCA TGC-3'	450	51
	CHRREV	S'-AGTGCTGCCGTACAGCACGT _3'		

b. Serological techniques

Serological procedures are useful for preliminary identification of purified cultures or bacteria in plant and soil samples if specific antibodies to commonly occurring serogroups are available. Standard indirect immunofluorescence and triple antibody sandwich ELISA protocols can be used for strain identification if it is known that the antibody reacts according to serogroup specificity in the particular procedure being used (22).

Lipopolysaccharides are important antigenic determinants of most Gram negative bacteria including the pectolytic erwiniae and antibodies to them can be used to differentiate among strains (5,47). The usefulness of serology for identification of pectolytic *Erwinia* to species and subspecies is limited, however. Although antibodies to lipopolysaccharides of *E. carotovora* subsp. *carotovora* and *atroseptica* are highly specific (5) the existence of many *E. carotovora* subsp. *carotovora* serogroups makes identification of this subspecies by serology impractical. Most *E. carotovora* subsp, *atroseptica* strains belong to sera group I and several serogroup-specific monoclonal antibodies are useful for identification of the subspecies (6, 27). However, at least three other serogroups (XVIII, XX, and XXII) occur among *E. carotovora* subsp. *atroseptica* strains, and although they occur infrequently, it limits serological identification of all strains in the subspecies. Serology of the other *E. carotovora* subspecies has not been studied.

It is estimated that about one third of the *E. carotovora* strains isolated from potato can be placed in one of forty serogroups described to date. Some strains from other hosts also belong to these serogroups but the serological diversity of strains from non-potato hosts is unknown. Homologous strains representing the four *E. carotovora* subsp. *atroseptica* serogroups (ICMP 9167, 9184, 9186, and 9188) and 36 *E. carotovora* subsp. *carotovora* serogroups (leMP 9168-9206) are in the International Collection of Micro-organisms from Plants, Landcare Research, Auckland, New Zealand. Polyclonal antisera produced against glutaraldehyde-fixed cells of homologous strains normally contain a high titre of antibodies to the lipopolysaccharide antigen and are useful for serotyping. Strains of *E. carotovora* can be placed into serogroups by the Ouchterlony double diffusion test (5). The test requires a dense aqueous suspension of a pure culture, which after treatment with phenol (~20 *,uVrnl),* is placed in a 3 rom well cut into an agar double diffusion plate (15 ml of 0.8% agar, 0.85% NaCl, 200 ppm NaN_3 in a 100 x 15 rom plastic Petri dish). An adjacent well is loaded in the same way with the phenol-treated cells of the homologous strain of the serogroup being tested. Serogroup-specific antibody is placed in a well equidistant (4 rom) from the two sample wells. Precipitin bands that form in 12-24 h between the sample and antibody wells indicate a serological reaction, but the serogroup can only be identified to that of the homologous strain and antibody used if the band formed by the test strain fuses with that fanned by the homologous strain.

Serological techniques using "0" antigens (11, 46, 47), purified membrane protein complex (59), or fimbrial-specific monoclonal antibodies (49) are very useful for rapid presumptive identification of suspected cultures of *E. chrysanthemi.* Several serological typing schemes of three or four serovars or serogroups have been suggested but these have not been reconciled with one another or adopted for further studies (11, 27). Polyclonal and monoclonal antibodies produced to the lipopolysaccharide of *E. chrysanthemi* do react with many strains of the species but their use for identification and detection is greatly hampered by their cross-reactivity with a lipopolysaccharide epitope of some common soil pseudomonads (57). A monoclonal antibody to a fimbrial epitope of *E. chrysanthemi* has potential for identification and perhaps detection of strains isolated from potato and some other hosts in Europe (49).

c. Commercial automated techniques

1. Carbon source utilizations

The commercially available Biolog system (Biolog, Inc., Hayward, CA) for substrate utilization is helpful for rapid identification of those species and subspecies included in the database. These currently include: *E. carotovora* subsp. *atroseptica,* E. *carotovora* subsp. *betavasculorum,* E. *carotovora* subsp. *carotovora, E. chrysanthemi,* and *E. cypripedii.* See Appendix C for description and details.

2) 168 rRNA gene sequencing

The MicroSeq Microbial Identification system is produced by PE Applied Biosystems. Identification service using the MicroSeq 16S rRNA Gene Kit is provided by:MIDI Labs (Newark, DE 19713). The commercial database currently contains 16S rRNA sequences for *E. carotovora* subsp. *betavasculorum, E. carotovora* subsp. *carotovora, E. cypripedii,* and *E. rhapontici* of the soft rot erwiniae group.

7. CULTURE PRESERVATION

Members of the soft rot *Erwinia* group vary considerably in viability when stored under various conditions. Several methods are available for the storage of *Erwinia* cultures. These include agar plate cultures, water cultures, glycerol cultures and lyophilized cultures. The method of choice depends on the duration of storage, the species or subspecies involved and the available equipment and/or cost.

Agar plate or slant cultures are a common way of storing soft rot *Erwinia* bacteria for a short time. Cultures can be stored at 4 DC for, at least for one month, on YDC agar slants (see b, p. 4). Grow strain for 1-2 days at 23-2rC, then refrigerate.

Cell suspensions of some soft rot *Erwinia* also preserve well in water at room temperature. However, some species, notably *E. chrysanthemi,* lose viability almost immediately when stored in water. Grow the culture on an appropriate agar medium and use a loop to scrape the cells into a screw-capped vial filled with sterile water. Cover the vial, label and store at room temperature.

Bacterial cell suspensions can also be stored at -70°C in Luria-Bertani (LB) (see 1, p. 41) - glycerol broth (LB broth containing 20% glycerol, mixed after autoclaving). Cultures are grown as described above for water cultures and cells are resuspended in the LB-glycerol medium in cryovials. These cells will remain viable for a very long time. When reviving a glycerol culture, it is advisable not to let the frozen culture thaw before streaking out. It is best to simply remove samples of frozen cells with a loop and streak onto the medium. A viable culture should start growing during overnight incubation. This method of culture preservation has the advantage that culture revival is quick but requires a -70°C freezer. A commercial product, "Protect", consisting of medium and beads in cryovials is also available as a bacterial preservation system (Protect, STC Technical Service Consultants Ltd, Heywood Lanes, UK). Bacterial cells are suspended in the medium, mixed with the beads in the vial and then excess medium removed. The vials are stored at -70°C and bacteria are recovered by removing individual beads which are streaked directly on a culture medium.

An alternate method for long-term culture preservation is lyophilization. Cells are suspended in 10% skim milk and freeze dried under vacuum. Prepare 10% skim milk, autoclave, and aliquot at about 400 III per sterile tube. Suspend bacterial cells from an overnight agar plate culture in the milk, vortex and use a sterile Pasteur pipette to transfer about 100 III of the suspension into a sterile lyophilization tube. Cap each tube with a cotton plug and freeze overnight. Use a freeze-drying machine to lyophilize the cultures and then seal the vials by melting the top with a torch. Lyophilized cultures can be stored at room temperature for many years.

8. LITERA TURE CITED

1. Alcorn, S. M., 1. V. arum, A. G. Steigerwalt, J. L. M. Foster, J. C. Fogleman and D. J. Brenner. 1991. Taxonomy and pathogenicity of *Erwinia cacticida* subsp. nov. Int. J. Syst. Bacterial. 41:197-212.

2. Burr, 1. J., and M. N. Schroth. 1977. Occurrence of soft-rot *Erwinia* spp. in soil and plant material. Phytopathology 67:1382-1387.

3. Cather, E. J., and K. Sivasithamparam. 1983. *Erwinia:* The 'carotovora' group. Pages 87-106 in: P. C. Fahy and G. J. Persley, eds., Plant Bacterial Diseases: A Diagnostic Guide. Academic Press, Australia.

4. Cuppels, D., and A. Kelman. 1974. Evaluation of selective media for isolation of soft-

rot bacteria from soil and plant tissue. Phytopathology 64:468-475.

5. De Boer, S. H., R 1. Copeman, and H Vruggink. 1979. Serogroups of *Erwinia carotovora* potato strains determined with diffusible somatic antigens. Phytopathology 69:316-319.

6. De Boer, S. Hand M. E. McNaughton. 1987. Monoclonal antibodies to the lipopolysaccharide of *Erwinia carotovora* subsp. *atroseptica* serogroup 1. Phytopathology 77:828-832.

7. De Boer, S. H. and L. 1. Ward. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. Phytopathology 85:854-858.

8. Dickey, R. S. 1979. *Erwinia chrysanthemi:* A comparative study of phenotypic properties of strains from several hosts and other *Erwinia* species. Phytopathology 69: 324-329.

9. Dickey, R S. 1981. *Erwinia chrysanthemi:* Reaction of eight plant species to strains from several hosts and to strains of other *Erwinia* species. Phytopathology 71:23-29.

10. Dickey, R S., and J. 1. Victoria. 1980. Taxonomy and emended description of strains of *Erwinia* isolated from *Musa paradisiaca* Linnaeus. Int. 1. Syst. Bacteriol. 30: 129134.

11. Dickey, R S., C. H. Zumpoff, and 1. K. Uyemoto. 1984. *Erwinia chrysanthemi:* serological relationships among strains from several hosts. Phytopathology 74: 1388-1394.

12. Dye, D. W. 1969. A taxonomic study of the genus *Erwinia.* II. The "carotovora" group. N. Z. 1. Sci. 12:81-97.

13. Dye, D. W. 1981. A numerical taxonomic study of the genus *Erwinia,* N. Z. J. Agric. Res. 24:223-229.

14. Dye, D. W., J. F. Bradbury, M. Goto, A. C. Hayward, R A Lelliott, and M. N. Schroth. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Rev. Plant Pathol. 59: 153-168.

15. Ercolani, G. L. 1984. Infectivity titration with bacterial plant pathogens. Annu. Rev. Phytopathol. 22:35-94.

16. Frechon, D., P. Exbrayat, O. Gallet, E. Guillot, V. Le Clerc, N. Payet and Y. Bertheau. 1995. Sequences nuceotidiques pour la detection des *Erwinia carotovora* subsp.

atroseptica. Institut National de la Recherche Agronomique, Institut National Agronomique Paris-Grignon, Sanofi Diagnostics Pasteur. Brevet 95: 12-803.

17, Gallois, A., R. Samson, E. Agaron, P. A. D. Grimont. 1992. *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory *(Chichorium intybus* L.). Int. 1. Syst. Bacteriol. 42:582-588.

18. Gerhardt, P. (Ed.). 1993. Methods for General and Molecular Bacteriology. Am. Soc. Microbiol. Washington, D. C.

19. Goto, M., and K. Matsumoto. 1987. *Erwinia carotovora* subsp. *wasabiae* subsp. nov. isolated from diseased rhizomes and fibrous roots of Japanese horseradish *(Eutrema wasabi* Maxim). Int. 1. Syst. Bacteriol. 37: 130-13 5.

20. Graham, D. C. 1972. Identification of soft rot coliform bacteria. Pages 273-279 in: H. P. Maas Geesteranus, ed., Proc. 3rd Int. Conf Plant Pathogenic Bacteria, Wageningen, the Netherlands.

21. Graham, D. C. and M. D, Harisson, eds. 1985. Report of the International Conference on Potato Blackleg Disease. Potato Marketing Board, Oxford.

22. Hampton, R., E. Ball and S. De Boer, eds. 1990. Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens: A Laboratory Manual. APS Press, MN.

23. Holt, 1. G., N. R. Krieg, P, H. A. Sneath, 1. T. Staley and S. T. Williams, eds. 1994. Bergey's Manual of Determinative Bacteriology. Ninth edition. Williams & Wilkins, Baltimore, MD.

24. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. 1. Bacteriol. 66:24-26.

25. Hyman, L. J., 1. K. Toth and M. C. M. Perombelon. 1998. Isolation and identification. Pages 60-71 in: M. C. M. Perombelon and 1. M. van der Wolf, eds., Methods for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* on potatoes: Laboratory Manual. Scottish Crop Research Institute. Occasional Publication No. 10. Ivergowrie, Dundee, DD2 5DA, Scotland, UK.

26, Hyman, L. 1., A. Wallace, M. M. Lopez, M. Cambra, M.T. Gorris and M. C. M. Perombelon. 1995. Characterization of monoclonal antibodies against *Erwinia carotovora* subsp. *atroseptica* serogoup 1: specificity and epitope analysis. 1. Appl. Bacteriol. 78:437444.

27. Janse, 1. D. and M. A. Ruissen. 1988. Characterization and classification of *Erwinia chrysanthemi* strains from several hosts in the Netherlands. Phytopathology 78:800-808.

28. Kado, C. I., and M. G. Heskett. 1970. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas* and *Xanthomonas.* Phytopathology 60:969-976.

29. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

30. Kelman, A., and R. S Dickey. 1989. Detection of *Erwinia carotovora* and *E. chrysanthemi* in potato. Pages 76-91 in: A. W. Saetler, N. W. Schaad and D. A. Roth, eds. Detection of Bacteria in Seed and Other Planting Material. APS Press, St. Paul, MN.

31. Klement. Z., K. Rudolph and D. C. Sands. 1990. Methods in Phytobacteriology. Akademiai, Budapest, Hungry.

32. Lelliott, R. A., and R. S. Dickey. 1984. Genus VII. *Erwinia* Winslow, Broadhurst, Buchanan, Krumweide, Rogers, and Smith 1920. Pages 469-476 in: N. R. Krieg and John G. Holt, eds., Bergey's Manual of Systematic Bacteriology. Vol. 1. Williams and Wilkins, Baltimore, MD.

33. Lum, K. Y., and A. Kelman. 1981. Infectivity titrations of *Pseudomonas solanacearum* on tomato. Phytopathology 71:891. (Abstr.)

34. Meneley. J. c., and M. E. Stanghellini. 1976. Isolation of soft-rot *Erwinia* spp. from agricultural soils using an enrichment technique. Phytopathology 66:367-370.

35. Mergaert, 1., L. Verdonck, K. Kersters, 1. Swings, 1.-M. Boeufgras, and J. De Ley. 1984. Numerical taxonomy of *Erwinia* species using API systems. 1. Gen. Microbiol. 130: 1893-1910. -

36. Miller, 1. M. and 1. W. Wright. 1982. Spot indole test: evaluation of four methods. 1. Clin. Pathol. 15:589-592.

37. Miller, T., and M. N. Schroth. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with selective medium. Phytopathology 62: 1175-1182.

38. Nassar, A., A. Darrasse, M. Lemattre, A. Kotoujansky, C. Dervin, R. Vedel and Y. Bertheau. 1996. Characterization of *Erwinia chrysanthemi* by pectinolytic isozyme polymorphism and restriction fragment length polymorphism analysis ofPCR-amplified fragments of *pel* genes. Appl. Environ. Microbiol. 62:2228-2235.

39. O'Neill, R., and C. Logan. 1975. A comparison of various selective isolation media for their

efficiency in the diagnosis and enumeration of soft rot coliform bacteria. J. Appl. Bacteriol. 39: 139-146.

40. Perombelon, M. C. M. 1971. A semi-selective medium for estimating population densities of pectolytic *Erwinia* spp. in soil and in plant material. Potato Res. 14: 158-160

41. Perombelon, M. C. M., and A. Kelman. 1980. Ecology of the soft rot erwinias. Annu. Rev. Phytopathol. 18:361-387.

42. Perombelon, M. C. M. and 1. M. Van Der Wolf 1998. Methods for the detection and quantification of *Erwinia carotovoa* subsp. *atroseptica* on potatoes. Scottish Crop Research Inst. Occasional Publication No. 10. Ivergowrie, Dundee, DD2 5DA, Scotland, UK.

43. Phillips, 1. A., and A. Kelman. 1982. Direct fluorescent antibody stain procedure applied to insect transmission of *Erwinia carotovora.* Phytopathology 72:898-901.

44. Pierce, L. and A. H. Mccain. 1992. Selective medium for isolation of pectolytic *Erwinia* sp. Plant Dis. 76:382-384

45. Ryu, E. 1937. A simple method of staining bacterial flagella. Kitasato Arch. Exp. Med. 14:218-219.

46. Samson, R. 1973. Les *Erwinia* pectinolytiqes. II. Recherches sur les anti genes somatiques *d'EJWinia carotovora* var. *chrysanthemi* (Burkholder) Dye 1969. Ann. Phytopathol. 5:377388.

47. Samson, R., R. Poutier, M. Sailly and B. Jouan. 1989. Caracterisation des *Erwinia chrysanthemi* isolees de *Solanum tuberosum* et d'autres plantes-hotes selon les biovars et serogroupes. EPPO Bull. 17: 11-16.

48. Sellwood, 1., and R. A. Lelliott. 1978. Internal browning of hyacinth caused by *Erwinia rhapontici.* Plant Pathol. 27: 120-124.

49. Singh, u., C. M. Trevors, S. H. DeBoer, and 1. D. Janse. 1999. A fimbrial-specific monoclonal antibody-based ELISA test for European potato strains of *Erwinia chrysanthemi* and comparison to PCR. Plant Dis. 84:443-448.

50. Skerman, V. B. D., V. McGown, and P. H. A. Sneath, eds. 1980. Approved list of bacterial names. Int. 1. Syst Bacteriol. 30:225-420.

51. Smid, E. 1., A. H. 1. Jansen, and L. G. M. Gorris. 1995. Detection of *Erwinia carotovora* subsp. *atroseptica and Erwinia chrysanthemi* in potato tubers using polymerase chain reaction. Plant Pathol. 44: 1058-1069

52. Stanghellini, M. E. 1982. Soft rotting bacteria in the rhizosphere. Pages 249-263 in: M. S. Mount and G. H. Lacey, eds., Phytopathogenic Prokaryotes. Academic Press, New York.

53. Starr, M. P. 1983. The genus *Erwinia.* Pages 1260-1271 in: Phytopathogenic Bacteria. M. P. Starr, ed. Chapter 102. Springer Verlag, New York.

54. Starr, M. P., and A. K. Chatterjee. 1972. The genus *Erwinia:* Enterobacteria pathogenic to plants and animals. Annu. Rev. Microbiol. 25:389-426.

55. Thomson, S. v., D. C. Hildebrand, and M. N. Schroth. 1981. Identification and nutritional differentiation of the *Erwinia* sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi.* Phytopathology 71:1037-1042.

56. Tsuyama, H., and M. Sakamoto. 1952. Isolation methods of soft-rot causing bacteria from the soil. Sci. Rep. Res. Inst. Tohoku Univ., Ser. D., BioI. 3:29-34.

57. Van Der Wolf, J. M., J. R. C. M. Van Beckhoven, E. De Boef, and H. J. M. Roozen. 1993. Serological characterization of fluorescent *Pseudomonas* strains cross-reacting with antibodies against *Erwinia chrysanthemi.* Neth. J. Plant Pathol. 99:51-60.

58. Waldee, E. L. 1945. Comparative studies of some peritrichous phytopathogenic bacteria. Iowa State College J. Sci. 19:435-484.

59. Yarkus, M. and N. W. Schaad. 1979. Serological relationships among strains *ofErwinia chrysanthemi.* Phytopathology 69:517-522.

9. Chemical List

Chemical **Source**

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O. Box 14508, S1, Louis, MO 63178.

Agar
Ammonia
Arabitol
Bromcresol purple
$CaCI_2"2H_20$
Casamino acid
Citrate
Crystal violet
$CuS0_4"5H_20$
Erythromycin

Glucose
H_zO_2
Hydrochloric
acid K_zHP0_4
KH_2P0_4
Keto-methyl glucoside
Lactose
$MgS0_4"7H_zO$
Melibiose
$NazC0_3"H_zO$
$NaN°_3$
Raffinose
Sorbitol
NaOH
NH_4CI
$(NH4)Z^S04$ P-
dimethylaminocinnamaldehyde
Peptone
Phenolphthalein diphosphate sodium
Sodium citrate
Sodium hypochlorite
Sodium polypectate Dr. M. Burger, Eton Ridge Road, Madison, WI. USA
Sodium dodecyl sulfate
Sucrose
Tetramethyl-p-phenylenediamine dihydrochloride
2,3,5 triphenyl tetrazolium chloride
Tryptone
Tryptophan

n, GRAM-NEGATIVE BACTERIA

B-3 *Pantoea*

D. L. Coplin and C. I. Kado

1. INTRODUCTION

The present genus *Pantoea* (7) corresponds to the *Erwinia herbicola/Enterobacter agglomerans* group as defined by Dye (5) and later by Ewing and Fife (6); it includes the phytopathogens *P. stewartii* subsp. *stewartii'* and *indologenes, P. ananas, P. citrea* and *P. agglomerans* pvs. *milletiae, gypsophilae* and *betae.* Other species in this genus represent non-pathogenic plant epiphytes and related soil bacteria, such as *P. agglomerans, P. punctata* and *P. terrea (9).*

The major pathogen in this genus is *P. stewartii* subsp. *stewartii,* which incites Stewart's bacterial wilt and leaf blight of com. Symptoms are water-soaked lesions and vascular wilting on sweet com seedlings and leaf blight of mature maize plants. A small number of former *E. herbicola* strains causing leaf spots of millets have been placed in *P. stewartii* subsp. *indologenes (12) P. ananas* (synonomous with *E. uredovora)* causes brown colored coalescing spots leading to rots ("marbling") of pineapple fruits and honeydew melons (15). It also survives as a epiphyte on uredospores of *Ustilago* smut of maize and the panicles of barley, buckwheat and rice. *P. citrea* causes the pink-disease of pineapple fruits (2). Infection is primarily through the juvenile florets of the fruit, which remain nearly asymptomatic until they are harvested, cored and canned. In fruits that ripen on the plant, the infected tissues will appear semitranslucent, but otherwise infection remains undetectable. *P. agglomerans* comprises a diverse group of common plant epiphytes. The phytopathogenic strains in this species cause abnormal growth; *P. agglomerans* pv. *milletiae* causes galls on *Wisteria* spp. and pvs. *gypsophilae* and *betae* cause galls on *Gypsophilia paniculata* and beets, respectively.

2. ISOLATION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

The methods for isolation and identification of species in the *Pantoea* group are essentially the same as those for *Erwinia* spp., so only particular differences will be discussed below. Most standard media and procedures used for isolating plant pathogenic bacteria can be used for the *Pantoea* group. Luria-Bertani (LB) agar, Nutrient agar (NA), Trypticase Soy agar (TSA) and casamino-acid peptone glucose (CPO) agar can be used for routine isolation. *P. citrea,* however, grows best on MGY medium (see 3, p. 75) (3). Isolation plates should be incubated at 25-29°C. Most species are salt-tolerant so that buffered saline (0.01 M potassium phosphate, pH 7.0; 0.8% NaCI) can be used as a diluent. Colonies will be entire, smooth and glistening, but not mucoid on LB and NA. LB agar supports production of the bright yellow carotenoid pigment. On CPO, *P. stewartii* colonies will be very mucoid and spreading and only slightly pigmented. Other species, such as *P. ananas,* may be semi-mucoid. On NA and TSA, colonies of *P. citrea* turn taupe with a

slight depression in the center as they age.

Only a few serniselective media have been developed for *Pantoea* species in this group and growth on MS medium is poor. To isolate *P. stewartii,* lesions from com leaves should be cut into fine strips and soaked in saline. Then the exudate streaked onto LB or CPG agar. Colonies will appear after two days at 29°C. Isolations can be made from the com flea beetle vector *(Chaetocnema pulicaria* Melsh.), collected from com by grinding individual beetles in a small amount of saline in a tissue homogenizer. Since *P. stewartii* is normally only found inside plant tissues and insect guts, where it is present in high concentrations, a selective medium is not usually necessary. However, when isolating *P. stewartii* from very old lesions Ivanoff's serniselective medium (8) can eliminate some contaminants. For isolation of *P. citrea,* a natural enrichment medium is canned pineapple juice from the grocery store, whose low pH and fruit components inhibit the growth of other bacterial species (2). *P. agglomerans* pv. *gypsophilae* may be isolated from galls afG. *paniculata* on a semiselective medium (10), which was developed by M. Sasser at the University of Delaware.

a. Recipes for general plating media.

1) Casamino-acid peptone glucose (CPG) agar

	perL
Bacto cas amino acid	1.0 g
Bacto peptone	10.0 g
Glucose	10.0 g
Agar, purified	15.9 g

2) Luria-Bertani agar (see 1), p, 41)

3) Nutrient agar (see a, p. 3)

4) Trypticase soy agar (BBL or Difco)

b. Recipes for semiselective agar media

1) Ivanoff's medium for *P. stewartii (8)*

This medium is useful for eliminating contaminants when isolating from very old com lesions or from flea beetles.

	perL
Glycerol	30.0 ml
Ferric ammonium citrate	10.0 g
NaCI	15.0 g
$NaS0_4$	2.5 g
K_2HP0_4	2.5 g

$CaCl_2$	0.1 g 0.1
$MgS0_4e7H_20$	g 300.0
H_20	ml

Stir and then mix separately:

1. Sodium taurocholate	3.0 g
H_20	200.0 ml
2. Agar	15.0 g
H_20	400.0 ml

Heat until agar is dissolved, combine all three solutions, adjust the pH to 7.0 with NaOH, bring the volume up to 1 L and autoclave for 20 min at 120°C.

2) Semiselective medium for *P. agglomerans* pv. *gypsophilae (10)*

	perL
D-trehalose	2.0 g
2, 6-diaminopurine	0.2 g
NaCI	15.0 g
K_2HP0_4	0.8 g
$KH_2{}^P04$	0.8 g
$MgS0_4e7H_20$	0.2 g
Yeast extract	10.0 mg
Agar, Bacto	20.0 g

After autoclaving and cooling, the pH is adjusted to 4.9 with 1 NH_4CI, then cycloheximide is added from a stock solution to a final concentration of 50 ug/ml.

3) MGY medium for *P. citrea (3).*

	perL
Mannitol L-	10.0 g
glutamate	2.0 g
K_2HP0_4	0.2 g
$MgS0_4e7H_20$	0.2 g
Yeast extract	0.25 g

Adjusted to pH 7.0 with 3 N NaOH.

4) Pineapple medium (Kado, unpublished). A naturally selective medium is canned pineapple juice whose low pH and fruit components inhibit the growth of other bacterial species.

Add 15 g agar to 1 L of juice.

3. DIFFERENTIATION OF COMMONLY ISOLATED SPECIES

Pan toea spp. share many characteristics with other members ofthe original genus *Erwinia.* They are Gram-negative, rod-shaped, facultatively anaerobic bacteria that are oxidase negative and catalase positive. Most strains produce pili except for *P. stewartii* and *P. citrea* and most are motile by peritrichous flagella. Acid is produced from arabinose, fructose, galactose, glucose, trehalose and N-acetylglucosamine. Gas is not produced from glucose. They utilize acetate, formate, fumarate, gluconate, lactate, malate and succinate, but not ex-methyl glucoside, melezitose, adonitol, dulcitol, benzoate, oxalate or propionate. The group is commonly differentiated from the other erwiniae by the production of a yellow pigment. However, *P. citrea* and a few strains of *P. agglomerans* do not produce yellow pigment. Other characteristics that distinguish *Pantoea* spp. from other erwiniae are: they do not degrade pectate, do not require growth factors, do not produce urease, lysine decarboxylase or ornithine decarboxylase, and do not produce HzS on Triple Sugar Iron agar.

In most cases, the KOH reaction (see a, p. 7), Hugh-Liefson (see 3, p. 9) test and pigment production, along with a knowledge of disease symptoms, are sufficient to tentatively place an isolate in the *Pantoea* group. Motility (see d, p. 46), nitrate reduction (see b, p. 46), indole production (see i, p. 61), HR and pathogenicity tests should be used to confirm initial identification. Further diagnostic tests to differentiate the species of *Pan toea* are given in Table 1 ~

P. stewartii subsp. *indologenes* differs from *P. stewartii* subsp. *stewartii* in that it can produce indole, utilize citrate, grow on cis-aconitate, and produce acid from glycerol, D-arabitol, cellobiose, maltose, lactose, arbutin and salicin; motility is variable.

4. DIAGNOSTIC MEDIA AND TESTS

a. Hypersensitive Reaction (see e, p.96)

P. stewartii and *P. agglomerans* pvs. *gypsophilae* and *betae* are known to have *hrp* secretion systems, but do not cause an HR when grown on rich media, such as LB agar. To obtain an HR, bacteria are transferred from CPG broth (see a, 1), p. 74) to IM liquid medium, grown for about 6 h, and then infiltrated into tobacco leaves at 10^8 CFU/mi. IM consists of 2 roM (NH4)ZS04' 1 roM KH~04' 1 roM $MgS0_4$'$7H_zO$, 100 mM MES, 0.1% casamino acids, and 1 % sucrose. The pH is adjusted to 5.5 with IN NaOH and the sucrose and $MgS0_4$ are autoclaved separately. It is not necessary to grow *P. citrea* on this medium to preinduce it to cause an HR.

b. Pigment on YDC (see b, p. 4)

c. H_2S from cysteine. (see h, p. 47).

d. Indole production (see i, p. 61).

Table 1. Determinative tests for the separation of *Pantoea* spp. from each other and from the nonpectolytic erwiniae.

Testa	*P. agglomerans* (herbicola strains)	*P. ananas*	*P. citrea*	*P. stewartii* subsp. *stewartii*
Tobacco hypersensitivity			+	
Yellow pigment		+		+
Taupe pigment on YDC			+	
Growth at 37°C	+	+	+	
H_2S from cysteine	+	V	+	
Indole production	V	+		
Nitrate reduction	+	V	+	
Gelatin liquification	+	+		
Production of 2,5 di-keto-D-gluconate			+	
Motility	+	+		
Utilization of:				
Citrate	+	+		
Malonate	+			
Tartrate		+		
Acid production from':				
Cellobiose	V	+		
Glycerol		+	+	
Lactose	V	+	+	
Maltose	+		+	
Melibiose		‡	+	+
Raffinose	V	+		+
Rhamnose	+	+		
Salicin	V	+		
Sucrose	+	+		+
meso-Inositol		+		
Mannitol	+	+		+
Sorbitol		+		V
Arbutin	Nl)	NQ		

+,80% or more strains positive; -PJ, 80% or more strains delayed positive; V, between 21-79% of strains positive; -,80% or more strains negative; ND, not determined,

Data from Cha et al. (2), Gavini et al. (7), Kageyama et al. (9), Mergaert et al. (12) and Wilson et al. (16).

"Saphrcphytic strains of *P. agglomerans* do not give an HR on tobacco, but pvs. *gypsophilia* and *betae* do. Prior growth of *P. stewartii* and *P. agglomerans* pv. *gypsophilae* on 1M medium is necessary.

'For acid production (see f, p. 47).

"P. stewartii subsp. *stewartii* cannot utilize lactose, but it is B-galactosidase positive.

e. Nitrate reduction (see b, 46).

f. Gelatin liquification (see e, p. 46).

g. Assay for glucose dehydrogenase (GDB) Activity in *P. citrea.* Bacteria are streaked on MGY (see 3, p. 75) agar containing 20 g glucose. Colonies are overlaid with 4 ml of 0.5% agar containing a mixture of methylene blue (65 ,ug/ml) and eosin yellow (400 g/ml). Upon incubation for 1-5 min at 30°C, a purple halo around individual colonies indicates GDH activity due to the production of 2-gluconate from glucose (3).

h. Motility (see d, p, 46).

ı. Carbon source utilization (see f, p, 96).

j. Acid production (see f, p. 47).

5. PATHOGENICITY TESTS

Pathogenicity tests with *P. stewartii* are conveniently done with 8 day-old com seedlings (four-leaf stage) grown in a greenhouse or growth chamber at 29°C with intense light and high fertility. Most early maturing sweet com varieties, such as Jubilee, Seneca Horizon and Earliking, are highly susceptible. The simplest inoculation technique is to stab the pseudostem just above the soil line with a toothpick or inoculating needle that has been dipped into a bacterial colony. Alternatively, the stems can be injected with a cell suspension (10^8 CFU/ml) using a syringe. Water-soaked lesions appear after 3 days and the plants should wilt between 5 and 8 days after inoculation. To specifically test for water-soaking ability, suspend cells at 10^7 to 10^8 CFU/ml in 100 mM phosphate buffer (pH 7.2) containing 0.2% Tween 40 and pipet 0.1 ml of inoculum into the whorl of8-day old seedlings without wounding. Water-soaking symptoms develop after 3 days.

Pathogenicity of *P. agglomerans* pv. *gypsophi/ae* on G. *panicu/ata* is tested by dipping cuttings into a bacterial suspension before rooting. Cuttings should then be grown at 20-25°C and kept moist by misting. Galls are observed in the crown region after 7-10 days.

Pathogenicity of *P. citrea* is assayed by growing cultures in 5 ml canned pineapple juice until stationary phase. The culture tube is then either autoclaved or placed in a boiling water bath for 10 min. A red to rusty red color is indicative of *P. citrea.* To verify pathogenicity, the culture is injected into slices offresh pineapple and allowed to incubate overnight at 30°C. Pathogenicity of *P. ananas* is tested by injection into the florets of fresh pineapple fruit and infection allowed to progress for 4-5 days at 30°C. Then the inoculated fruit is sliced open at the points of inoculation. A variegated black to brown color, known as marbling, should be observed around the infection site.

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular techniques

1. PCR

Two PCR tests are available for identification of *P. stewartii* based on primers complementary to the rRNA region. A ligase chain reaction using primers to the 16S rRNA was developed by Wilson et al. (17) for detection of this pathogen in plant material. A simpler PCR assay (K. Geider, pers. commun.) is based on primers to the ITS region. For the identification and detection of *P. agglomerans* pv. *gypsophilae* in gypsophila plants, a primer pair based on the *etzI* cytokinin biosynthetic gene has proven useful (11). Similarly, *P. citrea* can be identified with primers from the *gdhB* gene for glucose dehydrogenase (14). These primers are given in Table 2.

2) Plasmid Profiles

All strains of *P. stewartii* subsp. *stewartii* contain from 8 to 13 cryptic plasmids (4). Plasmid profiles can therefore be used to both identify this species and type strains, since very few other bacteria contain this many plasmids in every strain. Most strains contain conserved 4.2, 13,25,45,65, 74, 103, and 320 kb plasmids. Standard mini-prep procedures for plasmid DNA preparation will isolate all but the 320 kb plasmid. Electrophoresis should be carried out in 0.5% agarose gels in TA buffer (40 mM Tris, 2 mM EDTA, adjusted to pH 8.0 with acetic acid) at 5.5 V/cm, in order to separate closely-spaced large plasmids.

P. citrea contains a 5.3 kb plasmid, designated pUCD5000, whose replication and mobilization loci are similar to pSWI00 and pSW200 of *P. stewartii* subsp. *stewartii* (13). The remaining three loci ofpUCD5000 are required for promoting full color production in pink disease development.

For details on molecular techniques see Appendix A.

Table 2. Sequences of oligonucleotide primer pairs used for peR detection and identification of *Pantoea* spp.

An ELISA based on a polyclonal antiserum has been developed for detection of *P. stewartii* in com seed (1). Polyclonal antibodies in an indirect ELISA can be used to identify *P. agglomerans* pv. *gypsophilae* (10), but they do not distinguish between pathogenic and saprophytic strains. See Appendix B for details.

Target	Name	Primer Sequence	Product size bp	Reference
P. stewartii	Esl	5' -GGCAGCGAACTIGGCAGAGATGCC-3'		17
16S rRNAin	Es2	5' - TIGGTGCCTTCGGGAACCGTGA-3'		
ligase chain	Es3	5' -GCATCTCTGCCAAGTTCGCTGG-3'		
reaction	Es4	5' -GGCACGGTTCCCGAAGGCACCAAG-3'	46	
P. stewartii	ESIR 1	5' -CGAAGCGAGGACACACG-3'		K. [illegible]
rRNAITS	ESIR 2	5' -GCGCTTGCGTGTT ATGAG-3'	290	(personal communication)
	ESIR 2	5' -GCGCTTGCGTGTI ATGAG-3'		
	ES16	5' -GCGAACTTGGCAGAGAT -3'	920	
P. agglomerans		5' -GCAAAAGAACGCGGCTGG-3'		11
pv. *gypsophilae*		5' -GGGTCTCTTGTICCTGCC-3'	607	
etzI				
P. citrea	*gdhB*	5' -GAAGCGAATICCCCACTCGGAACATA-3'		14
glucose	Eco 1	5' -ATICTGAATTCTGCCGCGAATCTATGG-3'	2703	
dehydrogenase B	*gdhB*			
	Ec02			

b) Serological techniques

c) Commercial techniques

An ELISA test kit is available from AgDia Inc. (Elkhart, IN) to identify *P. stewartii.* See Appendix C for details.

7. CULTURE PRESERVATION

P. stewartii loses viability within 2 weeks when kept on slants or agar plates at 4°C, but other *Pantoea* spp. may keep about 4 weeks on NA slants. Most pantoeae will remain viable for several months in deep stab cultures in LB agar at room temperature. For working stock cultures, stationary phase cells grown in LB broth or Dye's liquid medium C (see f, p. 47) can be mixed with an equal volume of glycerol and conveniently stored at -20°C for up to several years. Lyophilization is

recommended for long term storage.

8. LITERA TURE CITED

1. Block, C. C., *I.* H. Hill, and D. C. Mc Gee. 1998. Seed transmission of *Pantoea stewartii* in field and sweet com. Plant Dis. 82:775-780.

2. Cha, *I.* -S., C. Pujol, A. R. Ducusin, E. A. Macion, C. H. Hubbard and C. I. Kado. 1997a. Studies on *Pantoea citrea,* the causal agent of pink disease of pineapple. *I.* Phytopath. 145:313-319.

3. Cha, J. -S., C. Pujol and C. I. Kado. 1997b. Identification and characterization *ofaPantoea Citrea* gene encoding glucose dehydrogenase that is essential for causing pink disease of pineapple. Appl. Environ. Microbiol. 63:71-76.

4. Coplin, D. L., R. G. Rowan, D. A. Chisholm and R. E. Whitmoyer. 1981. Characterization ofplasmids in *Erwinia stewartii.* Appl. Environ. Microbiol. 42:599-604.

5. Dye, D. W. 1969. A taxonomic study ofthe genus *Erwinia.* iii. The *"Herbicold'* Group. N. Z. *I.* Sci. 12:223-236.

6. Ewing, W. H., and M. A. Fife. 1972. *Enterobacter agglomerans* (Beyerinck) comb. nov. (the Herbicola-lathyri bacteria). Int. *I.* Syst. Bacteriol. 22:4-11.

7. Gavini, F., *I.* Mergaert, A. Beji, C. Mielcarek, D. Izard, K. Kersters, and *I.* De Ley. 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. Int. *I.* Syst. Bacteriol. 39:337-345.

8. Ivanoff, S. S. 1933. Stewart's wilt disease of com, with emphasis on the life history of *Phytomonas stewartii* in relation to pathogenesis. *I.* Agr. Res. 47:749-770.

9. Kageyama, B., M. Nakae, S. Yagi, and T. Sonoyama. 1992. *Pantoeapunctata* sp. nov., *Pantoea citrea* sp. nov., and *Pantoea terrea* sp. nov. isolated from fruit and soil samples. Int. *I.* Syst. Bacteriol. 42:203-210.

10. Manulis, S., Y. Gafui, E. Clark, D. Zutra, Y. Ophir and I. Barash. 1991. Identification of a plasmid DNA probe for detection of strains of *Erwinia herbicola* pathogenic on *Gypsophila paniculata.* Phytopathology 81:54-57.

11. Manulis, S., N. Kogan, L. Valinsky, O. Dror and F. Kleitman. 1998. Detection of *Erwinia herbicola* pv. *gypsophilae* in gypsophila plants by PCR. Eur. *I.* Plant Pathol. 104:85-91.

12. Mergaert, J., L. Verdonck and K. Kersters. 1993. Transfer of *Erwinia ananas* (synonym, *Erwinia uredovora)* and *Erwinia stewartii* to the genus *Pantoea* emend. As *Pantoea ananas* (Serrano 1928) comb. nov. and *Pan toea stewartii* (Smith 1898) comb. nov., respectively, and description of *Pantoea stewartii* subsp. *indologenes* subsp. nov. Int. J. Syst. Bacteriol. 43: 162-173.

13. Pujol, C. J. and C. I. Kado. 1998. Characterization of pucd5000 involved in pink disease color formation by *Pantoea citrea.* Plasmid. 40: 169-173.

14. Pujol, C. J. and C. I. Kado. 1999. *Gdhb,* a gene encoding a second quinoprotein glucose dehydrogenase in *Panotea citrea,* is required for pink disease. Microbiology 145: 1217-1226.

15. Wells, J. M., W. -So Sheng, M. J. Ceponis and T. A. Chen. 1987. Isolation and characterization of strains of *Erwinia ananas* from honeydew melons. Phytopathology 77:511-514.

15. Wilson, W. J., H. R. Dillard and S. V. Beer. 1999. Assessment of phenotypic variability in *Erwinia stewartii* based on metabolic profiles. Plant Dis. 83: 114-118.

17. Wilson, W. J., M. Wiedmann, H. R. Dillard and C. A. Batt. 1994. Identification of *Erwinia stewartii* by a ligase chain reaction assay. Appl. Environ. Microbiol. 60:278-284.

9. CHEMICAL LIST

Chemical	Source

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178.

2,6-diaminopurine (cat. no. D 1518)	
Bacto Agar	Difco
Biolog GN Microplate	Biolog (Hayward CA)
Canned pineapple juice	Dole brand
D-trehalose	
Eosin yellow (cat no. E6003)	
Gluconate (cat no. G1l39)	
Mannitol	
MES buffer (cat. no. M3023)	
Methylene blue (cat. no. Mb-l)	
Sodium glutamate	
Sodium taurocholate (crude from ox bile)	Difco
Trypticase Soy agar	Difco
Tween 40 (cat no. P1504)	

II. GRAM-NEGATIVE BACTERIA

C. *Pseudomonas*

A. Braun-Kiewnick & D. C. Sands

1. INTRODUCTION

The phytopathogenic pseudomonads cause numerous plant diseases with diverse symptoms including cankers, diebacks, blossom, twig, leaf or kernel blights, leaf spots *(Pseudomonas syringae* pathovars), soft or brown rots *(P. viridiflava, P. marginalis* pathovars), tumors or galls *(P. savastanoi* pathovars), and mushroom blights *(P. tolaasii, P. agarici).* Many pseudomonads are associated with plants as foliar epiphytes or rhizosphere inhabitants. Since precise identification may be complicated, a combination of using selective media, biochemical/nutritional, pathogenicity, and genetic tests is recommended. However, identification of many pseudomand pathogens is relatively simple and may necessitate only a few tests (24).

The genus *Pseudomonas* comprises one-celled bacteria which are Gram-negative, straight or curved rods, 0.5-1.0 x 1.5-4.0 urn in size. Cells are motile by one to several polar flagella and the G+C content of the DNA ranges from 58-71 mol%. No resting stages are known in this genus. Bacteria are catalase-positive and strict aerobes except for a few which denitrify (51). A characteristic feature of all species is the production of fluorescent pigments, which become visible on iron-deficient media, such as KB (31). The only exceptions are some strains of *P. syringae* pv. *persicae,* and the species *P. corrugata.* Genotypically the latter apparently belongs to *Pseudomonas* (1), even though it shares more phenotypic traits with *Burkholderia.* Except for *P. corrugata,* no pseudomonad grows at 41°C, utilizes D-arabinose as sole carbon source or accumulates poly-p-hydroxy butyrate (PHB) as carbon reserve material (51, 73).

In 1973, Palleroni and collaborators (50) proposed subdividing the genus *(Pseudomonas)* into five rRNA homology groups (rRNA groups I to V), based on the percentages of similarities of various *Pseudomonas* species by rRNADNA hybridizations. The heterogeneity among this old genus of fluorescent and non-fluorescent species reflected by the presence of distantly related species which have since been placed in existing or newly defined genera (30, 71). Former plant pathogenic *Pseudomonas* species of RNA groups II to IV have been reclassified to other genera including *Burkholderia, Ralstonia,* and *Acidovorax* (30). New molecular analyses and taxonomic rearrangements identified the rRNA group I species, including the type species *Pseudomonas aeruginosa* and other fluorescent species such as *P. jluorescens, P. putida,* and *P. syringae,* as members of a phylogenetically homogenous group referred to as *Pseudomonas* (sensu stricto) (30, 71). This classification is in agreement with phylogenetic information obtained from 16S rRNA sequence data. Molecular taxonomy based on 16S rRNA sequences now places the genus *Pseudomonas,* which has been referred to as the type I or fluorescent group of pseudomonads, in the group called *Pseudomonas* and relatives (71).

We include 14 plant pathogenic *Pseudomonas* species, ofwhichP. *syringae* is the most

economically important with more than 50 pathovars (74, 75), of which 26 of the most important ones are presented in Table 2. Others include *P. viridijlava,* a soft-rotting species, and *P. marginalis,* a brown rotting species on lettuce, alfalfa and parsnip and *P. savastanoi* a gall or tumor-producing species. The latter was proposed as a species *includingphaseolicola* and *glycinea* as pathovars (16). We accept the proposed *P. savastanoi,* but reject inclusion ofpv. *phaseolicola* and pv. *glycinea.* The organisms can easily be differentiated phenotypically, serologically, as well as by host reaction and should therefore be differentiated from *P. savastanoi* (61). We will describe *P. savastanoi* as a species in this book, while *phaseolicola* and *glyeinea* remain as pathovars of the species *P. syringae.* Additional phytopathogenic species include *P. eichorii,* which causes chicory wilt, *P. corrugata* the causal agent of tomato pith necrosis and *P. tolaasii* and *P. agariei,* the cultivated mushroom infecting species. The remaining species *P. amygdali* (on almonds), *P. asp/enii* (bacterial blight ofbird's nest fern), *P. caricapapayae* (on *Carica papaya* L.), *P. jicuserectae* (bacterial leaf spot on *Ficus erecta* L.), *P. fuscovaginae* (sheath brown rot disease of rice) and *P. meliae* (bacterial gall of china berry) are of minor importance. We include the three non-pathogenic species *P. putida, P. fluorescens, P. chlororaphis* (including *P. aureofaciens),* and the opportunistic plant and animal pathogen *P. aeruginosa,* in this chapter for comparative purposes.

The large *P. syringae* group of phytopathogens consists of a number of pathovars which in the past had been given species rank. However, these species, like most other plant pathogenic species, were so poorly characterized that they could not be included in the 1980 List of Approved Bacterial Names (62). Because of this, a pathovar system of nomenclature was devised (13) to conserve the former species names of significance to plant pathologists. Pathovar as an infrasubspecific term is explicitly a special purpose classification and not part of the taxonomic hierarchy (72) and should therefore be eliminated as the primary name of an organism as soon as sufficient data are obtained to justify species and subspecies ranking. Some fluorescent *Pseudomonas* species, including *P. marginalis, P. savastanoi,* and *P. syringae,* comprise several to many pathovars based on the distinctive pathogenicity to one or more plant host species (6, 13, 75). Currently, *P. marginalis* comprises the three pathovars *alfalfae, marginalis,* and *pastinacae. P. savastanoi* includes the three pathovars *savastanoi, fraxini,* and *nerii.*

2. ISOLATION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

Selective media containing growth requirements of only pseudomonads are desirable for epidemiological studies and for the identification of a causal agent of a plant disease by eliminating many other bacteria from growth. Some media have been described that were too inhibitory to the desired bacteria, not selective enough, or bacteria that were cultured on them became atypical. Therefore, it is best to use several selective media and a non-selective medium in parallel studies. The following are semiselective media that may be useful for the isolation of different pseudomonads:

a. Modified KB medium (Sands, unpublished)

Add the following to 950 ml ofKB medium (see d, pA) after autoclaving: 75 mg cycloheximide to reduce growth of fungi, 75 mg of penicillin, and 45 mg novobiocin to limit the growth of other bacteria. Prepare the antibiotics mixture by adding all three to 10 ml of95% ethanol and then diluting with 40 ml of sterile distilled water, before adding to the cooled (ca. 50C) KB medium.

This medium is useful for isolating all fluorescent pseudomonads from plants. Pseudomonads produce diffusable yellow, green, or blue fluorescent pigments on this iron deficient medium after 24 to 48 h of growth (see Plate 3, Fig. 2). Fluorescent colonies can be visualized by viewing plates under 366 nm (long wave length) UV light. Pathovars of *P. syringae* are normally blue and produce less pigment then do saprophytes.

b. **PCSM-Medium** (68) **for *P. cichorii* isolation**

	perL
KH_2P0_4	0.5 g
NazHP04e12 H_20	3.0 g
Sodium tartrate	8.0 g
(NH4)2S04	5.0 g
$MgS0_4e7H_20$	25.0 mg
NazMo04e2 H_20	24.0 mg
EDTA-Fe	10.0 mg
L-cystine	5 0. a ,ug
Phenethihcillin, potassium salt	50.0 mg
Sodium ampicillin	10. a mg
Cetrimide	10. a mg
Cycloheximide	25. a mg
Thiram-benomyl wettable powder	100. a mg
Methyl violet	1. a ml *
Phenol red	1.0 ml**
Agar	15.0 g

* Dissolve 10 mg in 2 ml of ethanol, then bring to 10 ml with water. ** Dissolve 200 mg in 10 ml of 0.05 M NaOH.

After autoclaving, cool to 50 ^{0}C, and add:

Potassium tellurite (caution toxic)	25 mg

Dissolve potassium tellurite in 5 ml of distilled water and filter sterilize. Add entire 5 ml to the autoclaved and cooled medium.

PCSM was developed for the isolation of *P. cichorii* from soil and plant debris. Recovery of this bacterium was higher on PCSM than on KB. Colonies of *P.*

ciehorii on PCSM can be identified using a stereomicroscope.

c. Media for the identification of soft-rotting pseudomonads (pectolytic + or -)

1) CVP (Crystal Violet Pectate) medium (11) (see a, p. 57)

A medium useful for the identification of pectolytic pseudomonads such as *P. marginalis* or *P. viridiflava.* Note: Pits produced in this medium by *Pseudomonas* spp. are more shallow than the deep cup-like pits caused by soft rotting *Erwinia* species (Plate 2, Fig. 5).

2) MP medium, a general purpose agar for detecting pectate lyases (20)

	perL
Citrus pectin	5.0 g
KH_2PO_4	4.0 g
N~HP04	6.0g
Yeast extract	1.0 g
Agar	15.0 g
$(NH4)2^{S0}4$	2.0 g
$FeSO_4.7\ H_20$	1.0 mg
$MgSO_4$	0.2 g
$CaCl_2$	1.0 mg
	perL
H_3BO_3	*10.0,ug*
$MnSO_4$	*10.0,ug*
$ZnSO_4$	*70.0,ug*
$CuSO_4$	*50.0,ug*
$Mo0_3$	10.0 *,ug*

Agar, yeast extract, and pectin are mixed dry, then added to the salt solution. The pH will be near 7.0 and needs no adjustment. Pectolytic activity is determined after 3-4 days growth at 27°C by flooding the plate with a 1% solution of hexadecyltrimethylammonium bromide which precipitates intact pectin. Halos of pectolysis are visible when viewed against a dark background. Note: the compound is toxic after prolonged exposure.

3) Hildebrand's media for pectate degradation (23)

A basal polypectate medium is adjusted to three pH levels. Ingredients for the basal medium are added in the following order:

Distilled water (heated to near boiling)	1000 m1
Bromthymol blue (1.5% in 95% ethanol)	1.0 m1
10% $CaCl_2'H_20$ solution (freshly prepared)	6.0ml

Sodium polypectate (add slowly)	22.0 g

This mixture is stirred with a mechanical stirrer while being kept warm on a hot plate or other means. After the sodium polypectate is dissolved, add 4% sterile agar solution, 100 ml to each medium (A, B, C) and adjust the pH as follows:

Medium A: Adjust to pH 4.5-4.7 with 1 N HCI and autoclave.
Medium B: Adjust to pH 6.9-7.1 with 1 N HCI and autoclave.
Medium C: Autoclave and then adjust to pH 8.3-8.5 with sterile 1 N NaOH.

Petri dishes must be poured before the temperature drops below 70°C. The plates are stored at room temperature for several days until the surface of the gel is dry. Masses of bacteria (2-4 mm in diameter) are spotted using a sterile toothpick or transfer loop (4 samples/plate) on the gel surface and the plates incubated for 1-6 days at 28 °C before the occurrence of pitting is ascertained.

d. Selective media for *P. syringae* pathovars

1) BCBRVB medium (Sands, unpublished)

KB (see d, p. 4)	950 ml

Autoclave, cool to 45°C, then add a mixture of the following antibiotics in 10 m1 70% ethanol diluted with 40 m1 sterile distilled water:

Bacitracin	10. a mg
Vancomycin	6. a mg
Rifampicin	0.5 mg
Cycloheximide	75. a mg
Benomyl	0.25 mg

The medium is useful for isolating from insects or plant debris due to high selectivity. It may be toxic to some strains. New chemicals and freshly prepared antibiotic solutions should be used.

2) M-71 Medium for *P. syrinae* pv. *g/ycinea* isolation (37)

	perL
Glucose	5. a g
Peptone	10.0 g
Casein hydrolysate	1. a g
Cycloheximide	50.0 g
Triphenyl tetrazolium HCI	50.0 g
Boric acid	1. a g
Agar	20.0 g

Tetrazolium is autoclaved separately in 5 ml water and added to cooled agar before pouring. Cycloheximide is added after autoclaving. The medium is semiselective and colonies of *P. syringae* pv. *glycinea* exhibit a characteristic colony morphology. Plating efficiency is very variable depending on the strain. More species should be tested with this medium.

3) BANQ medium for *P. syringae* pv. *glycinea* isolation (15)

	perL
D-quinic acid	2.0 g
D-serine	0.2 g
K_2HP0_4	2.0 g
KH_2P0_4	2.0 g
$MgS0_4e7H_20$	0.1 g
Boric acid	1.5 g
Yeast extract	30.0 mg
Chlorothalonil (as 1 :25 aqueous solution)	40.0 mg
Agar	15.0 g

Dissolve the $MgS0_4 \cdot 7H_20$ before adding to the other ingredients. Adjust pH to 7.0-7.2 and autoclave before adding 16 J,lg/ml ampicillin and 24 J,lg/ml novobiocin. Plating efficiency is high.

4) KBC medium (49) for isolating *P. syringae* pathovars *syringae,pisi* and *tomato* from plants or seeds.

After preparing 900 ml ofKB (see d, p. 4) add the following:

Boric acid, autoclaved 1.5% aqueous solution	100.0 ml
Cephalexin (stock solution of 10 mg/ml distilled water)	8.0 ml*
Cycloheximide (stock solution of 100 mg/ml 75% methanol)	2.0 ml*

Plating efficiency is high. Fluorescent pigment is produced but delayed 1-2 days.
*Note that amount in 2nd Edition of this manual was incorrect.

5) MSP (modified sucrose peptone) medium (46) for the isolation of *P. syringae* pv. *phaseolicola* from bean plants or seed.

This is a modified sucrose peptone agar (38) with added antimicrobial compounds and bromthymol blue.

	perL
Sucrose	20.0 g
Peptone	5.0 g
K_2HP0_4	0.5 g
$MgS0_4e7H_20$	25.0 mg

Agar	20.0 g

Adjust the pH to 7.2 to 7.4, autoclave, cool to 45°C and add a mixture of the following filter sterilized stock solutions

	perL
Cycloheximide (100 mg/ml 75% methanol)	2.0 ml
Cephalexin (10 mg/ml distilled water)	8.0 ml
Vancomycin (10 mg/ml distilled water)	1.0 ml
Bromthymol blue (15 mg/ml95% ethanol)	1.0 ml

Plating efficiency is good. Colonies of *P. syringae* pv. *phaseolicola* are yellow and domed shaped (levan positive) and produce a blue fluorescent pigment on this medium.

3. DIFFERENTIATION OF COMMONLY ISOLA TED SPECIES AND PA mOVARS

a. Pathogenic species and pathovars

General identification of the isolated strain usually begins with an examination of disease symptoms. Knowledge of the host and the type of symptoms produced often enables an investigator to make a preliminary judgement as to the identity of the causal agent since most of the plant pathogenic pseudomonads are host specific. A preliminary identification can also be made by the use of semiselective media in combination with disease symptomatology and host of origin. After a preliminary identification is made, various phenotypic and genetic tests can be done to further identify the organism on the species and pathovar level.

The tests in Table 1 are those which are useful in identifying species of the better known *Pseudomonas* pathogens on a biochemicallnutritionallevel. Species identification is mainly based on LOPAT characters (38), including Levan production on sucrose medium, Oxidase reaction, ~ectolytic activity on potato slices or pectate gel, Arginine dihydrolase activity, and hypersensitivity reaction (HR) on Tobacco leaves. Additional tests are based on the schemes presented by Hildebrand et al. (24) and in Bergey's Manual of Determinative Bacteriology (27). Many of the tests involve the use of substances for growth which often but not always are comparable to acid production. When testing for acid production always include known strains.

Table 1. Identification of *Pseudomonas* species.[a]

Characteristic	*P.marginalis*	*P. tolaasii*	*P. agarici*		*P. viridiflava*	*P. savastanoi*	*P. syringae*	*P. fuscovaginae*	*P. corrugata*
Diffusable fluroescent pigment	+	+	+	+	+	+	+	+	-
Non-diffusible pigment				-	V (blue-green)				-
Levan				-	-				-
Oxidase			+	+	-				+
Arginine dihydrolase				-	-				+
Pectolytic Activity				-	+				-
Tobacco HR			+	+	+				ND
Growth at 37°C				-	-				+
Nitrate to N_2				-	-				+
PHB[c]				-	-				+
Gelatin hydrolysis				-	+				+
Utilization of:									
2-ketogluconate				+	+			-	ND
Mannitol				+	+			ND	+
Geraniol				-				ND	ND
Benzoate				-				ND	-
Cellobiose				-	+			ND	-
Sorbitol				-				ND	-
Trehalose				-				ND	+
Sucrose				-				+	+
meso-tartrate				+				ND	ND
D(-)-tartrate				-	+			ND	V
D-arabinose				-				ND	+
L-rhamnose				-				ND	-
D-aspartate				+				ND	ND
Ice nucleation				-				-	-
IAA production				-				-	-

+, 80% or more strains positive; -, 80% or more strains negative; V, between 21-79% of strains positive; ND, not determined.

[a] Modified after Sands et al., 1970 (57), Hildebrand et al., 1988 (24); Holt et al., 1994 (27); and Young & Triggs, 1994 (74).

[b] Pathovars *delphinii*, *populans*, and *passiflorae* are negative.

[c] Poly β hydroxybutyrate (PHB)

Pathovar identification is more complicated than species identification, since it relies on more tests and host specificity. However, for correct pathogen identification and plant disease diagnosis, reliance upon host pathogenicity alone is unsatisfactory and suffers from the same problems as reliance upon any other single phenotypic or genetic property. The tests listed in Table 2 can be used to distinguish the most important pathovars of *P. syringae* with good accuracy. Use Tables 1 and 2 to identify a strain to a pathovar or cluster of pathovars and then do pathogenicity tests of both isolated and authentic (known reference) strains to confirm an identification. It is extremely important to follow the pathogenicity methods described in this chapter (p. 102), especially the use of an inoculum containing $< 10^7$ CFU/ml.

In addition to nutritionallbiochemical tests (24, 51, 57, 74) and toxin analysis (see n, p. 99) presumptive pathovar identification can be based on serological analyses (see d, p. 106) (48, 49, 58, 59) by using specific antibodies raised against the lipopolysaccharides of bacterial cell walls. Molecular techniques (see d, p.l 04) become of increasing importance for the identification and classification of bacteria, especially at the pathovar and strain level (6, 7). *P. syringae* strains within and between pathovars have been described using both phenotypic (11, 14,25, 55, 57, 74) or genetic characters (6, 8, 12, 44,45, 70, 71) or a combination of both (21,40). Most authors agree that strains within the pathovar *syringae* represent very diverse populations while strains of pathovars *tomato* (12) or *morsprunorum* (14, 40) were more host-specific and similar in their genetic structure.

b. Plant-associated saprophytic species

The fluorescent saprophytic pseudomonads associated with plants are included here for the purpose of making a correct identification of pathogens and can be assigned generally to one of three species, i. e. *P. fluorescens, P. putida,* or *P. ch/ororaphis.* The species *P. chlororaphis* now includes *P. aureofaciens,* which was formerly considered to be a separate species (27, 29). *P. aeruginosa* is an opportunistic plant and animal pathogen, that forms a tight cluster and is relatively easy to identify (Table 3). Most of the plant-associated strains belong to the *P. fluorescens-P. putida* complex of organisms. Although the classical means of separating these two species has been trehalose utilization and gelatin liquefaction *(P. fluorescens* positive, *P. putida* negative), many strains are isolated which are positive for one and negative for the other. Hence, clear distinction and identification of most strains, unless their properties happen to fit almost exactly the description of either *P. fluorescens* or *P. putida* or one of their subgroups, is difficult. However, when in doubt and precise identification of a *P. fluorescens* isolate is not necessary, the strain often can be assigned to *P. fluorescens* biovar V as this is the biovar which consists essentially of strains with the general character of *P. fluorescens* which remained unaccounted for after recognition of the other four biovars of *P. fluorescens. P. putida* contains two biovars A and B. Properties which can be used to assist in subdividing the *P. fluorescens-P. putida* complex and related species are listed in Table 3.

Antibiotics 2, 4-diacetylphloroglucinol (Phl) and phenazine-l-carboxylic acid (PCA) are frequently produced by saprophytic *P. fluorescens* or *P. aureofaciens* strains. Specific genes within the biosynthetic loci for phloroglucinol and phenazin are conserved among *Pseudomonas* strains worldwide.

4. DIAGNOSTIC MEDIA AND TESTS

a. Levan. Streak culture onto nutrient agar (see a, p. 3) to which 5% *(w/v)* sucrose has been added. White mucoid, dome-shaped colonies after 3 to 5 days incubation indicate a positive reaction. A positive control may be most any pathovar of *P. syringae. P. marginalis* and *E. carotovora* are negative.

b. Kovacs oxidase test (34). (see 7, p. 10) Most non-pathogenic pseudomonads are positive, whereas pathovars of *P. syringae* and *P. savastanoi* and *P. viridiflava* are negative (Plate 1, Fig. 2).

c. Pectolytic activity. Pectolysis of potato slices involves placing washed and alcohol-flamed potato slices (7-8 mm thick) into Petri dishes containing a sterile, moistened filter paper. A bacterial cell suspension is pipetted into a depression cut in the potato. Pectolysis beyond the point of inoculation after 24 h at 22°C indicates a positive response (plate 3, Fig. 1). Pectolytic activity can also be identified on CVP medium (see a, p. 57). *P. viridiflava, P. marginalis,* and, *E. carotovora* subsp. *carotovora* are positive. *P. syringae* is negative. Pectolytic *Erwinia* sp. produce deep cup-like pits on CVP, while pseudomonads produce shallower, wider pits. However, plant pathogenic bacteria produce a number of pectolytic enzymes making it difficult to use one medium that is best for all pectolytic pathogens. For instance, pectate lyases usually have alkaline pH optima and polygalacturonases have more acidic pH optima. A general purpose agar for detecting pectate lyases is MP medium (see 2), p. 87). For taxonomic identification, it is best to use both high and low pH media. Hildebrand's media A and C are recommended for this purpose (see 3), p. 87).

Table 2. Identification of the most common pathovars of *syringae*.

Ice nucleation		+	+	+	+	+		+							+	+	+	+									
Levan	+	+	+	+	+	+	+		+																		
Pectolysis[1]																											
β-glucosidase		+	+	+																							
Utilization of:																											
D-mannitol	+	+			+	+			+	+																	
Adonitol																											
Insitol	+	+	+	+	+	+	+		+	+	+	+															+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+		+	+														
Trigonelline	+	+	+		+	+	+	+	+	+	+	+	+														+
D-quinate	+	+	+		+	+	+																				+
Erythritol						+																					
L(+) tartrate								+																			
D(-) - tartrate			+						+																		
L-lactate																											
Anthranilate																											
DL-homoserine																											
Glutarate	+	+	+	+	+	+		+	+	+	+																
DL-glycerate											+																
Gelatin liquefaction	+	+	+	+		+		+		+	+		ND	ND													
Arbutin hydrolysis	+	+	+								+		ND	ND		ND											
Aesculin hydrolsis	+[D]		+			+		+			+	ND	ND	ND		ND											
Polygalacturonase	+[D]	+										ND	ND	ND		ND											
Pectate lyase	+[D]	+[D]								+		ND	ND	ND	+	ND											
	+	-	+	+	+	-	ND	-	+	-	+	+	ND	ND	-	+		+									

[1]pH at which pitting on pectate gel occurs, 4=pH 4.6/ 8=pH 8.5.

+, 80% or more strains positive; +[D], 80% or more strains delayed positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

Modified after Hildebrand et al., 1988 (24) and Young & Triggs, 1994 (74).

Table 3. Identification of plant-associated saproph[illegible] c fluorescent *Pseudmonas* species.

Characteristic	*P. aeruginosa*	*P. fluorescens*						*P. chlororaphis**			
Diffusible non-fluorescent pigment +											
Non-diffusible, non-fluorescent											
Pigment	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Levan	[illegible]	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Oxidase	[illegible]	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Arginine dihydrolase	[illegible]	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Pectolytic Activity	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Tobacco HR	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Growth @ 41 C	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Growth @ 4 C	[illegible]	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Nitrate to N_2	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Gelatin liquefaction	[illegible]	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Growth on:											
L-arabinose		+									
D-galactose		+									
Trehalose		+									
Saccharate		+									
Butyrate	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Valerate		V									
Azalate		-									
Sorbitol		+									
Meso-inositol	[illegible]	V	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Adonitol		+									
Propylene glycol	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Ethanol		-									
Geraniol		-									
Testosterone		-									
Phenyl acetate		-									
Butylamine		-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Nicotinate		-									
Trigonelline		V									
Lecithinase		+									

+, 80% or more strains positive; $+^{D}$, 80% or more strains delayed positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

* The species *P. chlororaphis* now includes *P. aureofaciens*, which was formerly considered to be a separate species (31).

[a] Modified after Hildebrand et al., 1988 (24) and Holt et al., 1994 (27).

d. Arginine dihydrolase activity (67). Test for the presence of two enzymes that permits certain pseudomonads to grow under anaerobic conditions. The enzymes generate ATP by the degradation of arginine to ornithine with the generation of CO_2 and NH_3. The two enzymes are arginine desmidase which degrades arginine to citrulline + NH_3, and citrulline ureidase which converts citrulline to ornithine + CO_2 + NH_3. It is the alkaline reaction of NH_3 production that is detected by the test.

A fresh culture is stabbed into a soft agar tube of Thornley's medium 2A, sealed with sterile mineral oil (1 ml) or melted agar and incubated at 28 °C. A color change from faint pink to red within four days is a positive reaction (plate 3, Fig. 3). *P. fluorescens*, *P. corrugata*, and *P. marginalis* are positive. *P. syringae* is negative.

Thornley's medium 2A (67)

	perL
Peptone	1.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Agar	3.0 g
Phenol red	1.0
Arginine HCI	mg
Adjust pH to a faint pink color (pH 7.2)	10.0 g

e. Tobacco hypersensitivity reaction (HR) (33). Identifies heterologous pathogenic bacteria based on the local cell death of tissue between veins of tobacco leaves when bacterial suspensions (> log 6 CFU/ml) are injected into the intercellular space of the leaf with a 25 gauge needle and syringe (33). Complete collapse of the tissue after 24 hours is recorded as positive (Plate 3, Fig. 4). *P. syringae* pathovars are positive. An exception to the above is the failure of the homologous tobacco pathogen *P. syringae* pv. *tabaci* to induce HR. Saprophytes allegedly do not induce this reaction, making it a quick and useful determinative test to differentiate saprophytes from plant pathogens.

f Carbon source utilization

Carbon sources are filter sterilized and added at 0.1 % (w/v) final concentration to autoclaved and cooled Ayers et al, mineral salts medium (2).

	perL
$NH_4H_2PO_4$	1.0 g
KCI	0.2 g
$MgSO_4$-7 H_2O	0.2 g
Bromthymol blue (1.6% v/v in 95% ethanol)	1.0 ml
Agar	12.0 g

The pH is adjusted to 7.2 prior to autoclaving. Bacteria are streaked onto the

medium, or patched on with replica plating methods, and incubated at 27°C for 3, 7 and 14 days. Growth is compared to plates containing no added carbon source.

g. Temperature relationships

Observe growth in tubes of yeast extract (5 gil) or Ayers et al. (1) mineral salts medium at 27, 37, and 41°C. No plant pathogenic pseudomonad can grow at 41°C. and only *P. savastanoi* grows at 3rC.

h. Nitrate reduction

Pseudomonads cannot reduce nitrogen, but some non-pathogenic species such as *P. jluorescens, P. chlororaphis,* and *P. aeruginosa* can use nitrate as a terminal electron acceptor and grow in the absence of oxygen.

	perL
Yeast extract	5.0
$KN0_3$	g
Noble agar	3.0
$NH_4H_2P0_4$	g
KCI	1.0
$MgS0_4e7\ H_20$	g
	1.0
	g
	0.2
	g
	0.2
	g

Dispense medium into tubes, autoclave and cool. Then inoculate with the strain in question and plug each tube with 3% Noble agar. Growth at 27°C after up to 5 days is recorded as a positive test for denitrification.

i. Fluorescent pigments on KB medium (see d, p. 4)

The KB medium is used for detection of fluorescein, a fluorescent green or blue, water-soluble, chloroform insoluble pteridine pigment (i.e. pyoverdine siderophores) (Plate 3, Fig. 2). After 24-48 hour growth at 27°C, colonies are examined for fluorescence with a long wave-length (366 nm) ultraviolet lamp. Impurities such as iron will repress pigment formation and quench the fluorescence of formed pigments.

J. Poly-~-hydroxybutyrate (PHD) accumulation (see a, p. 157).

PHB serves as an intercellular carbon source only in *P. corrugata.*

k. Arbutin hydrolysis (~-glucosidase activity) (9)

The arbutin medium detects P-glucosidase activity of *P. syringae* pathovars and strains which splits plant glucosides such as arbutin and salicin. The enzyme degrades glucosides in such a manner that the sugar residue cannot be used for growth. Occasional

strains which use one of these glucosides for growth are using the aglycone portion.

	perL
Arbutin	5.0
Peptone	g
Yeast extract	10.0 g
D-glucose	3.0 g
Ferric citrate	1.0 g
Noble agar	0.5 g
	12.0 g

Add the ingredients to 1000 ml distilled water, adjust the pH to approximately 7.0, and autoclave for 20 minutes. Plates of the medium are spotted with no more than two strains well separated and incubated at 25-28 ° C for up to 10 days. A positive result is indicated by growth and browning of the medium. Care must be used in reading the results when more than one strain is cultured on a plate as often strong positive results can obscure weak positive results.

The chromogenic substrate p-nitrophenyl-ji-glucoside (PNBG) also can be used to detect this enzyme in suspensions or extracts of a bacterium (22).

Most pathovars of *P. syringae* are positive, but *morspronorum, helianthi, cannabina, glycinea* and *phaseolicola* are negative as is *P. savastoni.*

I. Ice nucleation activity (INA) (41, 42, 43)

Many strains of *P. syringae, P. viridiflava,* and *P. jluorescens,* are ice-nucleation active (INA). These bacteria catalyze ice formation at temperatures above _10°C, some strains even at temperatures as warm as -1.5°C. If INA-positive bacteria induce ice formation at temperatures above - 5 ° C in frost sensitive plants that may lead to freezing injury/frost damage (42). While most strains of pvs. *syringae, pisi, glycinea, lachrymans,* and *coronafaciens* are INA positive, ice-nucleation has never been detected in *P. savastanoi* or *P. syringae* pv. *tomato.* A few strains of *P. syringae* pv. *phaseolicola* are positive exhibiting ice-nucleation at -10°C and many strains of *P. syringae* pv. *glycinea* express INA only at temperatures approaching -10°C (25). Thus, while a positive INA assay will not unambiguously identify a bacterial strain, it will in many cases eliminate certain species or pathovars from consideration as a causal agent. Most bacteria active in ice-nucleation at temperatures greater than -10 ° C, will express detectable nucleation frequencies at temperatures warmer than - 5 ° C as well.

Droplet freezing technique (43):

Grow strains on KB for 4-6 days at 18 to 24°C for best expression of INA. Single colonies are removed from agar plates with a toothpick and suspended in 0.1 ml of distilled water to yield a turbid suspension (> 10^8 CFU/ml). Appropriate dilutions in 10 roM

potassium phosphate buffer (pH 7.0) are divided into large numbers (approx. 20 to 40) of small droplets (10 *J,ll)* which are then cooled to a given temperature (typically -5 or -lOOC) on a test surface. The number of INA events is determined by visual observation. It is necessary to test a series of dilutions in order to obtain one or more dilutions for which some but not all of the drops freeze. The test surface is prepared by spraying aluminum foil with a 1% solution of paraffin in xylene, removing the xylene at 55°C in a circulating oven, folding the coated foil into a flat-bottomed "boat", and floating the "boat" on a methanolwater solution maintained at - 5°C or -10°C. An ethanol-refrigerated circulating bath is the constant heat sink for such assays. A colony is considered to contain nuclei active at 5 ° C or -10 ° C if droplets freeze within 30 sec. Frozen droplets are generally opaque and non-hemispherical unless a turbid bacterial suspension is being tested.

m, Indole acetic acid (IAA)

lAA is most useful for identification of the gall producing pseudomonad, *P. savastanai* (Table 1).

P. savastanoi is the only known *Pseudomonas* species that causes formation of hyperplastic growth, apparent as knots or tumors on young stems and branches, and occasionally on leaves and fruits in a range of plant species. The symptoms are similar to *Agrobacterium* infections. However, the pathogen prefers warmer climates and occurs on a limited number of hosts including:

olive *(Olea europaea)* pv. *savastanoi*
oleander *(Nerium oleander)* pv. *nerii* ash *(Fraxinus excelsior)* pv. *fraxini* forsythia *(Forsythia intermedia)* jasmin *(Jasminus spp.)*
privet *(Ligustrum japonicum)*

While the identification of *P. savastanoi* and its disease symptoms are quite simple, IAA extraction from plant knot tissue or bacterial cultures, purification and identification are more complicated and require a chromatography lab equipped with thin layer chromatography (TLC) or high performance liquid chromatography (HPLC)

n. Toxin tests

The use oftoxin bioassays or DNA-analysis oftoxin genes (see a, p. 104) may be helpful to differentiate pathovars of *P. syringae* (Table 2). Although, in most cases, toxins contribute to virulence of pathogens, they are in general not needed for pathogenicity.

I) Detection of syringomycin, syringotoxin, syringostatin, pseudomycin and pseudopeptin:

These toxins are cyclic lipodepsinonapeptides and are either small molecules (approx. 1200 Da) or large molecules (approx. 2500 Da). Syringomycin (19) and its amino acid derivatives syringostatin, syringotoxin (47) and pseudomycin are small molecules, while pseudopeptin is a large molecule. The toxins are the only known necrosis-inducing toxins produced by *P. syringae* pvs. *syringae, atrofaciens,* and *aptata* (Table 4) with both phytotoxic and antimicrobial activities (19). The toxins affect ATPase activity located in the plasmalemma. They interfere with the ion flux by ion channel formation in the plant membrane.

a. SRM (syringomycin minimal) medium for the detection of syringomycin production or its amino acid analogs syringotoxin and syringostatin (18).

Dissolve 4 g L-histidine and 20 g agar in 889 ml distilled water, adjust pH to 7.0-7.1 with 1 M NaOH and autoclave. Add the other ingredients from the following sterile stock solutions:

D-glucose	100 ml (10% w/v) autoclaved
$MgS0_4$' 7 H_20	0.8 ml (2.46 gllO ml) autoclaved
KH_2P0_4 (0.5 mol/I)	3.6 ml (0.68 gllO ml) autoclave
K_2HP0_4 (0.5 mol/I)	6.4 ml (0.87 gila ml) filter sterilized
$FeCl_3$	100 ,ul (0.27 gila ml) filter sterilized

b. SRM_w modified syringomycin minimal medium (including plant signal molecules for induction of syringomycin gene expression)

Add 100 zzmol/l arbutin (27 mg/l) and 0.1 % D-fructose to SRM medium

Pipette exactly the same amount of medium into each plate (eg. 15 or 20 ml/ per plate) for quantitative inhibition assays. Transfer purified test strains to SRM or SRM.A.F (2-4 strains/plate). Incubate plates for 5 days at 25°C. Mark the areas of colony growth and scape off the colonies with sterile cotton swabs. Kill remaining bacterial cells by exposure of plates to chloroform vapors (20 minutes). Evaporate chloroform vapors by leaving open plates under laminar flow hood for 20 minutes. Spray a 10^7 CFU/ml conidial suspension of the syringomycin sensitive fungus *Geotrichum candidum* onto plates, incubate for 24 hours at 25 ° C and record zones of inhibition. The assay can also be done on PDA plates with less sensitivity.

For the detection **ofsyringopeptins** *Bacillus megatherium* should be used as toxin indicator strain, since G. *candidum* is insensitive to syringopeptins

(30). Rhodotorula pilimanae can be used for the detection of both syringomycins and syringopeptins.

2) Detection of phaseolotoxin:

This toxin is unique to strains of *P. syringae* pv. *phaseolicola.* Its target molecule in the plant is the enzyme L-ornithine carbamyl transferase. Indicative for the toxin are chlorotic halos around infection centers on young bean leaves.

A simple agar plate bioassay using *E. coli is available* (57, 64).

3) Detection of coronatine:

The toxin is a polyketide molecule produced by strains of *P. syringae* pvs. *tomato, atropurpurea, glycinea, morsprunorum,* and *maculicola* and causes light-dependent chlorosis in green leaves, stunting, and hypertrophy. No bioassay with toxin-sensitive microbes is available.

a) HSC medium for optimal production of coronatine:

The HS broth medium described by Hoitink & Sinden (26) was optimized for coronatine production (52).

	perL
Glucose	20.0 g
NH_4CI	1.0 g
$MgS0_4.7H_20$	0.2 g
KH_2P0_4	4.1 g
$K_2HP0_4.3H_20$	3.6 g
$KN0_3$	0.3 g

add 20 *J.IM* filter-sterilized $FeCl_3$ solution after autoclaving.

b) Coronatine detection by the induction of hypertrophies on potato tuber discs:

Prepare filter-sterile culture extracts of test strains or use purified test strains directly. Keep potato disc in a Petri dish containing moistened filter paper. Transfer 5 mm diameter filter paper soaked in purified toxin solution (10 - 20 J.ll) or culture extract on potato discs or transfer strain in question directly onto potato disc. A hypertrophic response (formation of outgrowth) will be visible after 5 days at 23 DC (17,57).

4) Detection of tabtoxin/tabtoxinine-Il-lactam:

Tabtoxin is the precursor molecule for the mono cyclic p-Iactam antibiotic tabtoxinine-ji-lactam, The toxin is produced by strains of *P. syringae* pvs. *tabaci, coronafaciens,* and *garcae* and interferes with glutamine synthetase (39). It induces the formation of chlorotic halos around necrotic lesions. Chlorosis is lightdependent.

Tabtoxin identification by *E. coli* inhibition:

The same *E. coli* inhibition bioassay described for phaseolotoxin (57, 64) can be used for identification and toxin quantification. However, the *E. coli* inhibition is reversed by glutamine, since tabtoxinine- p-Iactam is a specific glutamine synthetase inhibitor. For quantification, the activity which produces a zone of inhibition of 25 mm diameter under standardized conditions is defined as one unit, which is equivalent to 5.5 nM of tabtoxin (57).

5) Detection of tagetitoxin:

The toxin is a cyclic hemithioketal molecule. It is only produced by strains of *P. syringae* pv. *tagetis* and is not detectable in culture filtrates. The toxin interferes with RNA polymerase in protein biosynthesis of chloroplasts, No microbial inhibition assay is available. However, the toxin can be rapidly detected by its ability to elicit apical chlorosis in plant tissues. Typical yellow halos are visible on leaves of compo sitae, such as sunflowers and marigold, 2-3 days after application of bacterial suspensions.

5. P A mOGENICITY TESTS (24)

The techniques of inoculation with pseudomonads are essentially the same as used with other genera of plant pathogenic bacteria. These include such methods as wound inoculation by a blade or needle, spray inoculation and incubation under moist conditions, wound inoculation by rubbing plant parts with a suspension of bacteria with an abrasive such as carborundum or corundum, forced intromission of bacteria into plant tissue with a pressure sprayer, and vacuum infiltration of bacteria into plant tissues. All of these techniques have value depending upon the nature of the disease and purpose of the experiment. The controversial aspect of pathogenicity tests generally revolve around the interpretation of the results. Many of the phytopathogenic pseudomonads have the potential to produce a variety of reactions such as necrosis, chlorosis, discoloration, eruptions, and the well-known hypersensitivity reaction on non-host plants when plants are grown under unusual conditions, or when high dosages of inoculum are used. Accordingly, some ofthese reactions on a non-host plant may be misinterpreted as a true pathogenic response.

Some principles to apply when making pathogenicity tests:

1. Grow pathogen-free plants under conditions most favorable for their growth and which most closely approximate the conditions for disease development in the field.
2. Select an inoculation technique which most closely simulates the natural method of inoculation or infection.
3. Use relatively low dosages of bacteria in inoculation (10^5 to 10^6 *CFU/ml)* when spraying or infiltrating plants. Grow bacteria and always determine the actual *CFU/ml* used for each inoculum prepared, as described (see 5, p. 226).
4. Symptoms obtained from plant inoculation should closely resemble those that occur in the field.

Observation of these principles reduces the chance that an erroneous conclusion may be drawn from a pathogenicity test. With respect to the first principle, plants may either lose their resistance or gain susceptibility depending upon the disease when grown under abnormal growing conditions. For example, infection of bean by *P. syringae* pv. *phaseolicola* with the production of halo blight symptoms is greatly reduced at temperatures greater than 28°C. Moisture can be very important with leaf infecting organisms that invade through natural openings. Free water for a period of time is necessary for the bacteria to penetrate natural openings.

The ability of many bacterial plant pathogens to cause an assortment of reactions on non-host plants when massive dosages are used (i, e. > 10^7 *CFU/ml)* has led to the misinterpretation of host ranges. Whereas there is a general recognition that high dosages may produce a hypersensitive reaction when intromitted into a non-host plant, it is not well understood that other symptoms are readily produced depending upon the variety of plant and the nature of the inoculation. These reactions are frequently very definitive and specific to a particular bacterial species and may even be used as a diagnostic tool to help in the identification of a bacterium. A distortion reaction is produced by *P. syringae.* pv. *pisi* when leaves of red kidney beans are inoculated by mixing 10^7 *CFU/ml* with corundum as an abrasive (Plate 3, Fig. 5). However, this form of necrosis never occurs if leaves are gently sprayed with a low dosage of the bacteria. *P. syringae* pv. *tabaci* produces very distinctive angular discolorations on red kidney bean and is therefore easily identified (Plate 3, Fig. 6).

Pressure spraying of inoculum into leaves with a device such as an artist's airbrush is another technique which gives results similar to corundum; it too has advantages and disadvantages. With too much inoculum, a hypersensitive reaction is obtained; whereas with the proper amount of inoculum a susceptible reaction is obtained.

Vacuum infiltration of bacteria into lima bean leaves is especially useful when identifying *P. syringae* pv. *phaseolicola.* Infection is very uniform, and water-soaked lesions are easily observed (Plate 3, Fig. 7).

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular techniques

Molecular DNNRNA techniques are rapidly overtaking serology, enzymology and metabolic analyses for the identification of phytopathogenic bacteria. The reason for this is the availability of sensitive and precise DNAIRNA screening techniques. Many of these are commercially available kits. However, serology is a powerful tool to detect expression of specific enzymes, surface antigens, and toxins essential for accurate identification (65,72). New techniques are developed every year and described in such journals as "Biotechniques". Some currently available techniques are listed below.

PCR primers are available for several pathovars of *P. syringae* (Table 4).

1) Lipodepsinonapetide toxin gene of *P. syringae* pvs. *syringae, atrofaciens* and *aptata (63):*

Either whole-cell suspensions of bacteria grown overnight in potato dextrose broth at 25°C or purified genomic DNA can be used. Use a standard protocol to isolate genomic DNA or use 10 ,ul of whole-cell suspensions after centrifugation and resuspension in sterile distilled water. Amplification is carried out using 100-200 ng of DNA, 0.5 *,uM* of primer Bland B2 (Table 4), 200 *,uM* of each dNTP, 1.5 mM $MgCl_z$, 1 x PCR reaction buffer (50 mM KCI, 10 mM Tris-Hel, pH 8.3), and 0.025 U of *Taq* DNA polymerase per ,ul in a 100 ,ul reaction. Use the following conditions:

- DNA denaturation at 94 ° C for 1.5 min
- Primer annealing at 62 ° C for 1.5 min
- DNA elongation at 72°C for 3.0 min for 35 cycles
- Add an additional extension at 72 ° C for 10 min after the cycling period

The amplified DNA (5 *,ul* ofPCR products) is then separated on 1 % agarose gels and stained with ethidium bromide to determine ifa 0.752 kb amplification product is present.

2) Phaseolotoxin gene of *P. syringae* pv. *phaseolicola*

A direct, fast and highly sensitive PCR method for specific identification of *P. syringae* pv. *phaseolicola* strains was developed by Prosen et al. (53) and modified and improved by Schaad et al. (60). Even the rare *{ox* - strains apparently contain the *{ox* gene cluster. The method involves the amplification of a DNA segment of the phaseolotoxin gene cluster, which is a valuable characteristic trait of

this pathogen, since it is not produced by any other *P. syringae* pathovar. The BIOPCR method (60) combines biological (growth on agar media) and enzymatic amplification properties and therefore detects even smaller numbers of the pathogen (2-3 CFU) in seed extracts, excluding dead cells.

PCR reactions consist of standard PCR with the external primer pair (P 5.1 and P 3.1, Table 4) under the following conditions:

- Initial incubation at 94 ° C for 2 min
- A manual "hot start" step at 80 ° C
- DNA denaturation at 94°C for 1 min for 25-30 cycles
- Primer annealing at 58°C for 1 min for 25-30 cycles
- DNA elongation at 72°C for 2 min for 25-30 cycles
- Final DNA extension at 72°C for 8 min

The nested PCR reaction follows by re-amplification of2 J,ll of 10-fold diluted PCR products with the internal primer pair (P 5.2 and P 3.2, Table 4) for 25 cycles. Amplifications are carried out in 0.5 mI thinwall tubes, in a final volume of 50 J,ll. Reaction mixtures contain: 10 mM Tris-HCI (pH 8.3),50 mM KCI, 1.5 mM $MgCI_2$, 0.001% gelatin, 80 *J,lM* each ofdNTP, 0.2 units of *Taq* DNA polymerase and 0.5 *J,lM* of each primer. The amplified PCR products (5 J,ll) are then separated on 1 % agarose gel and stained with ethidium bromide. The size of expected products is 0.5 kb for external primers and 0.45 kb for internal primers.

3) Coronatine gene of *P. syringae* pvs. *tomato, atropurpurea, glycinea;* and *morsprunorum:*

The 1.4-kb eft-gene (coronafacate ligase) seems required for coronafacid acid adenylation and ligation to coronamic acid forming the complete toxic coronatine molecule (3). Therefore, the eft-gene can be used for rapid PCR amplification and specific detection of coronatine-producing *P. syringae* pv. *tomato, atropurpurea, glyeinea, morsprunorum,* and *maculieola* (4, 5). The primer set amplifies diagnostic 650 bp PCR products from genomic DNA or whole-cells of the five different coronatine-producing bacteria. Use a standard procedure to isolate genomic DNA or use whole-cells of test strains and direct PCR. Amplification is carried out using 10 ng of DNA 10 *J,ll* of whole-cells diluted in water. The standard reaction mixture (50 *J,ll)* contains 25 pmol of each primer (Table 4),0.5 U of *Tth* DNA polymerase (from *Thermus thermophilus;* Pharmacia), 0.2 mM of each dNTP, 16 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8), 1.5 mM $MgCI_2$, 10 mM þmercaptoethanol, 160 J,lg of bovine serum albumin per mI, 5% dimethyl sulfoxide, and 1% Tween 20. Amplification under done at the following conditions:

Denaturation at 93 °C for 2 min in the first cycle, in subsequent cycles for 1 nun

Primer annealing at 67 °C for 2 min

- Elongation at 72 °C for 2 min

After 37 cycles (3.5 h) separate the PCR products (5 *,ul)* on a 1.5% agarose gel (1.5 to 2 hat 100 V) and determine if 0.65 kb amplification products are present.

4) Tabtoxin gene of *P. syringae* pv. *tabaci, garcae,* and *coronafaciens*

Gene probes of toxin gene fragments are available for Southern blots (32). However, no PCR protocol is available for rapid identification of tab toxin producing strains.

5) 168 rRNA

A highly selective PCR protocol based on 16s rRNA genes is useful for identification of *Pseudomonas* spp. in environmental samples (71). Using rep-PCR primers several species and/or pathovars can be identified (Table 4) (44, 45, 70). However, these techniques require considerably more expertise and expense.

See Appendix A for details.

b. Serological techniques

1) IAA which is produced only by *P. savastanoi* can be identified by enzymelinked imrnunosorbent assays (ELISA) with antibodies raised against purified IAA, which detect the extracted IAA compound in question.

2) Detection of coronatine toxin produced by *P. syringae* pvs. *tomato, atropurpurea, morsprunorum* by specific antibodies has been described (36) and may be used in an enzyme-linked immunosorbent assay (ELISA).

Several ELISA kits are available for identification of pseudomonads. AGDIA (Elkhark, IN) has a kit for *P. syringae* pv. *phaseolicola; D-Genos* (Angers, France) has kits for *P. syringae* pvs. *tomato* and *phaseolicola* and *P. savastonoi;* ADGEW (Scotland, UK) has kits for *P. syringae* pvs. *phaseolicola, pisi, glycinea,* and *lachrymans,* and *P. viridiflava.*

See Appendix B for details.

c. Commercial automated techniques

1) Carbon source utilization

Kits, such as the Biolog GN Microl'late" system (Biolog, Inc., Hayward, CA) are available for rapid identification at the species level and higher. The system provides a standardized method using biochemical tests to identify a broad range of enteric, non-fermenting, and fastidious Gram-negative bacteria. Biolog's Microl.og" computer software identifies the bacterium from its metabolic pattern in the GN MicroPlate, which is based on the utilization (oxidation) of 95 different carbon sources.

Not recommended for correct identification at the pathovar level. For pathovar identification of the most common *syringae* species, the biochemical/nutritional tests in Table 1 are recommended.

See Appendix B for details.

2) Fatty acid composition

The Microbial Identification System (MIDI, Microbial ill Inc., Newark, Delaware) which identifies microbes based on their cellular fatty acid compositions, using fatty acid methyl esters of whole cells and high resolution gas chromatography is available.

Not recommended for identification at pathovar level. Recommended for genus and species identification, if library databases are regularly updated.

See Appendix B for description and details.

3) 16S rRNA

According to Applied Biosystems, the new Micro'Seq" 16S rRNA Gene Kit (PE Applied Biosystems, New Jersey) offers a unique advantage. By sequencing the 16S rRNA gene, you can identify biochemically inert bacteria that traditional methods fail to detect. The MicroSeq kit makes it fast and easy, with a complete solution that includes one-step reagent modules, software, and database.

7. CULTURE PRESERVATION

Once a pure culture of an organism has been obtained, it is important to preserve the culture for future references in a culture collection. For long-term storage, transfer a single colony to 3 ml NB Y broth using a sterile toothpick. Incubate at 28 ° C for 1 - 2 days with shaking at 100 rpm. Aseptically add 700 ,ul ofthe obtained culture to 300 ,ul sterile glycerol (30%). Vortex thoroughly and store in freezer at -70 ° C or lower. A loopful of the culture can be streaked on solid plates as needed.

Another, storage method is provided by the Microbank" system (pro-Lab Diagnostics, Austin, TX). Microbank" is a sterile vial containing porous beads which serve as carriers to support microorganisms. Individual colored beads are packaged approximately 25 beads in a cryovial containing cryopreservative. The beads are acid washed and are of a porous nature allowing microorganisms to readily adhere onto the bead surface. After inoculation the cryovials are kept at -70°C for extended storage. When a fresh culture is required, a single bead is easily removed from the vial and used to directly inoculate a suitable bacteriological medium. Short term storage at - 20 ° C is adequate.

Table 4. PCR Primers for *Pseudomonas* spp.

Specificity	Primer Designation	
Pseudomonas genus-specific	Ps-for	
P. putida	XylR-F1	
	XylR-R1	
Phloroglucinol-producing species (*P. fluorescens*, etc.)	Phl2a	
Phenazine-producing species (*P. fluorescens*, *P. aureofaciens*, *etc.*)	PCA2a	
	PCA3b	
[illegible] and *P. syringae*	A1	
	B6	
many species and *P. syringae* pathovars	[illegible]	
many *P. syringae* pathovars	[illegible]	

many *P. syringae* pathovars	ERIC1R
	ERIC2

LITERATURE CITED

1. Achouak, W., L. Sutra, T. Heulin, 1. Meyer, N. Gromin, S. Degraeve, R. Christen, and L. Gardan. 2000. *Pseudomonas brassicacearum* sp. nov. and *Pseudomonas thivervalensis* sp. nov., two root-associated bacteia isolated from *Brassica napus* and *Arabidopsis thaliana.* Int. 1. Syst. and Evol. Microbiol. 50:9-18.

2. Ayers, S. H., P. Rupp, and W. T. Johnson. 1919. A study ofthe alkali-forming bacteria in milk. U. S. Dept. of Agric. Bull. 782.

3. Bender, C. L., D. Palmer, A. Penaloza-Vasquez, V. Rangaswamy, and M. Ullrich. 1996. Coronatine, a plasmid-encoded virulence factor produced by *Pseudomonas syringae.* Pages 213-217 in: Proceedings Amer. Soc. Microbiol. annual meeting.

4. Bender, C. L., S. A. Young, and R. E. Mitchell. 1991. Conservation of plasmid DNA sequences in coronatine-producing pathovars of *Pseudomonas syringae.* Appl. Environ. Microbiol. 57(4):993-999.

5. Bereswill, S., P. Bugert, B. Volksch, M. Ullrich, C. L. Bender, and K. Geider. 1994. Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. Appl. Environ. Microbiol. 60:2924-2930.

6. Bradbury, 1. F. 1986. Pages 154-187 in: Guide to plant pathogenic bacteria. CAB International Mycological Institute, Kew, United Kingdom.

7. Clerc, A., C. Manceau, and X. Nesme. 1998. Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae.* Appl. Environ. Microbiol. 64: 1180-1187.

8. Cooksey, D. A. and 1. H. Graham. 1989. Genomic fingerprinting of two pathovars of phytopathogenic bacteria by rare-cutting restriction enzymes and field inversion gel electrophoresis. Phytopathology 79:745-750.

9. Crosse, 1. E. and C. M. E. Garrett. 1963. Studies on the bacteriophagy of *Pseudomonas morsprunorum, P. syringae* and related organisms. 1. Appl. Bacteriol. 26: 159-177.

10. Cupples, C. I., and A. Kelman., 1974. Evaluation of selective media for isolation of softrot bacteria from soil and plant tissue. Phytopathology 64:468-475.

11. Denny, T. P. 1988. Phenotypic Diversity in *Pseudomonas syringae* pv. *tomato.* 1. Gen. Microbiol. 134: 1939-1948.

12. Denny, T. P., M. N. Gilmour, and R. K. Selander. 1988. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae.* J. Gen. Microbiol. 134: 1949-1960.

13. Dye, D. W., J. F. Bradbury, M. Goto, A. C. Hayward, R. A. Leiliott, and M. N. Schroth. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Rev. Plant Path. 59: 153-168.

14. Endert, E. and D. F. Ritchie. 1984. Detection of pathogenicity, measurement of virulence, and determination of strain variation in *Pseudomonas syringae* pv. *syringae.* Plant Dis. 68:677-680.

15. Fieldhouse, D. J., and M. Sasser. 1982. A medium highly selective for *Pseudomonas syringae* pv. *glycinea.* Phytopathology 72:706. (Abstr.).

16. Gardan, L., C. Bollet, M. Abu Giiorrah, P. A. D. Grimont, and F. Grimont. 1992. DNA relatedness among pathovars *ofP. syringae* subspecies *savastanoi* Janse (1982) and proposal of *Pseudomonas savastanoi* sp. nov. Int. J. Syst. Bacteriol. 42:606-612.

17. Gnanamanickam, S. S., A. N. Starratt, and E. W. B. Ward. 1998. Coronatine production in vitro and in vivo and its relation to symptom development in bacterial blight of soybean. Can. J. Bot. 60:645-650.

18. Gross, D. C. 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. J. Appl. Bacteriol. 58: 167-174.

19. Gross, D. C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae.* Ann. Rev. Phytopathol. 29:247-278.

20. Hankin, L., M. Zucker, and D. C. Sands. 1971. Improved solid medium for the detection and enumeration of pee to lytic bacteria. Appl. Microbiol. 22:205-209.

21. Hendson, M., D. C. Hildebrand, and M. N. Schroth. 1992. Relatedness of *Pseudomonas syringae* pv. *tomato, Pseudomonas syringae* pv. *maculicola* and *Pseudomonas syringae* pv. *antirrhini.* J. Appl. Bacteriol, 73:455-464.

22. Hildebrand, D. C. and M. N. Schroth. 1968. Removal of pseudo monads from plant leaves and measurement of their in vivo P-glucosidase synthesis. Phytopathology 58:354-358.

23. Hildebrand, D. C. 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens. Phytopathology 12: 1430-1436.

24. Hildebrand, D. C., M. N. Schroth, and D. C. Sands. 1988. *Pseudomonas.* Pages 60-80 in: N. W. Schaad, ed., Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd

edition. APS Press, St. Paul, MN.

25. Hirano, S. S. and C. D. Upper. 1985. Ecology and physiology of *Pseudomonas syringae*. Bio Technology 3: 1073-1078.

26. Hoitink, H. A. H. and S. L. Sinden. 1970. Partial purification and properties of chlorosis-inducing toxins of *Pseudomonas phaseolicola* and *Pseudomonas glycinea.* Phytopathology 60: 1236-1237.

27. Holt, 1. G., N. R. Krieg, P. H. A., Sneath, 1. T., Staley, and S.T. Williams (eds.). 1994. Bergey's manual of determinative bacteriology. Ninth edition. Williams & Wilkins, Baltimore, MD.

28. Iacobellis, N. S., P. Lavermicocca, 1. Grgurina, M. Simmaco, and A. Ballio. 1992. Phytotoxic properties of *Pseudomonas syringae* pv. *syringae* toxins. Physiol. and Mol. Plant Pathol. 40: 107-116.

29. Johnson, 1. L. and N. 1. Palleroni. 1989. Deosyribonucleic acid similarities among *Pseudomonas* species. Int. 1. Syst. Bacteriol. 39:230-235.

30. Kersters, K, W., Ludwig, M. Vancanneyt, P. De Vos, M. Gillis, and K-H. Schleifer. 1996. Recent changes in the classification of the pseudomonads: an overview. System. Appl. Microbiol. 19:465-477.

31. King, E. 0., M. K Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. 1. Clin. Med. 44:301-307.

32. Kinscherf, T. G., R. H. Coleman, T. M. Barta, and D. K Willis. 1991. Cloning and expression of the tabtoxin biosynthetic region from *Pseudomonas syringae.* 1. Bacteriol. 173 :4124-4132.

33. Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 199:299-300.

34. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178:703.

35. Kuske, C. R., K L. Banton, D. L. Adorada, P. C. Stark, K K Hill, and P. 1. Jackson. 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol. 64:2463-2472.

36. Leary, 1. v., S. Roberts, and 1. W. Willis. 1988. Detection of corona tine toxin of *Pseudomonas syringae* pv. *glycinea* with an enzyme-linked immunosorbent assay.

Phytopathology 78: 1498-500.

37. **Leben, C. 1972. The development ofa selective medium for *Pseudomonas glycinea.* Phytopathology 62: 674-676.**

38. Lelliott, R. A, E. Billing, and A C. Hayward. 1966. A determinative scheme for the **fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.**

39. Levi, C., and R. D. Durbin. 1986. The isolation and properties of a tabtoxin-hydrolysing aminopeptidase from the periplasm of *pseudomonas syringae* pv. *tabact.* PhysioL Mol. **Plant Pathol. 28:345-52.**

40. Liang, L. Z., P. Sobiczews[ki], J. M. Paterson, and A. L. Jones. 1994. Variation in virulence, **plasmid content, and genes for coronatine synthesis between *Pseudomonas syringae* pv. *morsprnnornm* and *Pseudomonas* *syringae* from *Pnlm,ls.* Plant Dis. 78:389-392.**

41. Lindow, S. E., D. C. Arrrj, and C. D. Upper. 1978. *Erwinia herbicola:* a bacterial ice **nucleus active in increasing frost injury to corn. Phytopathology 68:523-527.**

42. Lindow, S. E., D. C. Amy, and C. D. Upper. 1983. Biological control of frost **injury: Establishment and effects of an isolate of *Envinia herbicola* antagonistic to ice nucleation active bacteria on corn in the field. Phytopathology 73: 1102-1106.**

43. Lindow, S. E. 1990. Bacterial ice-JUlcleation activity. Pages 428-434 in: Z. Klement, K. **Rudolph, and D. C. Sands, eds., Methods in Phytobacteriology. Akademiai Kiado, Budapest.**

44. Louws, F. J., D. W. Fulbright, C. T. Stephens, and F. J. de Bruiin., 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *pseudomonas* pathovars and strains generated with repetitive sequences and PCR. App. and Environ. MicrobioL 60:2286-2295.

45. Manceau, C. and A. Horvois. 1997. Assessment of genetic diversity among strains of *pseU£1lJmonas syringae* by PCR-restriction fragment length polymorphism analysis ofrRNA operons with special emphasis on P. *syringae* pv. *tomato.* Appl. Environ Microbiol. **63:498-505.**

46. Mohan, S. K. and N. W. Schaad. 1987. Semiselective agar medium for isolating *Pseudomonas syringae* pv. *syringae* and pv. *phaseolicola* from bean seed. Phytopathology 77: 1390-1395.

47. Morgan, M K. and A K. Chatterj ee. 1988 Genetic organization and regulation of proteins associated with production of syringotoxin by *pseudomonas syringae* pv. *syringae.* **J. Bacteriol. 170:5689-97.**

48. Ovod, v., P. Ashorn, L. Yakovleva, and K. Krohn. 1995. Classification of *Pseudomonas syringae* with monoclonal antibodies against the core and O-side chains of the lipopolysaccharide. Phytopathology 85:226-232.

49. Ovod, v., K. Rudolph, and K. Krohn. 1997. Serological classification of *Pseudomonas syringae* pathovars based on monoclonal antibodies towards the lipopolysaccharide 0chains. Pages 526-531 in: K. Rudolph, T. 1. Burr, 1. W. Mansfield, D. Stead, A. Vivian, and 1. von Kietzell, eds., Developments in Plant Pathology, Vol. 9: *Pseudomonas Syringae* Pathovars and Related Pathogens. Kluwer Academic Publishers. Boston.

50. Palleroni, N. 1., R. Kunisawa, R. Contopoulou, and M. Doudoroff 1973. Nucleic acid homologies in the genus *Pseudomonas.* Int. 1. Syst. Bacteriol. 23:333-339.

51. Palleroni, N. 1. 1984. Genus *Pseudomonas.* Pages 141-199 in: N. R. Krieg and 1. G. Holt, eds., Bergey's Manual of Systematic Bacteriology, Vol. 1. Williams & Wilkins, Baltimore.

52. Palmer, D. A. and C. L. Bender. 1993. Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. *glycinea.* Appl. Environ. Microbiol. 59: 1619-1626.

53. Prosen, D., E. Hatziloukas, N. W. Schaad, and N. 1. Panopoulos. 1993. Specific detection of *Pseudomonas syringae* pv. *phaseolicola* DNA in bean seed by PCR-based amplification ofa phaseolotoxin gene region. Phytopathology 83:965-970.

54. Raaijmakers,1. M., D. M. Weller, and L. S. Thomashow. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environment. Appl. Environ. Microbiol. 63: 881887.

55. Roos, I. M. M., and M. 1. Hattingh. 1987. Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. Phytopathology 77:900-908.

56. Rudolph, K. 1990. Toxins as taxonomic features. Pages 251-267 in: Z. Klement, K. Rudolph, and D. C. Sands, eds., Methods in Phytobacteriology. Akademiai Kiado, Budapest.

57. Sands, D. C., M. N. Schroth, and D. C. Hildebrand. 1970. Taxonomy of phytopathogenic pseudomonads. 1. Bacteriol. 101:9-23.

58. Saunier, M., L. Maladrin, and R. Samson. 1996. Distribution of *Pseudomonas syringae* pathovars into twenty-three O-serogroups. Appl. Environ. Microbiol. 62:2360-2374.

59. Schaad, N. W., S. Suele. J, W. L. van Vuurde, H. Vruggink, A. M. Alvarez, A. A. Benedict,

L. de Wael, and O. van Laere. 1990. Serology. Pages 153-190 in: Z. Klement, K. Rudolph, and D. C. Sands, eds., Methods in Phytobacteriology. Akademia Kiado, Budapest.

60. Schaad. N. W., S. S. Cheong, S. Tamaki, E. Hatziloukas, and N. J. Panopoulos. 1995. A **combined biological and enzymatic amplification (BIO-PCR) technique to detect.** *pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. Phytopathology 85:243-248.

61. Schaad, N. W., A. K Vidaver, G. H. Lacy, K Rudolph, and J. B. Jones. *2000.* Evaluation **of proposed amended names of several pseudomo[nads] and xanthomonads and recommendations. Phytopathology 90:208-213.**

62. Skerman, V. B. D., V. McGawavi, and P. H. A. Sneath. *1980.* Approved list of bacterial **names. Int. J. Syst. Bacteriol. 30:225-420.**

63. Sorensen, K N., K H. Kim, and J. K Takemoto. 1998. PCR detection of cyclic Iipodepsinonapeptide-producin[g] *Pseudomonas syringae* pv. *syringae* and similarity of **strains. Appl. Environ. Microbiol. 64:226-230.**

64. Staskawicz, B. J. and N. J. Panopoulos. 1979. A rapid and sensitive microbiological assay **for phaseolotoxin. Phytopathology 69:663-666.**

65. Stead, D. E. 1992. Classification of *Pseudomonas syringae* pathovars by fatty acid protiling. Pages *381-390* in: Proceedings of Working Group on *Pseudomonas syringae.* **University** of Florence, **Florence, Italy.**

66. Tesar, M., C. Hoch, E. R. B. 'Jloore, and K N. Timmis. 1996. Westprinting: Development **of a rapid immunochemical identification for species within the genus *Pseudomonas sensu stricto.* System. Appl. Microbiol. 19:577-588.**

67. Thornley, M. J. *1960.* The differentiation of *Pseudomonas* from other Gram negative **bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. 1:37-52.**

68. Utematsu, T., A. T akatsu, and K Ohata. 1982. A medium for the selective isolation of ***Pseudomonas** cichorii.* **Ann. Phytopath. Soc. Japan. 48:425-432.**

69. Vancanneyt, M., S. Witt, W.-R. Abraham, K. Kersters, and H. L. Fredrickson. 1996. Fatty **acid content in whole-cell hydrolysates and phospholipid fractions of pseudomonads: a taxonomic evaluation. System. Appl. Microbiol. 19:528-540.**

70. **Weingart, H. and B. V** olksch. **1997. Genetic fingerprinting of *Pseudomonas syringae*** pathovars using ERlC-, REP-, and JS5Q-PCR. J. Phytopathology 145:339-345.

71. Widmer, F, R. 1. Seidler, P. M. Gillevet, L. S. Watrud, and G. D. DiGiovanni. 1998. A highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas (sensu stricto)* in environmental samples. Appl. Environ. Microbiol. 64:2545-2553.

72. Young, 1. M., 1. F. Bradbury, R. E. Davis, R. S. Dickey, G. L. Ercolani, A. C. Hayward, and A. K. Vidaver. 1991. Nomenclatural revision of plant pathogenic bacteria and list of names 1980-1988. Rev. Plant Path. 70:211-221.

73. Young, 1. M., Y. Takikawa, L. Gardan, and D. E. Stead. 1992. Changing concepts in the taxonomy of plant pathogenic bacteria. Ann. Rev. Phytopath. 30:67-105.

74. Young, 1. M. and C. M. Triggs. 1994. Evaluation of determinative tests for pathovars of *Pseudomonas syringae* van Hall 1902. 1. Appl. Bacteriol. 77: 195-207.

75. Young, 1. M., G. S. Saddler, Y. Takikawa, S. H. De Boer, L. Vauterin, L. Gardan, R. I. Gvozdyak, and D. E. Stead. 1996. Names of plant pathogenic bacteria 1864-1995 - The ISPP accepted list of bacterial names. Rev. Plant Path. 75:721-763.

I. CHEMICAL **LIST**

Chemical	**Source**
Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O. Box 14508, St Luis, MO 63178.	
Adonitol	
Aesculin	
Agar Agarose	Difco
p-alanine	
Ampillicin	
Anthranilate	
DL-arabinose	
Arbutin	
Arginine HCI	
L-arginine D-aspartate	
Azalate	
Bacitracin	
Benomyl	
Benzoate	Target Chemical Co.
Betaine	
Boric acid	
Bovine serum albumin	1.T. Baker

Bromthymol blue	
Butyrate	
Butylamine csct, CaClzeHzO	IT. Baker
Casein hydrolysate	Difco
Cellobiose	
Cephalexin	
Cetrimide	
Chlorothalonil (Bravo 500) Fungizid	Diamond Shamrock
Citrus pectin	
Citrulline	
Corundum	
Crystal violet	Buehler Ltd.
CUS04	
L-cystine	IT. Baker
Cycloheximide	
Dimethylsulfoxide	
EDTA-Fe	
Erythritol	
Ethidium Bromide	
FeS04e7H20	
$FeCl_3$	J.T. Baker
Ferric citrate	
D-fructose	
D-galactose	
Gelatin	
Geraniol D-glucose	
Glutamine	
Glutarate	
DL-glycerate	
Glycerol L-histidine	
H_3B0_3	
Hexadecyltrimethylammonium bromide	IT. Baker
DL-homoserine	
DL-P-hydroxybutryate	
Inositol	
m-inositol	
KCl	
K_ZHP0_4	IT. Baker
KzHP°4e3HzO	J.T. Baker
	J.T. Baker

KH_zP0_4	1. T. Baker
$KN0_3$ L-lactate	1.T. Baker
Lecithinase	
Levulinate	
Lysozyme	
D-mannitol	
Malonate	
p-mercaptoethanol	
Methylviolet	1. T. Baker
$MgCl_z$	1.T. Baker
$MgS0_4$	1.T. Baker
$MgS0_4e7H_zO$	
Mineral Oil (sterile)	J.T. Baker
$MnS0_4$	1.T. Baker
$Mo0_3$	
NaCI	1.T. Baker
N~HP04	
N~HP04e12HzO	1.T. Baker
N~Mo04e2HzO	
NaOCl	1.T. Baker
NaOH	1. T. Baker
NH_4CI	1.T. Baker
$(NH4)zH_zP0_4$	1.T. Baker
$(NH4)ZS04$	
Nicotinate Psnitrophenyl-jl-glucosidase Noble agar	Difco
Novobiocin	
L-ornithine	
Oxidase (dihydrochloride diamethyl tetraethyl-p-phenylenediamine)	
Paraffin	Kodak
Penicillin	
Peptone	Difco
Phenethicillin Phenol red	
Phenyl acetate	
Plate count agar poly-p-hydroxy butyrate	Difco
Potassium phenethicillin	
Potassium tellurite	
Proteose peptone #3	Difco
N-propanol	Mallinckrodt

Propylene glycol	
D-quinate D-	
quinic acid L-	
rhamnose	
Rifampicin	
Saccharate	
Safranine	Fisher
Salicin	
D-serine	
Sodium ampicillin	
Sodium citrate Sodium	
dodecyl sufate Sodium	
polypectate Sodium	Dr. M. Burger, Eton Ridge Road, Madison, WI. USA
tartrate D-sorbitol	
Sucrose	
Sudan black B	
D(-)-tartrate	
L-tartrate	
meso-tartrate	
Thiram-benomyl wettable powder	
Trehalose	Target Chemical Co.
Trigonelline	
Triphenyl tetrazolium HCI	
Tween 20	
Vancomycin	
Valerate	
Xylene	
Yeast extract	Mallinckrodt
$ZnS0_4$	Difco
	1. T. Baker

u GRAM-NEGATIVE BACTERIA

D. *Acidovorax* and *Xylophilus*

J. B. Jones, R. D. Gitaitis, and N. W. Schaad

1. INTRODUCTION

a. *Acidovorax*

The genus *Acidovorax* was originally proposed in 1990 by Willems et al. (27). In 1992, Willems et a1. (28) proposed transferring those plant pathogens previously classified as *Pseudomonas avenae* (14), *P. pseudoalcaligenes* subsp. *citrulli* (15), and *P. pseudoalcaligenes* subsp. *konjaci* (8) into this newly proposed genus as subspecies of *Acidovorax avenae.* Bacteria within this genus belong to the acidovorans rRNA complex in rRNA superfamily ITI (4) which are included in the beta subclass of the Proteobacteria (19). Willems et al. proposed a new family, *Comamonadaceae* (25), for those members of rRNA superfamily ITI. Willems et al. (27) provided two reasons for placing several *Pseudomonas* spp. within the genus, *Acidovorax.* These were (i) all *Acidovorax* species form a separate rRNA sub-branch within the acidovorans rRNA complex; and (ii) the genus *Acidovorax* can be differentiated phenotypically from the other taxa in the acidovorans rRNA complex. The *Acidovorax* spp., originally placed in this genus (27), are not phytopathogens and are found in soil, water, and clinical environments.

In the original designation ofrRNA groups by Palleroni et al. (12), section ill contained no phytopathogens. However, more recent DNA-rRNA hybridization analyses has revealed that *Pseudomonas avenae, P. cattleyae, P. pseudoalcaligenes* subsp. *citrulli, P. pseudoalcaligenes* subsp. *konjaci, P. rubrilineans,* and *P. setariae* were closely related to members of superfamily lIT and were assigned (28) to the acidovorans rRNA complex (4). All the phytopathogenic pseudomonads within this rRNA complex, with the exception of *P. setariae,* were on the 1980 approved list of bacterial names (18). Van Zyl and Steyn (23) were able to distinguish *P. setariae* and *P. rubilineans* from *P. avenae* based on numerical analysis of polyacrylamide gel electrophoregrams of soluble proteins. They stated that *P. setariae* should be revived as a species; furthermore they determined that *P. setariae* and *P. rubilineans* were closely related to each other. However, Willems et a1. (28), using DNA-DNA hybridization data, proposed that *P. setariae* and *P. rubilineans* be placed in *Acidovorax.* Fatty acid profile data supported the close relationship between *P. rubilineans* and *P. avenae (23).*

Phytopathogenic strains within superfamily III were not originally placed within the genus *Acidovorax. Pseudomonas avenae* had relatively low DNA-rRNA similarities with strains within *Acidovorax* and were no closer to *Acidovorax* than other members of the Comomonadaceae including strains within *Hydrogenophaga, Comomonas* or *Variovorax* (27). However, based on phenotypic characteristics the phytopathogenic bacteria that belonged within the acidovorans rRNA complex were determined to be more closely related to the genus *Acidovorax* than to the latter three genera (Table 1). Two main DNA groups of phytopathogenic strains have been

identified. The first group is composed of *A. avenae* subsp. *avenae* (including *P. setariae* and *P. rubiiineansy, A. avenae* subsp. *cattleyae,* and *A. avenae* subsp. *citrulli,* while the second is *A. konjaci.*

The bacterium is a strictly-aerobic, Gram-negative, straight to slightly curved rod-shaped, 0.2 to 0.8 by 1.0 to 5.0 urn and motile by a single polar flagellum (rarely by two or three polar flagella). All strains are oxidase positive. No pigment is produced on nutrient agar. The strains of *A. konjaci* tum certain media (i.e., King medium B, yP agar, potato glucose agar) deep brown. Plant pathogenic strains within this genus cause seedling blights, leaf spots, leaf blights, and fruit blotches. Due to their unique metabolism these bacteria are rather easy to distinguish from other plant pathogens.

b. *Xylophilus*

This genus contains a single plant pathogenic species, *X ampelinus (Xanthomonas ampelina),* the causal agent of bacterial blight of grape. The disease causes severe losses to certain cultivars of grape in Europe and South Africa. Plants often fail to produce new growth in the spring or are severely stunted and eventually die (5). Cankers develop on petioles and fruit stalks. The disease has not been reported in North America. Grape is the only known host. The organism spreads via pruning wounds and cuttings. Latent infections are common and because the organism is very fastidious it is difficult to isolate (13). The organism grows very slowly on general plating media.

The causal agent of bacterial blight of grape was first described as *Erwinia vitivora* (5). In 1969 Panagopoulos described the causal agent of bacterial blight of grape in Greece as *Xanthomonas ampelina* and discussed the importance of using Difco nutrient agar (see a, p. 3) to isolate the organism (13). Because the organism was not characteristic of the genus *Xanthomonas,* it was reclassified in 1987 and put in a new genus, *Xylophilus* (26). The bacterium is aerobic, Gram-negative, rod shaped and 0.4-0.8 x 1.0-2.0 urn,

AlthoughX *ampelinis* is yellow on NA" YDC, and GYGP, the organism is quite easy to differentiate from *Xanthomonas spp.* because 10 - 12 days incubation are required for colonies to reach 1 mm diameter. The colonies are round and only slightly mucoid on YDe. The organism is urease positive (all xanthomonads are urease negative), and has an optimum and maximum temperature for growth of 24 ^{0}C and 28^{0}, respectively. It produces acid from arabinose and galactose but not from flucose or maltose, nor is esculin hydrolyzed or gelatin liquefied; growth is improved by glutamic acid (2). All strains are oxidase negative and produce H_2S from cysteine (16).

2. ISOLATION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

a. *Acidovorax*

1) Recipes for differential media:

The three subspecies of *A. avenae* can be readily isolated from lesions by directly plating onto beef-yeast extract or YDC agar. *A. konjaci* can be isolated by crushing the lesion in sterile water and streaking a loopful of resulting suspension on yeast-peptone (YP) agar plates (8).

a) Beef-yeast extract

	perL
Beef extract	1.0
Yeast extract	g
Agar	2.0 g
Adjust to pH 7.4 then autoclave	20.0 g

This medium is used to isolate *A. avenae* subsp. *avenae, A. avenae* subsp. *citrulli,* and *A. avenae* subsp. *cattleyae* from plant tissue. Colonies appear beige in color.

b) YDC (see b, p, 4)

Colonies of *A. avenae* are easily differentiated from most plant pathogenic bacteria. Colonies are convex 2 - 3 mm in diameter after 2 days at 3032°C, dark beige in color, and become very sticky after 3 - 4 days.

c) YP agar (8)

	perL
Yeast extract	5.0 g
Peptone Agar	10.0 g
	15.0 g

This medium is used to isolate *A. konjaci* from plant tissue. The bacterium turns deep brown in 3 to 5 days.

2) Recipes for semiselective media:

a) Sorbitol neutral red (SNR) agar (22)

per 950 ml 3.0 g LOg LOg 0.3 g

K_zHP0_4	10.0 ml
NaH_zP0_4	
$KN0_3$	
$MgS0_4 \cdot 7H_zO$	
Neutral red, (0.2% aqueous solution 69% active)	

Agar	15.0 g

Autoclave at 121°C for 15 min, cool to 50°C and add a mixture of the following filtered sterilized stock solutions:

	perL
Cycloheximide (100 mg/ml 75% ethanol)	2.0 ml
D-sorbitol (10% aqueous)	50.0 ml

The medium is useful for isolating *A. avenae* subsp. *avenae* from soil and plant debris.

b) WFB 08 medium (7)

	perL
NaCl	5.0 g
NH_4P0_4	1.0 g
K_ZHP0_4	1.0 g
$MgS0_4 \cdot 7H_zO$	0.2 g
Phenol red	0.01 g
Methyl violet	
B	1.0 ml
(1.0% aqueous stock solution)	
Berberine	0.2 g
Pourite	5.0 Jll
P-Hydroxybutyric acid	3.0 g
Agar	15.0 g
Cycloheximide	0.1 g *

Adjust pH to 7.2-7.4 prior to autoclaving.

* Added aseptically after autoclaving.

This medium is used for isolation of *A. avenae* subsp. *citrulli* from seed.

c) **WFB 44 medium** (7)

	perL
NaCI	5.0 g
NH_4P0_4	1.0 g
K_ZHP0_4	1.0 g
$MgS0_4 \cdot 7H_zO$	0.2 g
Phenol red	0.01 g
Methyl violet	
B	1.0 ml
(1.0% aqueous stock solution)	
Berberine	0.2 g
Pourite	5.0 Jll
D(-) Tartaric acid	3.0 g

CMC (high viscosity sodium salt of carboxymethylcellulose)	26.0 g * 9.0
Agar	g 30.0 mg
Cefaclor	** 0.1 g **
Cycloheximide	

* Added slowly to salts solution while blending at high speed in Waring" blender, pH adjusted to 7.2-7.4, and poured hot.

** Added aseptically after autoclaving

This medium is used for isolation of *A. avenae* subsp. *citrulli* from seed and is diagnostic with development of sunken pits on the medium (7).

d) Modified Ethanol Bromcresol purple brilliant blue (mEBB) medium (16).

	perL
$NH_4H_2P0_4$	2.6
KH_2P0_4	g 0.8
$KHP0_4$	g
KCl	0.3 g
$MgS0_407H_20$	0.2 g
Yeast extract	0.2 g
Boric acid	0.3 g
Bromcresol purple (15 mg/ml stock)	0.5 g
Brilliant blue R (10 mg/ml stock)	0.6ml
	1.0 ml

Colonies of *A. avenae* subsp. *citrulli* are round, 1 - 2 mm diameter after 34 days at 36°C. They continue to enlarge and develop olive green centers and a fried egg morphology after 4 - 5 days. Colonies of most all other bacteria on mEBB medium are blue and remain small.

This semiselective medium is useful for isolation of *A. avenae* subsp. *citrulli* from cucurbit seeds.

b. *Xylophilus*

Isolation is most successful during the early spring or late fall (1. 1. Serfontein, personal communication). To isolate from stalks and stems, surface sterilize a 2-3 em section, remove outer tissue and place small pieces of tissue into a droplet of phosphate buffered saline (PBS) (pH 7.0). After 15 - 20 mins, macerate the tissue and streak onto Difco NA (13). A novel *in situ* enrichment technique works best (17). Place stem samples in a plastic bag containing moistened paper and incubate for 2-3 days at 20-24°C and isolate as above.

1) Difco nutrient agar (see a, p. 3).

Follow manufacturers directions. Add 2.0 ml stock cycloheximide (1.0 gila ml 75% ethanol) after autoclaving.

This medium should be used for isolating directly from tissue (13).

2) GYGP agar (N. W. Schaad and A. Sechler, unpublished)

	perL
Galactose	20.0 g
Yeast extract	7. a g
$CaC0_3$, finely ground	3. a g
Glutamic acid	4. a g
K_ZHP0_4	1.5 g
KH_ZP0_4	0.5 g
Agar	16.0 g

This medium is useful for growing the organism for characterization, but should not be used for isolation.

Some strains of *Xylophilus ampelinus* develop a yellow (light to dark, depending on the strain) non-diffusible pigment. Colonies are visible after 4-5 days at 24°C and are round, convex and 1 mm in diameter after 6-7 days, depending on the strain.

3. DIFFERENTIATION OF COMMONLY ISOLATED SPECIES AND SUBSPECIES

3. *Acidovorax*

Placement of the strains into the correct genus using phenotypic characteristics involves one major assumption, which is that the strains being characterized are considered plant pathogens. Once it is established that the bacterium is a plant pathogen, placement of unknown strains into the correct genus involves processing the strains using specific phenotypic characteristics. There are several phenotypic characteristics that are useful for differentiating the phytopathogenic *Acidovorax* spp. from other phytopathogenic, Gram-negative, aerobic, bacteria. All phytopathogenic *Acidovorax* species accumulate poly-jl-hydroxybutryate (PHB), are motile by 1 or occasionally 2 polar flagella, do not produce a fluorescent pigment or a yellow water insoluble pigment, and give a positive hypersensitive reaction (HR.) (Tables 1 and 2). None utilize arginine or betaine.

Differentiation of the strains within this group is difficult and relies for the most

part on pathogenicity tests. However, there are unique characteristics between the different subspecies of *avenae* (Table 3). Subspecies *avenae, cattleyae,* and *citrulli* utilize L-arabinose, D-galactose, and ethanolamine, whereas only subsp. *konjaci* strains utilize malonate. All but subsp. *citrulli* utilize L-threonine and are positive for nitrate reduction. Only subsp. *avenae* produces a white precipitate zone around colonies grown on lactalysate-meat extract medium or produces a precipitate in nutrient broth.

Table 1. Differentiation of plant pathogenic *Acidovorax andXylophilus* from other genera of *Comomonadaceae*

Characteristic	*Acidovorax*	*Xylophilus*	*Comomonas*	*Hydrogellophaga*	*Variovorax*
Cell morphology	Rods	Rods	Rods to spirilla	Rods	Rods
Flagella	Polar, 1 to 2	Polar, 1	Bipolar.I to 5	Polar, 1	peritrichous
Yellow insoluble pigment on nutrient agar		+		+	+
Growth at 41 *DC*	+		+		+
Carbon sources utilized for growth:				+	
Adonitol or L-arabitol					+
D-glucose	+			+	+
Glycolate		ND	+D	+	+
L-mandelate		ND	+D	+	+D
L-tyrosine	+	ND	+D	+D	
Isolated from infected plant	+	+			
Hypersensitive reaction	+	ND			

+, 80% or more strains positive; +D, 80% or more strains positive but delayed; -, 80% or more strains negative; ND, not determined.

Table 2. Comparison of phytopathogenic species *ofAcidovorax* with other phenotypically similar plant pathogens.

Characteristic	*Acidovorax*	*Pseudomonas*	*Ralstonia*	*Burkholde,.ia*
Flagella Growth	Polar, 1 to 2	Bipolar, 1 to 5	Polar, 1 to 4	Polar, 1 to 4
at 41 *DC*	+			
Accumulates poly-P-hydroxybutyrate Fluorescent	+		+	+
pigments produced by most strains Carbon		+		
sources utilized for growth:				
Arginine		+		+
Betaine				+
Citraconate	+	ND		V
L-arabinose	+"			+
Sucrose	_b	+	+	V
Ethanol	+			
n-PToponal	+			

+, 80% or more strains positive; V, between 21 - 79 % of strains positive; -, 80% or more strains negative. [a] All positive exceptA. *konjaci,*

[b] All negative exceptA. *avenae* subsp. *cattleyae.*

Table 3. Differentiation between *Acidovorax avenae* subspecies and.L *konjaci,*

Characteristic	*avenae*	*cattleyae*	*citrulli*	*A. konjaci*
Growth on:				
D-xylose	+		+0	
D-fucose	+	+	+0	+
D- mannitol or D-arabitol	+	+		+
Sorbitol	+	+		ND
Sucrose		+		
Lthreonine or L-histidine	+	+		ND
Nitrate reduction	+	+		+
Lactalysate or beef extract ppt.	+			
Starch hydrolysis	+0	+D		
Pathogenicity on:				
Watermelon		ND	+	
Canteloupe		ND	+	
Squash		ND	+	
Pumpkin		ND	+	
Com	+	ND		
Rice	+	ND		
Phaienopsis spp.		+		
Cattieya spp.		+		
Amorphophaiius konjac				+

+,80% or more strains positive; *+D,* 80% or more strains positive but delayed; ., 80% or more strains negative; ND, not determined

b. *Xylophilis*

There is only one species *X ampelinus*

4. DIAGNOSTIC MEDIA AND TESTS

a. *Acidovorax*

1) Poly-p-hydroxybutyrate accumulation (PHB) (see 4 a, p. 157, also)

Many aerobic, Gram-negative bacteria produce inclusions of poly-P-hydroxybutyrate. Detection of PHB granules (3,6,21) can be done by phase contrast or oil immersion with or without epifluorescence. PHB inclusions are most prominent on media of high carbon/nitrogen ratio. A known positive *(Ra/stonia solanacearum)* and negative *(Pseudomonas syringae)* should be included. Detection of the polymer is possible by several methods. Below are listed three methods described by Lelliott and Stead (10).

Technique 1:

Grow in shake-culture in a basal Ayers et al. medium (see f, p. 96) (1) containing 0.5% DL-p-hydroxybutyrate (sodium salt). When adequate growth (circa 2 days) has occurred, observe smears or wet preparations by phase contrast microscopy and look for circular, hyaline granules inside the cell.

Technique 2:
Flood a heat-fixed smear from inoculum prepared in technique 1. Flood the smear with Nile blue A (1% aqueous solution) for 10 minutes at 55°C. Wash briefly in tap water. Flood with 8% aqueous acetic acid for 1 minute to remove excess stain, wash again in tap water and blot dry. Add a drop of water, then cover with a cover slip and examine under epifluroescence at 450 nm for PHB granules which fluoresce bright orange.

Technique 3:
Prepare a 0.3% solution of Sudan black B in 70% ethanol. The solution should not be used for circa 12 h until dye has dissolved. Prepare a heat-fixed smear and flood with the dye. Incubate for 10 minutes. Drain and blot dry. Briefly flood with xylol or water to remove excess dye. Drain and blot dry. Counterstain for 10 seconds with 0.5% safranin. Wash offwith tap water, blot dry and view under oil immersion. PHB granules appear blue-black.

2) Carbon source utilization (see f, p. 96)

Carbon sources are filter sterilized and added at 0.1 % w/v final concentration to autoclaved and cooled «50°C) Ayers et al. (1) mineral salts medium, which also contains 1.2% purified agar. The pH is adjusted to 7.2. Bacteria are suspended in sterile water and streaked onto the medium. Growth is compared to plates containing no added carbon source. It is important to use purified agar.

3) Hypersensitive reaction (see e, p. 96)

A. avenae subsp. *avenae*, *A. avenae* subsp. *citrulli*, *A. avenae* subsp. *cattyleae*, and *A. konjaci* produce a hypersensitive reaction on tobacco.

4) Nitrate reduction

Acidovorax species do not ferment nitrogen, but some use nitrate as a terminal electron acceptor when placed in an anaerobic environment.

Technique 1:

Reagents

a) Nitrate medium (20) per L distilled water

	perL
KNO_3	1.0 g
Pepton e	5.0 g

Yeast extract	3.0 g
Oxoid agar NO.3	3.0 g
Adjust to pH 7-7.2	

Dispense 8 ml per test tube and autoclave for 15 min.

b) Follett and Ratcliff's reagent

	perL
Glacial acetic acid	50.0 ml
Distilled water	360.0 ml
Sulphanilic acid a-	0.25 g
naphthol	0.20 g
10% (v/v) aqueous 0.88 ammonia solution.	90.0 ml

Add the acetic acid to water, mix and heat to 50°C. Pour into dark, glass container which contains sulphanilic acid. When the acid is dissolved add ex-naphthol and dissolve. Cool to room temperature, add ammonia solution and store in dark at 4 ° C.

Procedure:
Grow test bacterium in the nitrate medium for 3-5 days. Add equal volume of Follett and Ratcliff's reagent of one inoculated and uninoculated tube and shake well. Development of an orange to orange-brown color is a positive reaction and indicates nitrate in the medium. If no reaction occurs within 10 minutes add a trace of cadmium powder and record reduction of nitrate beyond nitrite if no orange color develops in 10 min. If this color does develop, record absence of nitrate reduction. Non-inoculated tubes should develop color in presence of reagent and metal.

Technique 2 (see h, p. 97):
A medium containing yeast extract, 5 g/ml; $KN0_3$ 3 gil; Noble agar, 1 gil; and Ayers et al. (1) mineral salts, is dispensed in tubes, autoclaved, cooled, inoculated with the bacterium and covered with molten, sterile 3% Noble agar or sterile mineral oil. Growth at 27°C within 5 days is recorded as positive.

5) **Fluorescent pigment**

Medium B of King et al. (9), (d, p. 4) is commonly used for identifying the fluorescent green or blue, water soluble pigments. After growing the bacterium on the medium for 24 to 48 h at 25-28 ° C, the plates are examined for fluorescence with a long wave-length ultraviolet lamp. *Acidovorax* strains do not fluoresce on this medium.

6) **Precipitate reaction in nutrient broth** (14) **or lactalysate-meat extract medium** (7):

a) Nutrient broth (see a, p. 3). Seed tube of nutrient broth and incubate without shaking at 36°C. Presence of white precipitate is considered positive.

b) Lactalysate medium

	perL
Lactalysate	20.0 g
Agar	18.0 g
Beef extract (Difco)	5.0 g

Streak bacterial suspensions on the agar plate in a standard manner. Incubate at 36°C for 48-72 h. Colonies *ofA. avenae* subsp. *avenae* will be surrounded by an immediate area of clear haloes within an area of a white precipitate.

7) Starch hydrolysis

	perL
Nutrient broth (Difco)	8.0 g
Soluble potato starch	10.0 g
Bacto agar (Difco)	15.0 g

Add nutrient broth, agar and potato starch to 1 liter of water and autoclave. Pour plates. Inoculate a 24 to 48 h old culture on the agar surface. Incubate at 28 ° C for at least 4 days. Scrape away part of the bacterial growth and add Lugol's iodine. The medium turns blue when starch has not been hydrolyzed and clear where it has been hydrolyzed.

b. *Xylophilus*

An important diagnostic feature of *X ampelinis* is its very slow growth.

1) Growth on Difco nutrient agar (NA) at 24 and 30°C.

Prepare dehydrated NA according to manufacture or as described earlier (see 2), p. 3).

Colonies are barely visible after 7 days and only reach 1 mm after 12 - 15 days at 24°C. No growth occurs at 30°C.

2) Growth and pigment production on GYGP.

Produces yellow, round, convex colonies 1 mm in diameter at 24 ° C in 7-10 days.

3) Urease reaction (see e, p. 47).

All strains are positive.

4) Acid production from arabinose and galactose but not glucose and maltose.

(see f, p, 47).

5) Esculin hydrolysis (see d, p. 190).

All strains are positive.

6) Gelatin liquefaction (see e, p. 46).

All strains are positive.

7) Oxidase reaction (see 7, p. 10).

All strains are negative.

8) Production of H_2S from cysteine (see h, p. 47).

All strains are positive.

5. PATHOGENICITY TESTS

a. *Acidovorax*

Inoculation techniques vary based on pathogen and host. Inoculation techniques include wounding the plant tissue with a needle and applying inoculum to the wound site; spraying without wounding and applying inoculum to the plant surface and incubating under moist conditions; applying the bacterial suspension with carborundum which creates wounds on the plant tissue; infiltration into the plant tissue by using a syringe and needle or vacuum infiltration; and dipping the plant tissue into a bacterial suspension containing a wetting agent such as 0.02% Silwet L-77 which aids in penetration of the bacterium into the plant tissue.

A. avenae subsp. *citrulli.* Approximately 5-7 day old watermelon seedlings of 'CV' Charleston Grey are inoculated by injecting a suspension containing 1 X 10^6 CFU into the cotyledon using a syringe and 25 gauge (15). The seedlings are covered with a plastic bag and held at 30-32°C. A positive reaction is characterized by watersoaking after 3 - 5 days and tissue collapse and eventually death of the seedling after 5 - 7 days.

A. avenae subsp. *avenue,* Sweet com plants approximately 2-3 weeks postemergent are inoculated by injecting 0.1 to 0.2 ml of cell suspension containing 1 X 10^7 CFU/ml into the whorl using a needle and syringe. The plants are incubated in a greenhouse for two weeks and checked for water soaked linear lesions (22).

A. konjaci. *Amorphophalus konjac* (Koch) plants grown from tubers are inoculated by spaying fully expanded leaves before or after wounding the leaf surface with a needle. The bacterial suspension is prepared from a 24- to 48-h culture grown on yP plates at 28°C. Inoculated plants are maintained in a mist chamber for 24 h and then returned to the greenhouse which ranges in temperature from 20°C to 35°C. Water-soaked spots develop 2 to 3 days after inoculation and progresses to a blighting (8).

b. *Xylophilus ampelinus*

Grow cells as described (see 5, p. 226) in liquid NBY. Using an inoculum containing 1 X 10^7 CFU/mI, atomize leaves and inject nodes of current season growth of cuttings of cv. Granache or Red Globe and incubate in a lighted dew (11 h light cycle) chamber at 22 - 24 ° C (18). Water-soaked lesions will occur on leaves in 10-14 days and black lesions will occur on stems in 21 - 28 days.

6. MOLECULAR, SERIOLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular techniques

1) *Acidovorax*

PCR primers are available for general identification of *Acidovorax avenae*

Table 4. PCR Primers for *Acidovorax avenae*

Organism	Primer Designation	Sequence	Size (bp)	Reference
Acidovorax avenue	RST49 RST51	(5'GATGGCCGTGCCCTTC TTCATCCTCG3') (5'CATGGCCACGATGAGG ATCG3')	390	(11), G. V. Minsavage (personal communication)
Acidovorax species RST63 and *A avenue* subsp. RST64		(5'TCCGGCGGCGCGCTCA CCGTGGTGCTG3') (5'AGCGCGGCGGCGTAG GCGCGCGAG3')	210	G. V. Minsavage (personal communication)
Acidovorax avenue subsp. *citrllli*	WFB1 WFB2	(5'GACCAGCCACACTGGG AC3') (5'CTGCCGTACTCCAGCG AD')		(24)

F or additional details see Appendix A.

b. Serological Techniques

1) Immunofluorescence (IF) has been shown to be useful for identification of *A. avenae* subsp. *citrulli* (15). When combined with a semiselective agar medium such as mEBB or WFB 44, IF becomes even more reliable.

F or additional details see Appendix B.

c. Automated commercial techniques

1) Carbon source utilizations

The Biolog GN database contains three subspecies of *A. avenae.* It also includes *A. Konjaci* and non-pathogens *A. delafieldii* and *A. facilis. Xylophilus* is not included.

See Appendix C for description and details.

2) Fatty acid methyl esterase (FAME) profiles

A database containing most of the above is available from MlDI. See Appendix C for description and details.

7. CULTURE PRESERVATION

a. *Acidovorax*

F or short term storage, cultures should be maintained on YDC slants at room temperature because the organism dies rapidly when stored at 4°C (Schaad, unpublished). Long term storage should be at -80°C.

b. *Xylophilus*

Working cultures should be maintained on NA slants at 4 a C. Long term storage should be at -80°C, in liquid NBY with 20% glycerol (W. J. Botha, personal communication).

8. LITERA TURE CITED

1. Ayers, S., P. Rupp, and W. T. Johnson. 1919. A study ofthe alkali-forming bacteria in milk. U.S. Dept. Agric. Bull. 782.

2. Bradbury, 1. F. 1986. Guide to Plant Pathogenic Bacteria. CAB Int. Mycological Inst. Kew, England.

3. Burdon, K. 1. 1946. Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. 1. Bacteriol. 52:665-678.

4. De Vos, P. K., M. GOOf, M. Gillis, and 1. De Ley. 1985. Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. Int. J. Syst. Bacteriol. 35: 169-184.

5. DuPlessis, S. 1. 1940. Bacterial blight of vines (Vlamsiekte) in South Africa caused by *Erwinia vitivora* (Bacc.) DuP. Science Bull. 214. Dept. of Agric. and Forestry, Stellenbosch Ser. No. 39.

6. Forsyth, W. G. C., A. C. Hayward, and 1. B. Roberts. 1958. Occurrence of'pcly-B-butyric acid in aerobic Gram-negative bacteria. Nature 183:800-801.

7. Gitaitis, R. D. 1993. Development ofa seedbome assay for watermelon fruit blotch. Pages 9-18 in: 1. W. Sheppard, ed., Proceedings 1 st 1ST A Plant Disease Committee Symposium on Seed Health Testing. Ottawa, Canada, August 9-11, 1993.

8. Goto, M. 1983. *Pseudomonas pseudoalcaligenes* subsp. *konjaci* subsp. nov., the causal agent of bacterial leaf blight ofkonjac *Amorphophalus konjac* (Koch.). Int. 1. Syst. Bacteriol. 33: 539-545.

9. King, E. 0., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. 1. Lab. Clin. Med. 44:301-307.

10. Lelliott, R. A., and D. E. Stead. 1987. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications. Oxford, England.

11. Minsavage, G. v., R. 1. Hoover, T. A. Kucharek, and R. E. Stall. 1995. Detection of the watermelon fruit blotch pathogen on seeds with the polymerase chain reaction. Phytopathology 85: 1162. (Abstr.).

12. Palleroni, N. 1., R. Kunisawa, R. Contopoulou, and M. Doudoroff. 1973. Nucleic acid homologies in the genus *Pseudomonas.* Int. 1. Syst. Bacteriol. 23:333-339.

13. Panagopoulos, C. G. 1969. The disease "Tsilik Marasi" of grapevine: Its description and identification of the causal agent *(Xanthomonas ampeJina* sp. nov). Annales de L'Phytopath. Benaki. 9:59-81.

14. Rosen, H. R. 1922. A bacterial disease offoxtail *(Chaetochioa lutescens).* Ann. Mo. Bot. Gard. 9:383-402.

15. Schaad, N. W., G. Sowell Jr., R. W. Goth, R. R. Colwell, and R. E. Webb. 1978. *Pseudomonas pseudoalcaligenes* subsp. *citrulli* subsp. nov. Int. 1. Syst. Bacteriol. 28: 117125.

16. Schaad, N. W. and A. Sechler. 1999. An improved semiseleetive agar medium for *Acidovorax avenae* subsp. *citrulli.* Phytopathology 89: S68-69. (Abstr.).

17. Serfontein, S., 1. 1. Serfontein, W. L. Botha, and 1. L. Staphorst. 1997. The isolation and chacterization of *Xylophilus ampelinus.* Vitis. 36:209-210.

18. Skerman, V. B. D., V. McGowan, P. H. A. Sneath. 1980. Approved lists of bacterial names. Int. 1. Syst. Bacteriol. 30:225-420.

19. Stackebrandt, E., R. G. E. Murray, and H. G. Truper. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." Int. 1. Syst. Bacteriol. 38:321-325.

20. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff 1966. The aerobic pseudomonads: a taxonomic study. 1. Gen. Microbiol. 43: 159-271.

21. Stead, D. E. 1992. Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. Int. 1. Syst. Bacteriol. 42:281-295.

22. Sumner, D. R., and N. W. Schaad. 1977. Epidemiology and control of bacterial leaf blight of com. Phytopathology 67: 1113-1118.

23. Van Zyl, E. P. L. and Steyn. 1991. Taxonomy of the phytopathogenic *Pseudomonas* species belonging to the *Acidovorans* rRNA complex. System. Appl. Microbiol. 14: 165168.

24. Walcott, R. R. and R. D. Gitaitis. 2000. Detection *ofAcidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and polymerase reaction. Plant Dis. 84:470-474.

25. Willems, A., 1. De Ley, M. Gillis, and K. Kersters. 1991. *Comomonadaceae,* a new family encompassing the acidovorans rRNA complex, including *Variovorax paradoxus* gen. nov., comb. nov., for *Alcaligenesparadoxus* (Davis 1969). In1, 1. Syst. Bacteriol. 41:445-450.

26. Willems, A., M. Gillis, K. Kersters, L. Van de Broecke, and 1. De Ley. 1987. Transfer of *Xanthomonas ampelina* Panagopoulos to a new genus, *Xylophilus* gen. nov., as *Xylophilus ampelinus* Panagopoulos (1969) comb. nov. In1, 1. Syst. Bacteriol. 37:422-430.

27. Willems, A., E. Falsen, B. Pot, E. Jantzen, B. Hoste, P. Vandamme, M. Gillis, K. Kersters, and 1. De Ley. 1990. *Acidovorax,* a new genus for *Pseudomonasfacilis, Pseudomonas delafieldii,* E. Falsen (EF) group 13, EF group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delafieldii* comb. nov., and *Acidovorax temperans* sp. nov. Int. 1. Syst, Bacteriol. 40:384-398.

28. Willems, A., M. Goor, S. Thielemans, M. Gillis, K. Kersters, and 1. De Ley. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli, Acidovorax avenae* subsp. *cattleyae,* and *Acidovorax konjaci.* In1, 1. Syst. Bacteriol. 42: 107-119.

9. CHEMICALS LIST

Chemical	Source
Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., *P.D.* Box 14508, S1, Louis, MO 63178.	
Bacto agar	Difco
Bacto peptone	Difco
Berberine	
DL-P-hydroxybutyrate, sodium salt	
Beef extract	Difco
Brilliant Blue R	
Boric acid	
Bromcresol purple	

Cycloheximide
Calcium carbonate, finely ground
Galactose
Glutamic Acid
Lactalysate
Methyl. violet B
«-napthol
Neutral red
Nile Blue A
Noble agar
Oxoid agar No. 3
Phenol red
Pourite
Safranin
Silwet
Sudan black B
Sulphanilic acid
D-tartaric acid
Yeast extract

051 Specialties, Inc., 39 Old Ridgebury Road, Danbury, Ct 06810

n. GRAM-NEGATIVE BACTERIA

E. *Burkholderia*

W. **Chun and** J. B. Jones

1. Introduction

Burkholderia, proposed as a genus in 1992 (32), contained plant pathogens (5) of the rRNA-DNA homology group II pseudomonads ofPalleroni et al. (20). Plant pathogenic species included in this reclassifcation were *B. caryophylli* (carnation wilt and stem crack), *B. cepacia* (sour skin and bulb rot of onions), *B. gladioli* (gladiolus corm rot), and *B. solanacearum* (bacterial wilt). Also included were the animal pathogens *B. mallei* and *B. pseudomallei,* and a human isolate, *B. pickettii.* However, *B. solanacearum* and *B. pickettii* along with *Alcaligenes eutrophus* were later transferred to a new proposed genus *Ralstonia* (32). Recently, the rice seedling blight pathogens, *P. plantarii* (3) and *P. glumae* (26) were added to *Burkholderia (32).* A pathovar of *B. gladioli* was recently reported as a pathogen of cultivated mushrooms (11, 12). *P. marginata* (spot and basal rot of Gladiolus leaves) (17) is considered a synonym of *B. gladioli* (14). Thus, current taxonomy recognizes 18 species in the genus *Burkholderia,* of which 8 are known plant pathogens (Table 1).

Table 1. Recognized plant-pathogenic species of *Burkholderia.*

Bacterium	Host(s)	Disease	References
Burkholderia andropogonis	Sorghum, com, bougainvillea, Mucuna, Trifolium, Euchlaena, Vicia, Medicago, Dolichos, Lespedeza, Phaseolus, Saccharum	Varies with host	(13)
B. caryophylli	Carnation	Wilt, stem crack, stem rot	(5)
B. cepacia	Onion	Sour skin, bulb rot	(5, 32)
B. gladioli	Gladiolus	Corm and pseudobulb rot	(14, 17)
B. gladioli pv. *agaricicola*	Mushrooms	Soft rot	(10, 11)
B. glumae	Rice	Sheath rot, grain rot	(8,24)
B. graminis	Wheat	Root-associated	(27)
B. plantarii	Rice	Seedling blight	(3)

The burkholderiads all have the following characteristics; Gram-negative, straight or curved rods (0.5 to 1.0 by 1.5 to 4.0 Ilm), polar or multitrichous flagella, catalase-positive and accumulates poIy-P-hydroxybutyrate (PHB). Most are strict aerobes and do not produce

flourescent pigment under iron limiting conditions. Members of this group display a high degree of diversity of catabolic activities and biological properties. Most notable are the production of antibiotics by *B. cepacia* (1, 18) that are involved in the bacterium's ability to function as a biological control agent against many fungal (13, 16, 18,21,33), bacterial (1), and nematode (19) pathogens of plants.

Plant disease symptoms produced by *Burkhoideria* include rots, wilts, and severe necroses depending on individual host plants and in some instances, cultivars. Several species can be found in close association with plant roots (26, 27).

2. ISOLATION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

a. Plant Material

1) Isolation from diseased tissue. Wash plant material thoroughly with tap water and soak 1 to 2 minutes in 10% bleach. Rinse thoroughly in tap water. When lesions or discoloration in the plant tissues are evident, small pieces (3 mm x 3 mm x 6 mm) can be excised using sterile scalpels and forceps and ground with a glass rod in a small volume of water. The extracts can then be streaked out on a general plating medium such as YDC (see b, p. 4) or on a differential or semiselective media.

2) Isolation from roots. Rhizoplane bacteria can be isolated from roots by after rinsing with tap water to remove loosely held soil. Roots can then be soaked in saline or 0.1 M phosphate buffer containing 0.001 % Tween 20, and the bacteria collected from the wash by centrifugation. Sonication for 30 minutes will aid removal of bacterial cells that are attached to root tissue. Again, resuspend the bacteria in saline and streak on appropriate media.

b. Recipes for selective and nonselective media

1) PCAT selective medium for *B. cepacia* (7)

	perL
Azelaic acid	2.0 g
Tryptamine	0.2 g
$MgS0_4 87H_20$	0.1 g
K_2HP0_4	4.0 g
KH_2P0_4	4.0 g
Yeast extract	200.0 mg
Agar	15.0 g
Chlorothalonil	1 ml

(Bravo 500, 1 :24 aq suspension)

Disssolve $MgS0_4$"$7H_20$ first, add azelaic acid followed by heating and stirring until dissolved. Add remaining ingredients and adjust pH to 5.7. Autoclave 15 minutes, 121 $_a$C

2) CB medium (30)

	perL
Plate count agar	23.5 g
or	
Tryptone	5.0 g
Yeast extract	2.5 g
Dextrose	1.0 g
Agar	15.0 g

Aqueous stock solutions containing 0.1 % C-390 (9-chloro-9-(4-diethylaminophenyl (-lO-phenylacridan) or 7.5% polymyxin p sulfate are prepared and 1 m1 of each added to 1 L of the medium.

The medium is recommended for isolation of *B. cepacia* strains from environmental water and water-related samples.

3) S-PG Medium for isolation of *B. glumae (24)*

	perL
KH_2P0_4	1.3 g
N~HP04	1.2 g
$(NH4)2^{S0}4$	5.0 g
$MgS0_4 \cdot 7H_20$	0.25 g
N~Mo04·2H20	24.0 mg
EDTA-Fe D-sorbitol Methyl violet Phenol red Agar	10.0 mg 10.0 g 1.0 mg 20.0 mg 15.0 g

After autoclaving, add 1 m1 of filter sterilized stock solutions of the following:
L-cystine (l mg/l00 m1 stock)
Pheneticillin, potassium salt (5 g/100 m1 stock)
Ampicillin, sodium salt (1 g/100 m1 stock)
Cetrimide, (1 g/100 m1 stock)

Burkholderia glumae colonies appear as circular, convex, entire colonies

that are either reddish-brown (TypeA), or opalescent with a purple or purple-red center (Type B). Other *Burkholderia* spp. and pseudomonads may grow on the medium but with different colony morphologies.

3. DIFFERENTIATION OF COMMONLY ISOLATED SPECIES

Species of *Burkholderia* can be differentiated on the basis of physiological tests (Table 2).

4. DIAGNOSTIC MEDIA AND TESTS

a. Gram reaction

1) KOH Test (23) (see a, p. 7)

2) Gram stain (see b, p. 7)

b. Oxidase test (see 7, p. 10)

Transfer 24 hold NA (see a, p. 3) cultures with a platinum or plastic loop or toothpick to a filter paper impregnated with 1 % tetramethyl-p-phenylenediamine dihydrochloride. A purple color developing within lOs is considered a positive reaction.

c. Growth at different pH

Adjust the pH of a suitable growth medium (LB see 1), p. 41) or NBY (see c, p. 4) broth, with I N HCI to obtain a pH of 4 before autoclaving. Adjust the pH to 8 and 9 with sterile IN NaOH after autoclaving. Inoculate media with a fresh culture and observe for growth over 24 h.

d. Growth at 40 °C

Inoculate NBY (see c, p. 4) containing 0.5% dextrose. Incubate at the desired temperature. Turbid cultures within 5 days are recorded as positive.

e. Growth in 3% NaCI (9)

Inoculate NBY broth + 3% NaCl. Observe daily for growth. If growth does not occur in 3% NaCl, the maximum NaCl concentration at which growth will occur can be determined by repeating the test with a range ofNaCI concentrations from 1% - 3%.

Table 2. Characteristics used in the identification of species of *Burkholderia.*

Characteristics							
Oxidase		+	+	v	+	ND	ND
Growth at pH 4	ND		+	+	ND		+
Growth at pH 8	ND		+	+	ND	V	
Growth at pH 9	ND				ND		
Growth at 40^0 C		+	+	+		+	+
Growth in 3% NaCl	ND		V		ND	+	
Arginine dihydrolase		+				+	V
Gel hydrolysis	+			V	+	+	+
Starch hydrolysis	ND				+		
Pectate hydrolysis			+	+	ND		
Colonies wrinkled, yellowish							
Utilization of							
p-alanine		V	V	V	ND	+	+
Arginine		+	+	+	+	+	+
Betaine		+	+	+	+	+	+
Glycine			V	+	ND	ND	ND
Isoleucine		V	+	+	ND	V	
L-valine		+	+	+	ND	+	+
Adonitol	+	+	+	+	+	+	
Benzoate	V		V	+	ND		
Cellobiose	ND	+	+	+	ND	ND	ND
Lactose	+		V	V	+		
Levulinate			+		ND	+	
N-propanol	ND	V	+	V	ND	+	V
L-rhamnose	ND	V			+		V
D-sorbitol	+	+	+	+	+	+	+
Sucrose		+	+	V	+		V
D-tartrate				+	ND		V
Trehalose	+	V	V	+	+	+	
D-xylose		+	+	+	+	+	0

+,80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

f. Arginine dihydrolase test (9).

Inoculate 5 ml of arginine broth with a fresh culture and incubate for 24 h. Add 0.25 ml of Nessler's reagent. Development ofa brown color indicates arginine hydrolysis.

	perL
Peptone Yeast	5.0 g
extract	5.0 g
K_2HP0_4	2.0 g
Dextrose	50.0 g
Arginine mono hydrochloride	3.0 g

Nessler's Reagent: Dissolve 5 g potassium iodide in 5 ml freshly distilled water. Add cold saturated mecuric chloride solution until a slight precipitate remains permanently after thorough shaking. Add 40 ml9 NNaOH. Dilute to 100 ml with distilled water. Allow to stand for 24 h. Protect from light.

g. Gelatin hydrolysis

Inoculate a plate of gelatin agar and incubate for 3 days. Flood the surface with 5 to 10 ml of acid mercuric chloride solution. Clear zones indicate areas of gelatin hydrolysis.

Acid mercuric chloride: Mix 12 g of mercuric chloride with 80 ml distilled water. Add 16 ml cone. HCl. Shake well until solution is complete.

Gelatin agar

	perL
Gelatin	4.0 g
Distilled water	50.0 ml
Nutrient agar	23.0 g

Soak the gelatin in water and add to molten nutrient agar. Mix and autoclave 15 min at 121°C. Distribute into plates.

h. Starch hydrolysis (9)

Inoculate starch agar plates and incubate plates for 5 days. Flood plates with Lugol's iodine. Clear, colorless zones indicate starch hydrolysis. Note: some *Burkholderia* spp. produce restricted zones so colonies should be scraped away for easier reading of results.

Lugol's Iodine

	per 100 ml
Iodine	5.0 g

Potassium iodide (KI) 10.0 g

Dissolve iodine and KI in 10 m1 of H_20. Adjust to volume with distilled water. For use, dilute 1/5 with distilled water.

Starch agar

	perL
Nutrient agar (Difco)	23.0 g
Soluble potato starch	10.0 g
Distilled water	1.0L

1. Carbohydrate utilization

Carbon sources are filter sterilized and added at 0.1 % final concentration to autoclaved and cooled mineral salts medium consisting of: K_2HP0_4, 7 g; KH_2P0_4, 2 g; $MgS0_4 \cdot 7H_20$, 0.1 g; and (NH4)2S04, 1 g; per liter of distilled water (pH 7). Purified agar is used to solidify medium. Streak bacteria onto the plate and observe for growth over 14 days. Compare with plates containing no carbon source.

5. PATHOGENICITY TESTS

The inoculation technique depends on both the host tissue and the pathogen. Methods used such as syringe inoculations, wounding, spraying, forced introduction or vacuum infiltration are all viable methods. Prepare inoculum, as described (see 5, p. 226).

F or stem inoculations of dicotyledonous plants with vascular pathogens, wound the axillary bud by inserting a dissecting needle approximately 4 mm deep. Immediately place 5 to 10 microliters of a bacterial suspension on the wounded spot. The inoculum should be taken in automatically within 10 minutes. Incubate the plant under the appropriate conditions.

For monocotyledonous plants, clip the entire plant approximately 1-2 inches above the apical meristem. Immediately cover the cut shoot with a cotton ball soaked with a bacterial suspension and secure over the shoot with a piece offoil. Remove the cotton after one day.

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular techniques

1) PCR.

Reliable PCR primers are available for several species (Table 3).

Table 3. rca Primers for *Burkholderia* spp.

Specificity	Primer Designiation	Seguence	Size (bp)	Reference
B.	Pf	(5' AAGTCGAACGGTAACAGGGAJ')	410	4
andropogonis	Pr	(5' AAAGGAT ATT AGCCCTCGCC3')		
B. gladioli	CMG-23-1	(5' ATAGCTGGTTCTCTCCGAAJ')	388	
	G-23-2	(5'CCTACCATGCAY AT AAAT3')		6
	CMG-16-1	(5' AGAGTITGATCMTGGCTC3')	468	
	G-16-2	(5 'CGAAGGATATT AGCCCTC3')		
B. cepacia	CMG-23-1	(5' ATAGCTGGTTCTCTCCGAAJ')	388	
	CM-23-2	(5'CTCTCCTACCATGCGYGC3 ')		6
	CMG-16-1	(5' AGAGTITGATCMTGGCTC3')	468	
	CM-16-2	(5 'CGAAGGATATT AGCCCTC3')		
B. glumae	1416S:	(5' GAGAGAATCGAGCCATGAAC)	873	A Hasebe,
	1414A:	(5' GAGCGCATCCAGAACGAAGT)		pers. Comm
	1417S:	(5' GACTCACACCAGGCAGGAAGT)	925	N. W. Schaad
	1417A:	(5' ATTTCGGACTCGGTATGCAGC)		
	1418S:	(5' GCGATATGGCAAGACGCAAA)	571	
	1418A:	(5' AGTCAT ACCCTTTGTCAGCGT)		
	1419S:	(5' ACCCGTTTTGATAGAGGTGCG)	628	
	1419A:	(5' ACGAAGGTCTGCTGGTAAATCC)		

F or details see Appendix A.

b. **Serological techniques**

Antisera to heat killed cells have been shown to be useful in agglutinations and agar diffusion tests for presumptive identification of *B. glumae (29).*

F or details see Appendix B.

c. **Commercial automated techniques**

a. **Carbon utilizations**

Commercially available kits such as the Biolog GN Microplate'Y, or API test strips provides standardized and rapid performance of many physiological tests. Particularly useful are the Biolog MT plates which allow design of custom carbohydrate utilization tests. In most instances, members of this group are readily identified by the Microlog II software (Biolog, Inc.).

F or details see Appendix C.

7. CULTURE PRESERVATION

Standard long term storage methods are applicable to the *Burkholderia* (see 7, p. 30). Temporary stock cultures can be stored refrigerated on YDC (see b, p. 4) plates for 3-6 months. Bacteria may also be stored refrigerated, resuspended in sterile water or saline for up to 1 year. Growth on YDC is recommended for preparing water and saline storage stocks.

8. LITERATURE CITED

1. Aoki, M., K. Uehara, and K. Koseki. 1991. An antimicrobial substance produced by *Pseudomonas cepacia* B5 against the bacterial wilt disease pathogen, *Pseudomonas solanacearum.* Agric. and BioI. Chern. 55:715-722.

2. Arie, T., S. Namba, S. Yamashita, Y. Doi, and T. Kijima. 1987. Biological control of Fusarium wilt of bottle gourd by mix-cropping with welsh onion or Chinese chive inoculated with *Pseudomonas gladioli.* Ann. Phytopathol. Soc. Jpn. 53:531-539.

3. Azegami, K., K. Nishiyama, Y. Watanabe, I. Kadota, A. Ohuchi, and C. Fukazawa. 1987. *Pseudomonas plantarii* sp.nov., the causal agent of rice seedling blight. Int. 1. Syst. Bacteriol. 37: 144-152.

4. Bagsic, R. D., M. Gegam, X. Li, and A. C. Hayward. 1995. Letter in Applied Microbiol. 21:87-92.

5. Ballard, R. W., N. 1. Palleroni, M. Doudoroff, R. Y. Stanier, and M. Mandel. 1970. Taxonomy of the aerobic pseudomonads: *Pseudomonas cepacia, P. marginata, P. allicola, andP. caryophylli.* J. Gen. Microbiol. 60:199-214.

6. Bauernfeind, A., I. Schneider, R. Jungwirth, and C. Roller. 1999. Discrimination of *Burkholderia gladioli* from other *Burkholderia* species detectable in cystic fibrosis patients by PCR. 1. Coo. Microbiol. 36:2748-2751.

7. Burbage, D. A. and M. Sasser. 1982. A medium selective for *Pseudomonas cepacia,* Phytopathology 72: 706. (Abstr.).

8. Cottyn, B., M. T. Cerez, M. F. van Outryve, Barroga, 1., 1. Swings, and T. W. Mew. 1996. Bacterial diseases of rice. I. Pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. 80:429-436.

9. Cowan, S. T. 1974. Cowan and Steel's Manual for the Identification of Medical Bacteria. Second ed, ed. S. T. Cowan. Cambridge University Press, Great Britain.

10. Gill, W. M. and A. L. J. Cole. 1992. Cavity disease of *Agaricus bitorquis* caused by

Pseudomonas cepacia. Can. 1. Microbiol, 38:394-397.

11. Gill, W. M. and A. Tsuneda. 1997. The interaction of the soft rot bacterium *Pseudomonas gladioli* pv. *agaricico/a* with Japanese cultivated mushrooms. Can. 1. Microbiol, 43:639-648.

12. Gillis, M., V. Tran Van, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kewrster, T. Healin, and M. Fernandez. 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an ememded description of the genus and the proposition of *Burkholderia vietnamiensis* sp. nov. for Nz-fixing isolates from rice in Vietnam. Int. 1. Syst. Bacteriol. 45:274-289.

13. Hebbar, K. P., A. G. Davey, 1. Merrin, T. 1. McLoughlin, and P. 1. Dart. 1992. *Pseudomonas cepacia,* a potential suppressor of maize soil-borne diseases-seed inoculation and maize root colonization. Soil Biol, Biochem. 24:999-1007.

14. Hildebrand, D. C., N. 1. Palleroni, and M. Doudoroff 1973. Synonymy of *Pseudomonas gladioli* Severini 1913 and *Pseudomonas marginata* (McCulloch 1921) (Stapp 1928). Int. 1. Syst. Bacteriol. 23:433-437.

15. Hildebrand, D. c., M. N. Schroth, and D. C. Sands. 1988. Pseudomonas. Pages 60-80 in: N. W. Schaad, ed., Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd Edition. APS Press, St. Paul, MN.

16. King, E. B. and 1. L. Parke. 1993. Biocontrol of *Aphanomyces* root rot and *Pythium* damping-off by *Pseudomonas cepacia* AMMD on four pea cultivars. Plant Dis. 77: 1185-1188.

17. McCulloch, L. 1921. A bacterial disease of gladiolus. Scienc 54: 115-116.

18. McLoughlin, T. 1.,1. P. Quinn, A. Bettermann, and R. Bookland. 1992. *Pseudomonas cepacia* suppression of sunflower wilt fungus and role of antifungal compounds in controlling the disease. Appl, Environ. Microbiol. 58: 1760-1763.

19. Novitski, C., T. 1. McLoughlin, and H. Atkinson. 1994. Method for protecting plants from nematodes using *Pseudomonas cepacia* stains. U.S. Patent No. 5,264,210.

20. Palleroni, N. 1., R., Kunisawa, R. Contopoulou, and M. Doudoroff 1973. Nucleic acid homologies in the genus *Pseudomonas.* Int. 1. Syst. Bacteriol. 23:333-339.

21. Parke, 1. L., R. E. Rand, A. E. Joy, and E. B. King. 1991. Biological control of *Pythium* damping-off and *Aphanomyces* root rot of peas by application of *Pseudomonas cepacia* or *P. fluorescens* to seed. Plant Dis. 75:987-992.

22. Scarlett, C. M., J. T. Fletche, P. Roberts, and R. A. LeIliott. 1978. Tomato pith necrosis caused by *Pseudomonas corrugata n. sp.* Ann. Appl. BioI. 88: 105-114.

23. Suslow, T. v., M. N. Schroth, and M. Isaka. 1982. Application ofa rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology 72:917-918.

24. Tushima, S., S. Wakimoto, and S. Mogi. 1986. Selective medium for detecting *Pseudomonas glumae* Kurita *et* Tabei, the causal bacterium of grain rot of rice. Ann. Phytopath. Soc. Japan 52:253-259.

25. Uematsu, T., D. Yoshimura, K. Nishiyama, T. Ibaraki, and H. Fujii. 1976. Pathogenic bacterium causing seedling rot of rice. Ann. Phytopathol. Soc. Japan 42:464-471.

26. Urakami, T., C. Ito-Yoshida, H. Araki, T. Kijima, K. Suzuki, and K. Momagata. 1994. Transfer of *Pseudomonas plantarii* and *Pseudomonas glumae* to Burkholderia as *Burkholderia* spp. and description of *Burkholderia vandii* sp. nov. Int. J. Syst. Bacteriol. 44:235-245.

27. Viallard, v., 1. Poirier, B. Cournoyer, J. Haurat, S. Wiebkin, K. Ophel-Keller, and J. Balandreau. 1998. *Burkholderia graminis* sp. nov. a novel species ofrhizospheric *Burkholderia* and reassessment of *Pseudomonas phenazinium, Pseudomonas pyrrocinia* and *Pseudomonas glathei* into *Burkholderia.* Int. 1. Syst. Bacteriol. 48:549-563.

28. Wakimoto, S., K., Hirayae, K. Tsuchiya, Y. Kushima, N. Furuya, and N. Matsuyama. 1986. Production of antibiotics by plant pathogenic *Pseudomonas.* Ann. Phytopathol. Soc. Japan 52:835-842.

29. Wakimoto, S., S. M. Araka, and K. Tsuchiya. 1987. Serological specificity of *pseudomonas glumae,* the pathogenic bacterium of grain rot disease of rice. Am. Phytopath. Soc. Japan 53:150-158.

30. Wu, B. 1. and S. T. Thompson. 1984. Selective medium for *Pseudomonas cepacia* containing 9-chloro-9 (4-diethylaminophenyl) -10-phenylacridan and polymixin P sulfate. Appl. Env. Microbiol. 48:743-741.

31. Yabuuchi, E., Y. Kosako, 1. Yano, H. Hotta, and Y. Nishiuchi. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: Proposal of *Ralstonia picketii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis, 1969) comb. nov. Microbiol. Immunol. 39:897-904.

32. Yabuuchi, E., K. Yoshimasa, H. Oyaizu.L Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and

M. Arakawa. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 36: 1251-1275.

33. Zaki, K. M. and I. 1. A. Heydari. 1998. Control of cotton seedling damping-off in the field by *Burkholderia (Pseudomonas) cepacia.* Plant Dis. 82:291-293.

4. CHEMICAL LIST

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178.

Chemical	Source
Agar, Bacto	Difco
Agar, Technical Grade	Difeo
Casein acid hydrolysate	Difco
Biolog Plates	Biolog, Inc., 3938 Trust Way, Hayward, CA 94545
Chlorothalonil (Bravo 500)	SDS Biotech Co., Painesville, OR
DL-p-hydroxybutyrate, sodium salt	
Plate count agar	Difco

II. GRAM-NEGATIVE BACTERIA

F. *Ralstonia*

T. P. Denny and A. C. Hayward

1. INTRODUCTION

Ralstonia solanacearum (Pseudomonas solanacearum) is pathogenic on several hundred plant species in over 50 families (22, 24). Hosts include peanut, potato, tomato, tobacco, banana, and economically important trees and shrubs. Usually soil-borne, *R. solanacearum* normally infects via the roots, moves systemically through the xylem and causes wilting symptoms that are often lethal. For bananas, some strains are insect vectored and the floral raceme is the primary site of infection; subsequent disease may include wilt symptoms or, as in the case ofBugtok on cooking banana in the Philippines, may be limited to fruit and localized vascular discoloration. Latent infections are also well documented and potentially very important in pathogen dissemination. For example, movement oflatently-infected seed potatoes within Europe helped create an outbreak of bacterial wilt (brown rot) on potato in the mid 1990's (26).

Until recently, *R. solanacearum* was categorized as a nonfluorescent pseudomonad despite evidence that it was completely unrelated to the fluorescent pseudomonads. For example, *R. solanacearum* is in rRNA homology group II whereas the fluorescent pseudomonads are in rRNA homology group I (41, 44), and total DNA-DNA hybridization between strains in these homology groups is essentially zero (42). In 1992, Yabuuchi et al. (65) transferred seven species in rRNA homology group II, including *'Po solanacearum'* into the new genus *Burkholderia.* However, within rRNA homology group II *Burkholderia cepacia* and related bacteria cluster separately from *'Burkholderia solanacearum'* and *'Burkholderia pickettii'* (16, 44). These distinct and separate sub clusters were confirmed by analysis of the 16S rRNA gene sequences (34,62). Based on these findings and additional chemotaxonomic data, Yabuuchi et al (66) established the genus *Ralstonia* to accommodate *R. solanacearum, Ralstonia pickettii,* and *Ralstonia eutropha (Alcaligenes eutrophus).* We accept the proposed name of *R. solanacearum,* as have most researchers in this field. All three names should be used when searching for all the pertinent literature on bacterial wilt.

Although similar in phylogeny and chemotaxonomic properties, *Ralstonia* species differ markedly in pathogenicity, host relationships, and other phenotypic properties. *R. pickettii* has been isolated from human clinical and environmental sources, including respiratory therapy solutions, deionized water, and aqueous disinfectants, and is capable of intracellular growth in the free-living amoeba *Acanthamoeba* spp. (38). *R. pickettii* is included in this chapter because it has been isolated from soil and plant material (57) and is similar in phenotype to *R. solanacearum* biovar 3.

Two other plant pathogens clearly belong in *Ralstonia,* but no formal proposal has been made to transfer them to this genus. The agent of Sumatra disease on clove, currently recognized

as *Pseudomonas syzygii* (50) is similar to *R. solanacearum* in Biolog tests, fatty acid analysis, serology, and DNA hybridization (11, 50, 60). The blood disease (BD) bacterium of banana, which causes foliar wilting of banana and red discoloration of the fruit in Java and Sumatra, for which the invalid name *'Pseudomonas celebensis'* (1]) has been used, is even more closely related to *R. solanacearum.* There are serological relationships and significant levels of DNA DNA hybridization between *R. solanacearom, P. syzygii* and the BD bacterium (3, 50) and between *R. solanacearum* and *R. pickettii* (48). Comparison of 16S rRNA gene sequences indicates that both *P. syzygii* and the BD bacterium are phylogenetically embedded in the *R. solanacearum* species complex (62). Recent phenotypic, genetic, and pathogenicity studies have shown that the Bugtok pathogen is indistinguishable from *R. solanacearum* race 2 (biovar 1) strains causing Moko disease on dessert bananas in the Philippines (11,49).

Differences in phenotype and genotype in *R. so/anacearum* might be anticipated from the great diversity of host plants affected by this pathogen, its wide geographical distribution, and the range of environmental conditions conducive to bacterial wilt. Pathotypic variation is expressed in the race classification based primarily on host range (Table 1), whereas differences in metabolic activity have been used to define five (or possibly six) biovars. There is no strict correlation between races and biovars, except that race 3 strains are usually in biovar 2. Studies at the genetic level have shown that there are two distinct divisions within *R solanacearum.* Restriction fragment length polymorphism (RFLP) analysis first revealed the two genetic divisions (8, 9), which later were found to correlate perfectly with the division of the species based on differences in the 16S rRNA gene sequences (62). There is a good correlation between the two divisions and biovars; biovars 1 and 2, which are metabolically less versatile (43) are contained in Division IT, and the metabolically more versatile biovars 3, 4 and 5 in Division 1. *R solanacearum* strains with wide host range are found in both divisions.

Table 1. Characteristics of races and their relationship to other subdivisions of *R. solanacearum*

Rac	Host Range	Geographical Distribution	Biovar	RFLP Division'
1	wide	Asia, Australia.	3,4	I
		Americas	1	II
2	banana	Caribbean, Brazil,		II
	other *Musa* spp	Philippines		
3	primarily potato	Worldwide'	2[3]	II
4	ginger	Asia	3,4	I
54	mulberry	China	5	I

1 Based on restriction fragment length polymorphism (RFLP) analysis (8,9).

1 Originating in the Andes, but disseminated worldwide on latently infected potato tubers.

3 Typical race 3 strains are sometimes referred to as biovar 2A Strains from the Amazon basin have been placed in a new biovar, designated by various authors as 2T or N2 (23).

4 Although originally designated as race 4, the prior designation of the ginger strains as race 4 takes precedence.

The following characteristics are typical of *R solanacearum:* Gram-negative rods (0.5 to 0.7 by 1.5 to 2.5 urn), oxidase and catalase positive, accumulate poly-P-hydroxybutyrate (PHB), and reduce nitrate. There is no growth at 40°C, little or no growth in broth with 2% NaCl, and cultures are negative for arginine dihydrolase, gelatin liquefaction, and starch or esculin hydrolysis. Colonies are nonfluorescent, but a diffusible brown pigment is often produced on complex media. Unlike *Burkholderia* species, cellular fatty acids of *Ralstonia* species lack ornithine lipids OL-1 and OL-2, and <1% of the total cellular fatty acid is C19:0 cyclopropanoic acid (60,66). Two colony types are often observed on complex medium containing 0.5% glucose or sucrose; one type is fluidal (mucoid) due to copious production of extracellular polysaccharide (BPS), whereas the other is dry (butyrous). Flagella are polar when present, but motility (and possibly flagellation) of strains varies with the colony type and culture age (5, 7). In complex liquid broth, mucoid strains have the highest percentage of motile cells when cultures have grown to about 1 x 10^8 cells per ml (OD_{600}ro:O.1); stationary-phase cultures have very few motile cells. Cells from wilted plants are also initially nonmotile, but motile cells become common after 4 to 6 hours in fresh medium (36).

2. ISOLATION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

Isolation of *R solanacearum* from fresh, symptomatic plants is usually not difficult, due to the high density of the pathogen in the tissues. Isolation of the pathogen from nonsymptomatic plants is more difficult due to the low number of viable cells present. As expected, isolation from soil is the most problematic due to the presence of other microorganisms, many of which multiply more rapidly than does *R solanacearum.*

Several semiselective media have been described for the isolation of *R. solanacearum (12*14, 18, 28). None is entirely satisfactory because they do not support growth of all *R solanacearum* strains and/or do not suppress growth of all related or unrelated Gram-negative bacteria. The best known and most widely used media are SM-1, SMSA, and a modified SMSA. Although SMSA and its modification (12) both contain glycerol, the replacement of glycerol with glucose may be advantageous because it enables more rapid growth of *R solanacearum,* thus avoiding some ofthe problems with competing and antagonistic bacteria. On SMSA incubation times of 48-72 hours at 28°C are optimum and growth and colony size are equivalent to those on TTC medium (31); colonies remain small and take longer to appear on SM-l (1. G. Elphinstone, personal communication).

Susceptible host plants can be exploited to make isolations from soil or to assess population numbers by the most probable number technique (45). Potato seedlings grown from excised seed tuber sprouts provide potato tubers suitable for the detection of *R solanacearum* in soil (17). Similarly, tomato seedlings can be used to enrich populations of the pathogen in potato tuber extracts (12).

a. Recipes for differential media.

1) YDC (see b, p. 4)

Colonies are mucoid and beige to light brown in color.

2) CPG and TIC media (31).

	perL
Casamino acid (casein hydrolysate)	1.0 g
Peptone	10.0 g
Glucose	5.0 g
Agar	17.0 g

CPG contains the four ingredients shown. To make TTC medium, cool the medium to 55°C and add 5 ml ofa 1% stock solution of2, 3, 5-tripheny tetrazolium chloride. The stock can be filter sterilized or autoclaved for 5 min at 121°C, and stored at 4°C or frozen.

b. Recipes for semiselective media.

1) SM-l medium (18); to TIC medium add the following after autoclaving.

	perL
Merthiolate tincture	5 to 50 Ill*
Crystal violet	50 mg** 100
Polymyxin p sulfate	mg** 20
Tyrothricin	mg** 5 mg**
Chloromycetin	50 mg**
Cycloheximide	

**Dissolve in 5 ml of 70% ethanol 30 min prior to use.

*Merthiolate tincture contains 1 part merthiolate per 1000 parts of 50% alcohol. Determine the best concentration to suppress local microflora, as suggested (18, 19).

2) Modified SMSA medium (12-14)

Prepare 1 L of TTC medium, except substitute glycerol (5 ml per L) for the glucose. Cool to 50°C and add the following antibiotics dissolved in 70% ethanol:

Crystal violet 1%	0.5 rnl	(final cone. 5 mg/L)
PolymyxinB sulfate 1%	10.0 ml	(final cone. 100 mgIL)
Bacitracin 1 %	2.5 ml	(final cone. 25 mgIL)
Chloromycetin 1 %	0.5 ml	(final cone. 5 mg/L)
Penicillin 0.1%	0.5 ml	(final cone, 0.5 mg/L)

When inhibition of fungal contaminants is desirable, add:

Cycloheximide 1%, in 70% ethanol	2.5 m1	(final conc. 100 mgIL)

Modified SMSA or other selective media can be used to improve detection of low pathogen populations in soil or plant material by PCR (28), because inhibitors of PCR are diluted while target populations are increased. This 'BID-PCR' technique is similar to that of Schaad et al. (54).

3. DIFFERENTIATION OF COMMONLY ISOLATED SPECIES AND SUBSPECIES.

a. Differentiation of species.

Some properties of *Ralstonia* spp. and related bacteria are shown in Table 2. In contrast to *R. solanacearum,* colonies of *P. syzygii* are slow growing, small, and tenaciously attached on TTC medium; the poor growth of *P. syzygii* on this and other commonly used media does not reflect any complex nutritional requirements for growth. All strains grow slowly on a simple mineral medium containing a limited range of carbon/energy sources, including dextrose, dicarboxylic amino acids, amines, and certain organic acids (11).

Table 2. Differentiation of two *Ralstonia* spp. from *Pseudomonas syzygii* and the Blood Disease Bacterium of banana [1].

	P. syzygii	BD Bacterium	*R. solanacearum*	R. *pickettii*
Colonies on TIC medium	tenacious, minute	viscid, <5 rnrn	Fluidal, >5 rnrn	ND[2]
Motility			v	+
Growth at 37°C		+	+	+
Growth at 41°C				+
NaCI tolerance	<1%	<1.5%	<2.0%	ND
Nitrite from nitrate	v		+	+
Gas from nitrate			v	+
TobaccoHR	v	+	+3	NO
Plant pathogenicity and host associations	Clove	Banana	*Solanaceae, Musaceae,* etc.	Bacteraemia in humans; intracellular growth in *Acanthamoeba* spp

1 Based in part on Eden-Green (11). 2
ND, not detemrined.
3 Systemic infection with stains from tobacco.

b. Differentiation of biovars (21, 27)

Each of the five biovars of *R. solanacearum* can be differentiated based on utilization of single alcohols and carbohydrates (Table 3).

The mineral medium of Ayers et al. (2) is supplemented with peptone, agar, and a pH indicator.

	perL
$NH_4H_2PO_4$	1.0
KCI	g
$MgSO_4 \cdot 7H_2O$	0.2g
Difco Bacto peptone	0.2g
Agar	LOg
BroDlothyrnolblue	3.0 g
	80.0 mg

The pH is adjusted to 7.0 - 7.1 (an olivaceous green color) by dropwise addition of 40% sodium hydroxide solution. The medium is heated to melt the agar, dispensed into bottles or tubes, sterilized by autoclaving at 121°C for 20 to 30 min, and cooled to 55 to 60°C.

Prepare 10% aqueous solutions of the test carbohydrates (see Table 3). Sterilize dulcitol by autoclaving at 110° C for 20 min. Filter sterilize the other carbohydrates. Sufficient carbohydrate solution is added to the wann basal medium to give a final concentration of 1% (e.g., 10 D1l of 10% solution to 90 D1l basal medium). After mixing, about 3 ml of the molten medium is dispensed into sterilized culture tubes (150 mm x 10 mm internal diameter) and allowed to solidify.

Inoculum is prepared by adding severalloopfuls of bacteria from 24 to 48 h old cultures on CPG or TTC plates to 3 to 5 ml sterile distilled water to make a suspension containing about 10^8 CFU/D1l.

* Add about 20 JlL of the bacterial suspension to the surface of the medium in each tube * Incubate the inoculated tubes at 28 to 32°C
* Examine the tubes at 3, 7, 14, and 28 days after inoculation for change in pH (indicated by a color change; examine from the top of the medium downward)

With dextrose and hexose alcohols, a change to yellow (acid pH, <6) indicating oxidation of the carbohydrate occurs within 3-5 days; those biovars capable of oxidizing the disaccharides could take a few days longer to give a clear positive result. The inoculated tubes should be compared with a noninoculated control tube to observe the change in color (in SODle cases there could be a slight change to alkaline pH in tubes containing carbohydrates that are not oxidized).

Table Differentiation of *Ralstonia solanacearum* biovars

Test	Biovars 1	2	3	4	5
Utilization of:					
Dextrose	+	+	+	+	+
Mannitol			+	+	+
Sorbitol			+	+	
Dulcitol			+	+	
Trehalose	+		+	+	+
Oxidation of: [1]					
Lactose		+	+		+
Maltose		+	+		+
D(+) cellobiose		+	+		+
Nitrite from nitrate	+	+	+	+	+
Gas from nitrate			+	+	+

+. 80% or more strains positive; -, 80% or more strains

ı Disaccharides are oxidized to bionic acids, but are not utilized as a source of

Further subdivision of'biovar 2 can be made with additional tests using the sugars D-ribose, trehalose, and meso-inositol as the carbon sources. Bacterial wilt of potato in temperate and subtropical regions and at high altitudes in the tropics worldwide is caused by biovar 2 (race 3) strains with the phenotype D-ribose negative, trehalose negative, and meso-inositol positive. This phenotype is RFLP group 26 in the classification of Cook et al. (8). A distinct phenotype ofbiovar 2 occurring in parts of Chile and Colombia, South America, is D-ribose negative, trehalose positive, and meso-inositol negative (23,25), and corresponds with RFLP group 27; most of these isolates do not produce nitrite from nitrate, a property universal among all other biovar 2 strains. A third phenotype ofbiovar 2, which occurs mainly in Peru and Brazil, is D-ribose positive, trehalose positive, and meso-inositol positive. This phenotype, which has been referred to as biovar N2 (9) or biovar 2T to reflect its lowland tropical origin (14), corresponds with RFLP groups 29-31, 33,36, and
39 (9).

4. DIAGNOSTIC MEDIA AND TESTS

a. Poly-J}-hydroxybutyrate (PHD) accumulation

The accumulation of cellular organic reserve materials, such as PHB, is favored under conditions of nitrogen starvation. Two media ordinarily used to promote PHB accumulation by *R. solanacearum* are: (1) nutrient agar (see a), p. 3) plus 5% sucrose and (2) a mineral medium consisting of(NH.t)2S04, 0.2 gIL; KCI, 0.2 gIL, $MgS0_4 \cdot 7H_20$, 0.2

gIL that is supplemented with DL-P-hydroxybutyrate (5 gIL) and adjusted to pH 7.2. Bacteria are cultured for 24 to 48 h before testing for PHB inclusions by either technique A or B below. Experience has shown that technique A, which does not always differentiate PHB granules satisfactorily (33), is inferior to technique B, and should be used only when a simple light microscope is all that is available.

1). Technique A ((i):

a) Prepare Sudan Black B solution (0.3 gin 100 m1 of 70% ethanol). After most of the dye has dissolved, shake the solution at intervals, then allow to stand overnight before use. The solution can be stored for several months at room temperature in a tightly closed container.
b) Make a bacterial smear on a glass slide; air-dry and heat-fix.
c) Flood the entire slide with Sudan Black B solution and leave undisturbed for 10 to 1 ~ min.
d) Drain off excess solution, blot dry, and clear slide with xylol (xylene) in a Coplin jar or by adding from a dropper bottle.
e) Blot the cleared slide to dryness and counterstain with safranine (0.5% aqueous solution) for 5 to 10 seconds. Avoid over staining. Wash in water, blot and dry the slide and examine under oil immersion with a light microscope. The PHB granules are dark blue-black. PHB granules also show up well in the electron microscope as egg-shaped bodies.

Cells that have accumulated PHB granules do not usually Gram stain well. Since endospores cannot be easily differentiated from polymer granules by this method, it should only be used when it is known from other evidence that the culture being observed is of a non-spore-forming bacterial species.

2) Technique B:

The superior method for detecting PHB granules is to stain with a 1 % aqueous solution of Nile Blue A (warm the solution if needed to dissolve the dye and then filter). Flood a heat-fixed smear with the Nile Blue A solution and hold at 55^0 C for 10 min. Wash the slide briefly with tap water and then flood with 8% aqueous acetic acid for 1 min to remove excess stain. Wash again in tap water and blot dry. Moisten again with a drop of water and apply a cover slip to prevent extraction of the dye if using immersion oil. Examine using an epifluorescence microscope equipped with a 450-490 nm excitation filter, a 510 nm dichroic mirror, and a 520 nm barrier filter (e.g. Nikon B-2A or B-2E filter cubes); PHB granules fluoresce bright orange (40).

3) Technique C: UV detection using NB medium (46)

PHB granules are produced by many aerobic, Gram-negative bacteria. Colonies ofPHB-positive bacteria fluoresce bright orange to yellow under longwave (366-nm) ultraviolet radiation when grown on a medium containing hydroxybutyrate and Nile Blue dye. Colonies of fluorescent pseudomonads do not fluoresce on this medium, and fluorescent granules are not visible microscopically.

	perLLO
$NH_4H_2P0_4$	g
KCI	0.2g
$MgS0_4$-$7H_20$ DL-~-	0.2
hydroxybutyrate, sodium salt Difco	g
Proteose Peptone No. 3	5.0
1 % Nile blue solution	g
1 NNaOH	20.0 g
Distilled water	1.0
Agar	ml
	4.5 ml
	900.0 ml
	17.5 g

The pH is about 7.0. After autoclaving add 100 ml ofa filter-sterilized or autoclaved solution of 20% glucose. Care should be taken not to autoclave the glucose solution for more than 15 min, as this will affect growth of some nontluorescent pseudomonads.

b. Nitrate reduction and gas from nitrate

More than 90% of *R solanacearum* strains in biovars 3, 4, and 5 produce gas from nitrate, but this ability is rare in biovars 1 and 2. Almost all *R. solanacearum* strains can grow anaerobically in the presence of nitrate, or produce nitrite from nitrate; strains unable to do so are almost entirely limited to biovar 2 (25). The choice of medium is critical, since in some media the production of gas from nitrate is erratic or absent. Reliable results can be obtained using the medium of van den Mooter et al. (63).

Nitrate reduction medium (63)

	perL
KH_2P0_4, anhydrous	0.5 g
$KJIP0_4$, anhydrous	0.5 g
$MgS0_4$-$7H_20$	0.2 g
Glycerol	2.0ml
$KN0_3$	3.0 g
Yeast extract (Difco)	5.0 g
Agar, Noble	1.0 g

The pH is about 6.9. Melt the agar and dispense 3 to 4 ml into culture tubes (150 rom x 10 rom internal diameter) either capped or plugged; screw-capped tubes of similar dimension may also be used. Autoclave at 121°C for 20 to 30 min. Store the tubes at room temperature or at 4°C (but see caution below).

Stab inoculate duplicate tubes of the semisolid medium (to the base of the tube) two or three times using a thin, straight wire loaded at the point with inoculum from an agar plate. Seal one of the tubes by adding 2 to 3 ml of molten 3% water agar. Incubate the tubes at 28 to 32°C. After 3 to 7 days, test the unsealed tube for the presence of nitrite by adding starch iodide and diluted hydrochloric acid reagents prepared according to Skerman (58) as described below.

Starch iodide solution

Starch	0.4 g 2.0
Zinc chloride (ZnCIJ	g 100.0
Distilled water	ml

Dissolve $ZnCl_2$ in 10 ml water. Boil and add the starch while still hot. Dilute to 100 ml with water, allow to stand for one week, and filter. Add an equal volume of a 0.2% solution of potassium iodide (KI) before use.

Hydrochloric acid

Concentrated hydrochloric acid (HCI)	16.0 ml
Distilled water	84.0 ml

Procedure - Add 50 ,ul of each reagent to the unsealed tube prepared above. A blue color indicates the presence of nitrite.

The test depends on the formation of nitrous acid and its subsequent reaction with potassium iodide to liberate iodine that turns the starch blue. The test is not entirely specific. Control tests should be made on a non-inoculated tube. Several other reagents are available to test for production of nitrite from nitrate (33).
A negative reaction for nitrite could indicate either that the nitrate has not been reduced, or that the nitrate has been reduced beyond nitrite. To differentiate between these possibilities, a speck of zinc dust is added to the tubes in which a weak or no reaction for nitrite has occurred. If the nitrate has not been reduced, then a blue color will develop after addition of the zinc dust. A weak reaction that does not intensify after addition of zinc dust indicates that most of the nitrate has been reduced beyond nitrite; a weak reaction that intensifies after the addition of zinc dust indicates that a little of the nitrate was reduced to nitrite.

The tube sealed with agar should be examined daily for 1 week for the presence of gas bubbles trapped in the medium or beneath the agar seal. The reaction is sometimes weak and slow to appear. A stronger reaction may be obtained if the isolates are subcultured several times through a medium containing nitrate to enhance the activity of the nitrate reductase enzyme (59). False positives can sometimes occur if the medium has been stored at 4°C, because bubbles appear in the medium when it is subsequently incubated; this problem is avoided by melting the medium and allowing it to reset before use.

c. Carbon source utilization (43, 59)

Prepare the mineral salts medium of Ayers et al. (2) (see f, p. 96) in one half the final volume of water and an equal volume of2.4% purified agar (e.g., agarose) in water; autoclave separately and then combine and cool to 50°C. Add filter-sterilized solutions of different carbon sources to 0.1 % *(w/v)* final concentration. Adjust the pH to 7.2 if necessary and dispense into Petri dishes. Bacteria are streaked onto the medium, or patched on with replica methods, and incubated at 28 to 32°C. The amount of growth is determined after 3, 7, and 14 days, and compared to plates containing no added carbon source.

The following general-purpose defined minimal medium is recommended for *R. solanacearum* and related bacteria. The important features of the medium are its low salt concentration (one fourth of that used in the minimal medium for *E. co/i)* and the presence of sodium citrate.

lOX stock solution	per 500 ml
KH_2PO_4	3.75 g
K_2HPO_4	8.75 g
$(NH_4)_2SO_4$	6.25 g
Sodium citrate	0.70 g
$MgSO_4 \cdot 7H_2O$	0.13 g

Prepare the lOX stock solution and either autoclave it or add a small amount of chloroform to the bottle to inhibit microbial growth; store this solution at room temperature. For use, dilute the stock solution 10-fold in deionized tap water (not glass distilled or from a milliQ-type water purifier, because water from those sources lacks micro elements), autoclave, and allow to cool. Before use, add 20% *(w/v)* filter-sterilized glucose or other carbon source to give a final concentration of 0.5%.

To make agar medium, dilute the stock solution 5-fold in one half the final volume of water and prepare an equal amount of double-strength agar; autoclave separately and then combine. Cool to 55°C, add sterile glucose or other carbon source to 0.5% (and other

amendments if any), and pour.

5. PAmOGENICITY TESTS

R solanacearum is one of the easier bacteria to test for pathogenicity and, when desired, to quantify relative virulence. The keys to success are to use a freshly grown bacterial culture, prepare inoculum with mucoid colonies (i.e., from those with copious EPS slime), inoculate succulent young plants, and then incubate the plants at the appropriate temperature. To produce inoculum, grow *R solanacearum* on TTC plates for 48 h at 30°C so that the white or pink, EPS-producing colonies can be differentiated from any red, EPS-negative colonies that might be present. Suspend one or more EPS+ colonies in sterile water and adjust the suspension to give an OD_{600} = 0.1 (approximately 1 x lOs CFU/ml) and then dilute in water to the desired cell density (which varies with the inoculation method). Determine CFU/ml, as described (see 5, p. 226).

For plants with large enough stems, the easiest inoculation method is to make a puncture wound into the pith at a leaf axil and apply a drop of inoculum. For plants like tomato and eggplant, this is best done by using a disposable tip from a mechanical pipettor to make the wound and then inserting a fresh pipet tip containing 10 or *20,uL* of inoculum (1 X 10^6 CFU/ml) into the hole. Incubate the plants in a greenhouse or growth chamber at 30 to 32°C during the day and 25°C at night (28°C day and 16°C night for the cool temperature race 3 strains from potato), relative humidity> 85%, with 12 h light and 12 h dark periods. After the inoculum is taken up by the plant (3 to 10 h or overnight), the pipet tip is removed and the hole that remains is left exposed. Keep the plants well watered, but avoid wetting the foliage. Wilt symptoms should begin to appear within 4 to 10 days. Alternatively, a lower leaf can be excised about 0.5 em from the stem and a droplet of inoculum (e.g., 2,uL containing 2 x 10^5 cells) deposited on the cut surface of the petiole.

More natural inoculation methods require that bacteria be applied to plant roots. Since these methods require large volumes of inoculum, the bacteria are usually grown in liquid minimal medium, collected by centrifugation and suspended in water. Tomato plants can be inoculated by pouring sufficient inoculum onto the soil in the pot containing a young plant (of the same age as for stem inoculation) to give about 1 x 10^7 CFU/gram soil. Intentionally wounding the roots is not essential, but to improve infection consistency (or to reduce the volume of inoculum needed), it is common to wound some of the roots by drawing a knife through the soil on one side of the plant just before inoculation. After inoculation, water plants from the bottom to prevent washing the bacteria out of the soil.

To quantify relative virulence of bacterial strains or resistance of plant cultivars, record the percentage of leaves wilted (or the number of plants completely wilted) on each plant on a daily basis and then calculate the average percentage wilt on each day for each treatment (10). Treatments can be examined statistically by comparing the average time required for 50% of the leaves of individual plants to wilt using the nonparametric Wilcoxon two-sample test (pROC NPAIRIWAy) in the Statistical Analysis Systems package (SAS Institute, Cary, NC, USA).

The rating system of Winstead and Kelman (64) has also been widely used. Observe the wilt symptoms developing from 5 to 21 days after inoculation and record disease ratings using the following scale: l=no symptoms; 2=one leaf wilted; 3=two to three leaves wilted; 4=four or more leaves wilted; 5=whole plant wilted (dead plant). Calculate the wilt intensity 21 days after inoculation, using the following formula:

$$I = [\sum (n \times v) \div (V \times N)] 100$$

where I = wilt intensity (%); n = number of plants with respective disease rating; v_i = disease rating (1,2,3,4 or 5); V = the highest disease rating (5); and N = the number of plants observed.

Another measure of resistance that can be used after inoculating the plants' roots is to determine the number of viable bacteria within the stem at a point half way between the apex and the soil (47). This method is more laborious and, because it requires that plants be sacrificed, one must either increase the number of plants per treatment and/or collect data only on selected days. However, it is reported to permit evaluation of relative resistance in the absence of wilt symptoms (e.g., during cool weather).

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular Techniques

1) Polymerase chain reaction

There has been significant progress in using PCR to detect and to identify *R solanacearum* from plants and soil, and the most useful primers are shown in Table 4. Except for the annealing temperature used, amplification reactions are similar to most PCR protocols. Two pairs ofPCR primers have been reported that permit amplification of a fragment specifically from *R. solanacearum* (20, 56) or from this pathogen and the most closely related bacteria (28,39, 57). The specificity of primer pair pehA#3/#6, which amplifies part of the polygalacturonase gene from all *R solanacearum* tested, has not been fully tested (15). One thousand or fewer *R. solanacearum* per ml of sample cells can be detected with some of these primers when using pure cultures or purified DNA, but the detection limit is 10 to
1 DO-fold higher when using plant extracts (20), and may be much higher when using soil (28). Although promising, these primers have not been exhaustively tested for sensitivity and specificity, so they should be used with due caution.

Table 4. PCR primers useful for *Ralstonia solanacearum* and closely related bacteria.

Primer	Primer sequence (5' to 3')	Anneal temp.	No. bases Amplified	Specificity'	Reference
Y2 aLII	CCCACTGCTGCCTCCCGTAGGAGT GGGGGTAGCTIGCTACCTGCC		287-288	*R. sol.* + related	57
759 760	GTCGCCGTCAACTCACTTICC GTCGCCGTCAGCAATGCGGAATCG		281	*R. sol.* + related	28,39
PS96-H PS96~	TCACCGAAGCCGAATCCGCGTCCATCAC AAGGTGTCGTCCAGCTCGAACCCGCC		148	*R. sol.*	20, 56
pehA#3 pebA#6	CAGCAGAACCCGCGCCTGATCCAG ATCGGACTIGATGCGCAGGCCGTI		504	*R. sol.*	1 5

ı Primer pairs Y2IOLII and *759n60* amplify the fragments of the indicated size from *R. solanacearum, P. syzygii,* and the BD bacterium, but not from *R. pickettii; R. eutropha,* or other bacteria. Primer pair PS96-Hl-I amplifies DNA only from *R. solanacearum,* Primer pair pehA#3/#6 amplifies DNA from *R solanacearum* but not from *R. pickettii;* amplification from *P. syzygii* and BD bacterium were not tested.

See Appendix A for details.

b. Serological techniques

The possibility of using serological methods for detection and identification of *R. solanacearum* has been studied in many laboratories (see Seal and Elphinstone [55]), but they have not been developed to the point of being reliable for identification. Polyclonal antibodies made to *R solanacearum* cells have good sensitivity in methods like enzyme-linked imrnunosorbent assays (ELISA), and immunofluorescence is now an accepted method for screening plant tissues for latent infections (29). However, because the polyclonal sera used to date cross-react with closely related organisms like *R pickettii* (51), positive samples must be confirmed by secondary tests (cultural, pathogenicity, or PCR). In contrast, almost all the monoclonal antibodies produced are too selective, and do not react with all strains of *R solanacearum.* A possible exception is the monoclonal PSI (1), which appears to recognize an epitope inR *solanacearum* EPS I (37) and reacted with all the diverse EPS-positive strains tested in an ELISA. Further tests are required before PS 1 can be recommended for routine identification, but this should be facilitated by the commercial availability of this monoclonal antibody (Agdia, 30380 Country Road #6, Elkhardt, IN 46514). max.).

See Appendix B for details.

c. Commercial automated techniques

1) Fatty acid methyl ester analysis (FAME)

The most complete FAME analysis of *R. solanacearum* and closely related bacteria was performed by Stead (60,61), and the methods described here are from that study. Strains of *R solanacearum* and the BD bacterium are grown on Trypticase Soy Broth (BBL) at 28°C for 24 ± 1 hour and 48 ± 2 hours, respectively. *P. syzygii* is cultured on casein salts agar (7.5 g acid casein hydrolysate [Oxoid], 2.0 g sucrose, 0.25 g $MgS0_4$-$7H_20$, 0.5 g KJIP04, 0.25 g ferric ammonium citrate, 15 g agar, 1 liter distilled water) at 28°C for 6 days. Cells are harvested, cellular lipids are saponified, and the fatty acids methylated according to MIDI protocols (52). FAMEs are then extracted, purified, and injected into a gas chromatograph fitted with an appropriate column.

The fatty acid composition of *R solanacearum* (races 1 to 3, biovars 1 to *4), P. syzygii,* and BD bacterium are very similar, but further tests are required to determine if they can be reliably differentiated by FAME analysis alone (60). Janse (30) reported similar data for *R. solanacearum.* These plant pathogenic bacteria, along with *Burkholderia* species, are differentiated from the fluorescent pseudomonads, and from *Comamonas* and *Acidovorax* species, by the presence of 14:0 3-0H, 16:1 2-0H, 16:0 2-0H, and 18:1 2-0H. The absence of 16:0 3-0H distinguishes *R solanacearum* and its related plant pathogens (and *R. pickettii)* from *R eutropha* and *Burkholderia* species (30, 60).

2) Biolog

Several laboratories have reported that Biolog GN plates are useful for identifying *R so/anacearum, P. syzygii,* and BD bacterium. However, only *R. solanacearum* (with A and B subgroups) is included in the current Biolog database. In one study, Black and Sweetmore (4) examined *R. solanacearum, P. syzygii,* and BD bacterium. They modified the manufacture's recommended procedure and determined that 92% of the *R solanacearum* strains were correctly identified, and *P. syzygii* was generally identified as *R solanacearum* (subgroup A). However, the BD bacterium was consistently misidentified as *Acinetobacter calocaceticus.* Assignment of *R so/anacearum* strains to biovars was not possible. Black and Sweetmore recommend that, if possible, researchers modify their database by removing the atypical *R. solanacearum* B subgroup and adding the metabolic profiles for *P. syzygii* and BD bacterium. In a second study, Li and Hayward (35) compared *R solanacearum* (biovars 1 to 4) to *R pickettii* and *Burkholderia* species. Using modified techniques, 96% of the strains tested were correctly identified to species level; 100% of 15 *R. so/anacearum* strains were correctly identified. *R pickettii* had the metabolic profile most like that of *R solanacearum,* but it clustered at only 66% similarity.

See Appendix C for description and details.

7. CULTURE PRESERVATION

a. Water suspensions

R. solanacearum can be stored for several years in distilled or deionized water (or tap water boiled to eliminate chlorine) without significant loss ofviru1ence or change in phenotype (14,32). Cultures should be streaked first on TTC medium and well-isolated fluidal colonies restreaked on CPG plates because some strains are sensitive to the formazan pigment produced from TTC. Two loopfuls of bacteria from a composite of about six individual 48 to 72 h-old colonies are transferred to 5 to 8 ml of sterile water in screw cap test tubes. Suspensions should be turbid (10^7 to 10^8 CFU/ml). Suspensions should be stored near 20°C (15°C min. and 28 °C max.) and restreaked every six months; repurify the culture if nonmucid mutants become numerous. Cultures have maintained viability for 8 to 10 years in water.

b. Lyophilized

R. solanacearum tolerates lyophilization very well, and this method was used for decades by Dr. Luis Sequeira to maintain his collection. To prepare the suspension medium for freezing, autoclave separately 14% (w/v) Bacto peptone and 14% (w/v) sucrose in water and then combine equal volumes of the sterile solutions. Remove cells from a fresh CPG plate and make a very dense (e.g., 1 x 10^{10} CFU/ml) suspension in a small quantity of the suspension medium. Use a Pasteur pipette to dispense the bacterial suspension into the bottom of two or more sterile lyophilization ampules (about 10% of the total volume in the ampule) keeping the neck area of the ampule clean. Freeze the samples in a dry ice-ethanol bath and attach the ampules to a freeze-drying machine. When lyophilization is complete, flame seal the neck of each ampule under vacuum. Store the ampules at room temperature protected from the light. Some lyophilized cultures are known to have remained viable for 30 years.

c. Cryostorage

Freezing (less than -70°C) *R. solanacearum* in a cryoprotectant is the most convenient method of long term storage with minimal phenotypic changes. Prepare screw-capped freezer vials for use by adding 0.6 ml of 20% (v/v) glycerol in water to each, capping loosely, and autoclaving for 20 min. Allow the tubes to cool to room temperature, cap tightly, and store at 4°C until needed. To stock a strain, add 0.5 ml ofCPG broth to each of two vials. Transfer to each vial the cells from one half a heavily streaked two-dayold (at 30°C) CPG or TTC plate; thoroughly mix, seal tightly, and place in the ultralow freezer. One vial serves as the working stock and the second vial as a backup stock in a separate ultralow freezer to reduce loses if one ofthe freezers fails. To recover a strain, remove a vial from the ultralow freezer and quickly, while the contents remain frozen, use a sterile wooden applicator or hypodermic needle to scrape off a small quantity of the

sample from the surface of the frozen stock and streak this onto a TTC plate.

8. LITERATURE CITED

1. Alvarez, AM., J. Berestecky, J. I. Stiles, S. A Ferreira, and A A Benedict. 1993. Serological and molecular approaches to identification of *Pseudomonas solanacearum* strains from *Heliconia.* Pages 62-69 in: G. L. Hartman and AC. Hayward, eds., Bacterial Wilt. Proceedings of an international conference held at Kaohsiung, Taiwan, 28-31 October 1992. ACIAR Proceedings No. 45. Australian Centre for International Agricultural Research, Canberra, Australia.

2. Ayers, S. R., P. Rupp, and W. T. Johnson, Jr. 1919. A study of the alkali-forming bacteria in milk. U. S. Department of Agriculture Bulletin 782: 1-39.

3. Baharuddin, B., K. Rudolph, and F. Niepold, 1994. Production of mono specific antiserum against the blood disease bacterium affecting banana and plantain. Phytopathology 84:570575.

4. Black, R., and A. Sweetmore. 1993. Identification and characterization of *Pseudomonas solanacearum* using metabolic profiles. Pages 32-44 in: G. L. Hartman and A C. Hayward, eds., Bacterial Wilt. Proceedings of an international conference held at Kaohsiung, Taiwan, 28-31 October 1992. ACIAR Proceedings No. 45. Australian Centre for International Agricultural Research, Canberra, Australia.

5. Brumbley, S. M., B. F. Carney, and T.P. Denny. 1993. Phenotype conversion in *Pseudomonas solanacearum* due to spontaneous inactivation of *PhcA*, a putative *LysR* transcriptional activator. J. Bacteriol. 175: 5477-5487.

6. Burdon, K. L. 1946. Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. J. Bacteriol. 52:665-678.

7. Clough, S. J., A. B. Flavier, M. A Schell, and T. P. Denny. 1997. Differential expression ofviru1ence genes and motility in *Ralstonia (Pseudomonas) solanacearum* during exponential growth. Appl. Environ. Microbiol. 63:844-850.

8. Cook, D., E. Barlow, and L. Sequeira. 1989. Genetic diversity of *Pseudomonas solanacearum:* Detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. Mol. Plant-Microbe Interact. 2: 113121.

9. Cook, D. and L. Sequeira. 1994. Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. Pages 77-93 in: A. C. Hayward and G. L. Hartman, eds., Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas solanacearum.* CAB

International, Wallingford, UK.

10. Denny, T. P. and S. R. Baek. 1991. Genetic evidence that extracellular polysaccharide is a virulence factor of *Pseudomonas so/anacearum.* Mol. Plant-Microbe Interact. 4: 198-206.

11. Eden-Green, S. J. 1994. Diversity of *Pseudomonas solanacearum* and related bacteria in south east Asia: new directions for Moko disease. Pages 25-34 in: A. C. Hayward and G. L. Hartman, eds., Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas solanacearum.* CAB International, Wallingford, UK.

12. Elphinstone, J. G., J. Hennessy, J. K. Wilson, and D. E. Stead. 1996. Sensitivity of different methods for the detection of *Pseudomonas solanacearum* in potato tuber extracts. EPPO/OEPP Bulletin 26:663-678.

13. Engelbrecht, M. 1994. Modification of a semi-selective medium for the isolation and quantification of *Pseudomonas solanacearum.* ACIAR Bacterial Wilt Newsletter 10:3-5

14. French, E. R., L. Gutarra, P. Aley, and 1. Elphinstone. 1995. Culture media for *Pseudomonas solanacearum* isolation, identification and maintenance. Fitopatologia 30:126-130.

15. Gillings, M., P. Fahy, and C. Davies. 1993. Restriction analysis of an amplified polygalacturonase gene fragment differentiates strains of the phytopathogenic bacterium *Pseudomonas solanacearum.* Lett. Appl. Microbiol. 17:44-48.

16. Gillis, M., T. Vanvan, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin, and M .P. Fernandez. 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N-2-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. 45:274-289.

17. Graham, J. and A B. Lloyd. 1978. An improved indicator plant method for the detection of *Pseudomonas so/anacearum* race 3 in soil. Plant Dis. Reptr. 62:35-37.

18. Granada, G. A. and L. Sequeira. 1983a. A new selective medium for *Pseudomonas solanacearum.* Plant Dis. 67: 1084-1088.

19. Granada, G. A and L. Sequeira. 1983b. Survival of *Pseudomonas solanacearum* in soil, rhizosphere, and plant roots. Can. J. Microbiol. 29:433-440.

20. Hartung, F., R. Werner, H. P. Muhlbach, and C. Buttner. 1998. Highly specific PCRdiagnosis to determine *Pseudomonas so/anacearum* strains of different geographical origins. Theor. Appl. Genet. 96:797-802.

21. Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum.* 1. Appl. Bacteriol. 27:265-277.

22. Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum.* Annu. Rev. Phytopathol. 29:65-87.

23. Hayward, A. C. 1994. Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. Pages 123-135 in: A. C. Hayward and G. L. Hartman, eds., Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas solanacearum.* CAB International, Wallingford, UK.

24. Hayward, A. C. 1995. *Pseudomonas solanacearum.* Pages 139-151 in: U. S. Singh, R. P. Singh, and K. Kohmoto, eds., Pathogenesis and Host Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Bases. Volume I: Prokaryotes. Elsevier Science, Inc. Tarrytown, N. Y.

25. Hayward, A. C., H. M. EI-Nashaar, U. Nydegger, and L. De Lindo. 1990. Variation in nitrate metabolism in biovars of *Pseudomonas solanacearum.* J. Appl. Bacteriol. 69:269-280.

26. Hayward, A. c., 1. G. Elphinstone, D. Caffier, 1. D. Janse, E. Stefani, E. R. French, and A. 1. Wright. 1998. Round table on bacterial wilt (brown rot) of potato. Pages 410-430 in: P. Prior, C. Allen, and 1. Elphinstone, eds., Bacterial Wilt Disease: Molecular and Ecological Aspects. Proceedings of the Second International Bacterial Wilt Symposium. SpringerVerlag, Berlin.

27. He, L. Y., L. Sequeira, and A. Kelman. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant. Dis. 67:1356-1361.

28. Ito, S., Y. Ushijima, T. Fujii, S. Tanaka, M. Kameya-Iwaki, S. Yoshiwara, and F. Kish., 1998. Detection of viable cells of *Ralstonia solanacearum* in soil using a semiselective medium and a PCR technique. 1. Phytopathol. 146:379-384.

29. Janse,1. D. 1988. A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. EPPO/OEPP Bulletin. 18:343-351.

30. Janse, 1. D. 1991. Infra- and intraspecific classification of *Pseudomonas solanacearum* strains, using whole cell fatty acid analysis. Syst. Appl. Microbiol. 14:335-345.

31. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

32. Kelman, A. and L. H. Person. 1961. Strains of *Pseudomonas solanacearum* differing in pathogenicity to tobacco and peanut. Phytopathology 51: 158-161.

33. Lelliott, R. A. and D. E. Stead. 1987. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications, Oxford, UK.

34. Li, x., M. Dorsch, T. del Dot, L. 1. Sly, E. Stackebrandt, and A. C. Hayward. 1993. Phylogenetic studies of the rRNA group II pseudomonads based on 16S rRNA gene sequences. J. Appl. Bacteriol. 74:324-329.

35. Li, X. and A. C. Hayward. 1993. The use of the Biolog identification system for the rapid identification of plant pathogenic pseudomonads. Pages 45-48 in: G. L. Hartman and A. C. Hayward, eds., Bacterial Wilt. Proceedings of an international conference held at Kaohsiung, Taiwan, 28-31 October 1992. ACIAR Proceedings No. 45. Australian Centre for International Agricultural Research, Canberra, Australia.

36. Mao, G. Z. and L. Y. He. 1998. Relationship of wild type strain motility and interaction with host plants in *R. solanacearum.* Pages 184-191 in: P. Prior, C. Allen, and J. Elphinstone, eds., Bacterial Wilt Disease: Molecular and Ecological Aspects. Proceedings of the Second International Bacterial Wilt Symposium. Springer-Verlag, Berlin.

37. McGarvey, J. A., C. J. Bell, T. P. Denny, and M. A. Schell. 1998. Analysis of extracellular polysaccharide in culture and in planta using immunological methods: new insights and implications. Pages 157-163 in: P. Prior, C. Allen, and J. Elphinstone, eds., Bacterial Wilt Disease: Molecular and Ecological Aspects. Proceedings of the Second International Bacterial Wilt Symposium. Springer-Verlag, Berlin.

38. Michel, R.,and B. Hauroder. 1997. Isolation of an *Acanthamoeba* strain with intracellular *Burkholderiapickettii* infection. Zbl. Bact. 285:541-557.

39. Opina, N., F. Tavner, G. Hollway, J. F. Wang, T. H., Li, R. Maghirang, M. Fegan, A. C. Hayward, V. Krishnapillai, W.F. Hong, B.W. Holloway, and J. N. Timrnis. 1997. A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum).* AsiaPacific J. Molec. Biol, Biotechnology 5: 19-30.

40. Ostle, A. G. and J. G. Holt. 1982. Nile blue A as fluorescent stain for poly-P-hydroxybutyrate. Appl. Environ. MicrobioL 44:238-241.

41. Palleroni, N. J. 1984. Family 1. Pseudomonadaceae. Pages 141-219 in: N. R. Krieg and J. G. Holt, eds., Bergey's Manual of Systematic Bacteriology Williams and Wilkins, Baltimore.

41. Palleroni, N. J., R. W. Ballard, E. Ralston, and M. Doudoroff 1972. Deoxyribonucleic acid homologies among some *Pseudomonas* species. 1. Bacteriol. 110: 1-11.

43. Palleroni, N. 1. and M. Doudoroff 1971. Phenotypic characterization and deoxyribonucleic acid homologies of *Pseudomonas solanacearum.* J. Bacteriol. 107:690-696.

44. Palleroni, N. 1., R. Kunisawa, R. Contopoulou, and M. Doudoroff 1973. Nucleic acid homologies in the genus *Pseudomonas.* Int. J. Syst. Bacteriol. 23:333-339.

45. Pfender, W. F., D. I. Rouse, and D. 1. Hagedorn. 1958. A "Most probable number" method for estimating inoculum density of *Aphanomyces euteiches* in naturally infested soil. Phytopathology 48: 126-128.

46. Pierce, L. and M. N. Schroth. 1994. Detection of *Pseudomonas* colonies that accumulate poly-P-hydroxybutyrate on nile blue medium. Plant Dis. 78:683-685.

47. Prior, P., S. Leclerq, S. Bart, A. Darrasse, and G. Anais. 1996. Resistance to bacterial wilt in tomato as discerned by spread of *Pseudomonas (Burkholderia) solanacearum* in stem tissues. Plant Pathology 45:720-726.

48. Ralston, E., N. 1. Palleroni, and M. Doudoroff 1973. *Pseudomonas pickettii,* a new species of clinical origin related to *Pseudomonas solanacearum.* Int. J. Syst. Bacteriol. 23: 15-19.

49. Raymundo, A. K., Y. Aves-Il.agan, and T. P. Denny. 1998. Analysis of genetic variation in a population of banana- infecting strains *ofRalstonia so/anacearum.* Pages 56-60 in: P. Prior, C. Allen, and 1. Elphinstone, eds., Bacterial Wilt Disease: Molecular and Ecological Aspects. Proceedings of the Second International Bacterial Wilt Symposium. SpringerVerlag, Berlin.

50. Roberts, S. 1., S. 1. Eden-Green, P. Jones, and D. 1. Ambler. 1990. *Pseudomonas syzygii* sp. nov., the cause of Sumatra disease of cloves. Syst. Appl. Microbiol. 13:34-43.

51. Robinson-Smith, A., 1. G. Elphinstone, and S. M. D. O. Forde. 1995. Production of antibodies to *Pseudomonas solanacearum,* the causative agent of bacterial wilt. Food & Agric. Immunol. 7:67-79.

52. Sasser, M. 1990. Identification of bacteria through fatty acid analysis. Pages 199-204 in: Methods in Phytobacteriology. D. Sands, Z. Klement, and K. Rudolph, eds. Akademia Kiado, Budapest.

53. Schaad, N. W., A. Takatsu, and 1. C. Dinense. 1978. Serological identification of strains of *Pseudomonas solanacearum* in Brazil. Pages 295-300, Vol. I., in: Station de Pathologie

Vegetale of'Phytobacteriol, eds., Proceedings of the IV Int. Conf on Plant Pathogenic Bacteria. Angers, France.

54. Schaad, N. W., S. S. Cheong, S. Tamaki, E. Hatziloukas, and N. 1. Panopoulos. 1995. A combined biological and enzymatic amplification (Bio-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. Phytopathology 85:243248.

55. Seal, S. E. and 1. G. Elphinstone. 1994. Advances in identification and detection of *Pseudomonas so/anacearum.* Pages 35-57 in: A C. Hayward and G. L. Hartman, eds., Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas so/anacearum.* CAB International, Wallingford, UK.

56. Seal, S. E., L. A, Jackson, and M. 1. Daniels. 1992. Isolation of a *Pseudomonas* so/anacearum-specific DNA probe by subtraction hybridization and construction of speciesspecific oligonucleotide primers for sensitive detection by the polymerase chain reaction. Appl. Environ. Microbiol. 58:3751-3758.

57. Seal, S. E., L. A. Jackson, 1. P. W. Young, and M. 1. Daniels. 1993. Differentiation of *Pseudomonas so/anacearum, Pseudomonas syzygii, Pseudomonas pickettii* and the blood disease bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. 1. Gen. Microbiol. 139: 1587-1594.

58. Skerman, V. B. D. 1967. A Guide to the Identification of the Genera of Bacteria. 2nd ed .. Williams and Wilkins, Baltimore, Maryland.

59. Stanier, R. Y., N. 1. Palleroni, and M. Doudoroff 1966. The aerobic pseudomonads: a taxonomic study. 1. Gen. Microbiol. 43:159-271.

60. Stead, D. E. 1992. Grouping of plant-pathogenic and some other *Pseudomonas* spp by using cellular fatty acid profiles. Int. 1. Syst. Bacteriol. 42:281-295.

61. Stead, D. E., 1. E. Sellwood, 1. Wilson, and 1. Viney. 1992. Evaluation ofa commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. 1. Appl. Bacteriol. 72:315-321.

62. Taghavi, M., C. Hayward, L. 1. Sly, and M. Fegan. 1996. Analysis of the phylogenetic relationships of strains of *Burkho/deria so/anacearum, Pseudomonas syzygii,* and the blood disease bacterium of banana based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. 46: 10-15.

63. van den Mooter, M., M. Steenackers, F. Maertens, F. Gossele, P. De Vos, 1. Swings, K. Kersters, and 1. De Ley. 1987. Differentiation between *Xanthomonas campestris* pv.

graminis (ISPP List 1980), pv. *phleipratensis* (ISPP List 1980) emend., pv. *poae* Egli and Schmidt 1982 and pv. *arrenatheri* Egli and Schmidt 1982, by numerical analysis of phenotypic features and protein gel electrophoregrams. 1. Phytopathol. 118: 135-156.

64. Winstead, N. N. and A. Kelman. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum.* Phytopathology 42:628-634.

65. Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H, Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa. 1992. Proposal of *Bur kholderi a* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 36:1251-1275.

66. Yabuuchi, E., Y. Kosako, I Yano, H. Horta, and Y. Nishiuchi. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1986) comb. nov, and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. 39:897-904.

9. CHEMICAL LIST

Chemicals	Source

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178

Acetic acid	
Agar	Difco, Detroit, MI
Ammonium dihydrogen phosphate	
Ammonium sulfate	
Bacitracin	
Bromthymol blue	
Casein acid hydrolysate (Casamino acids)	
D(+)Cellobiose	
Chloromycetin	
Crystal violet	
Cycloheximide	
Dipotassium hydrogen phosphate	
Dulcitol	
Esculin	
Ethanol	
Ferric ammonium citrate	
Glucose (dextrose)	
Glycerol	

Hydrochloric acid DL-p-hydroxybutyrate, sodium salt meso-Inositol	
Lactose	
Magnesium sulfate (heptahydrate)	
Maltose	
Mannitol	
Merthiolate tincture	Obtainable from any pharmacy
Nile Blue A sulfate (Basic Blue 12)	
Penicillin	
Peptone	Difco
Potassium chloride	
Potassium dihydrogen phosphate	
Potassium nitrate	
Polymyxin B sulfate	
Proteose Peptone No. 3	Difco
D(+) Ribose	
Safranin	
Sodium citrate	
Sodium hydroxide	
Sodium succinate	
Sorbitol	
Starch	
Sucrose	
Sudan Black B	
Thirnersal (see Merthiolate tincture)	
D(+)Trehalose	
Tyrothricin	
Triphenyl tetrazolium chloride	
Trypticase soybroth	BBL
Xylol (Xylene)	
Yeast extract	Difco
Zinc chloride	

II. GRAM-NEGATIVE BACTERIA

G. *Xanthomonas*

N. W. Schaad, J. B. Jones, and G. H. Lacy

1. INTRODUCTION

All *Xanthomonas* species discussed in this manual are plant pathogens and so far as is known they are found only in association with plants or plant materials. Over 100 xanthomonads have been recognized as distinct plant pathogens. However, because few species were easily distinguished from the type species *Xanthomonas campestris* and/or each other by the usual biochemical tests used in The Eighth Edition of Bergey's Manual of Determinative Bacteriology, only five species of *Xanthomonas, albilineans, ampelina, axonopodis, campestris, andjragariae,* were listed in the 1980 Approved List of Bacterial Names (43). All other species of *Xanthomonas* were designated as pathovars of X. *campestris.* The term "pathovar" was proposed in 1978 (57) as a means of preserving the names of plant pathogens, for which adequate phenotypic data were not available for differentiating them from all other species. Most names of xanthomonads were retained as pathovar epithets within the species X. *campestris.* The term pathovar has no taxonomic standing in the International Code of Nomenclature of Bacteria (45). The term was meant to be temporary, so that the names and authorities would be saved for future elevation back to species rank. In this chapter we will continue to follow the species and pathovar designations.

Since 1980, several changes have been proposed related to the nomenclature and taxonomy of xanthomonads. The current 'Index of the Bacterial and Yeast Nomenclatural Changes' published in the *International Journal of Systemic Bacteriology* between 1980 and 1992 (30) includes the following species of *Xanthomonas: albilineans, axonopodis, campestris, citri, fragariae, maltophilia, oryzae, phaseoli,* and *populi.* We accept the elimination of X. *ampelina* and its reclassification as *Xylophilus ampelinus* (55). We agree that X. *citri* can be differentiated from X. *campestris* pv. *campestris* (17), however, additional data is needed to determine its proper species epithet Also, we accept the elevation of X. *campestris* pv. *oryzae* to the rank of species with the inclusion of pathovars *oryzae* and *oryzicola* (48). We do not support the inclusion of X. *maltophilia* (49) due to its low DNA-DNA homology to *Xanthomonas (47).* Vauterin et al. (51, 52) have made the most sweeping proposals for reclassification of X. *campestris* based on original and cited fatty acid, nutritional, and *DNNDNA* analysis. We agree with many of their proposed changes. However, we will instead adhere, in most part, to recommendations of Schaad et al. (42) as follows:

The following proposed names (52) are not included:

X. arboricola (Vauterin et al., 1995)
X. axonopodis (Starr and Garces), Vauterin et al., 1995, all proposed pvs. of *X. bromi* (Vauterin et al., 1995)
X. codiaei (Vauterin et al., 1995)
X. hortorum (Vauterin et al., 1995)

X. melonis (Neto et al., 1984), Vauterin et al .. 1995
X. sacchari (Vauterin et al., 1995)
X. theicola (Uehara et al., 1980), Vauterin et al., 1995
X. translucens pvs. *arrhenatheri, graminis, phlei, phleipratensis* and *poae* (Vauterin et al., 1995)
X. vasicola (Vauterin et al., 1995) with pvs. *holcicola* and *vasculorum* type "B". *X. vesicatoria* type "B". (ex Doidge, 1920), Vauterin et al., 1995

We include the following species in this chapter:

X. albilineans
X. axonopodis
X. *campestris,* including pvs. *campestris, aberrans, armoracieae, barbarae, incanae,* and *raphani*
X. cassavae (type "A" strains only)
X. cucurbitae
X. fragariae
X. hyacinthi
X. oryzae, including pvs. *oryzae* and *oryzicola*
X.pisi
X.populi
X. translucens including pvs. *translucens, cerealis, hordei; secalis,* and *undulosa*

The following pathovars should remain in X. *campestris* until adequate data are available: *alfalfae, arrhenatheri, begoniae, carotae, cassavae* (type "B"), *citri, corylina, dieffenbachiae, glycines, graminis, hederae, holcicola, juglandis, malvacearum, manihotis, melonis, nigromaculans, pelargonii, poae, phaseoli, phlei, phleipratenis, phyllanthi, poinsettiicola, pruni, ricini, sesbaniae, tamarindi, theicola, vasculorum, vesicatoria* (type "A"), *vignicola* and *vitians.* For additional pvs., see Young et al. (57).

Phenotypic description. Xanthomonads are Gram-negative, aerobic, rods (0.4-0.7 x 0.7-1.8 urn) with a single polar flagellum. They are catalase positive and weak producers of acids from carbohydrates. Colonies of most species and pathovars are mucoid, convex, and yellow on YDC agar (Plate 4, Fig. 1). Most produce copious amounts of extracellular polysaccharide, the source of "xanthan gum" (21), on media containing glucose. The unique yellow membrane-bound non-water-soluble pigments produced by all but three specieslpathovars, are brominated arylpolyene esters (xanthomonadins) (46) soluble in petroleum ether, methanol, and benzene. They have absorption maxima in methanol at wavelengths of 420,441, and 468 nm. Xanthomonads are easily differentiated from the other genera of aerobic, Gram-negative rods such as *Pseudomonas, Acidovorax, Burkholderia,* and *Ralstonia* (see p.6), and other yellow pigmented bacteria such as *Flavobacterium* and *Pantoea* spp. (Table 1).

Table 1. Phenotypic characters useful for differentiating *Xanthomonas* from *Pseudomonas* and other yellow pigmented bacteria such as *Flavobacterium.*

Character"	*Xanthomonas*	*Pseudomonas*	*Flavobacterium*	*Pantoea*
Flagellation	**I, polar**	>1, polar	None	Peritrichous
Xanthomonadin	Yes	No	No	No
Fluorescence	No	Yes	No	No
Litmus milk	Alkaline"	Alkaline	Unchanged	Not Determined
Growth at 40°C	*ve«*	No	Yes	Yes
Levan from sucrose	Yes	Yes	No	No
H_2S from cysteine	Yes	No	No	No
Oxidase	Negative	Negatived	Positive	Negative
Fermentative	No	No	No	Yes
Growth on:				
0.1% TTC	No	Yes	Yes	Yes

a For flagellation, fluorescence, levan, H_2S, oxidase, fermentation, and TIC, see pp. 10,97,93,47, 10,9, 154, respectively.
b X. *fragariae* is unchanged
c X. *fragariae* will not grow at 40°C d
P. *cichorii* is positive

2. IDENTIFICATION OF XANTHOMONADIN PIGMENT

Many yellow-pigmented bacteria are usually isolated from plant tissues and soil debris. It is not always easy to distinguish the colonies of *Xanthomonas* spp. visually from colonies of saprophytic bacteria by color. One way of helping to confirm the identification of *Xanthomonas* spp. is to identify the yellow xanthomonadin pigment of *Xanthomonas* (20, 21, 46).

Identification of xanthomonadins can be accomplished as follows:

a. Streak a colony onto NA (see a, p. 3) (NA should not contain any additional carbohydrates because the resulting copious slime will interfere with chromatography of the pigments). (A known xanthomonad should be included as a control).

b. After 48 h growth, scrape the bacteria from the surface and add to 3 ml of spectrophotometry grade methanol in a test tube with a screw cap. Enough bacteria should be added to the methanol to give a turbidity equivalent to near 10^{10} CFU/ml, (approximately 0.5 OD at 600 nm).

c. Place the capped tube in a boiling water bath until the pigment has been removed from the bacteria (solution becomes yellow) centrifuge at 1,000 g for 15 min.

d. Decant supernatant and evaporate the methanol extract in a water bath at 50-60°C until the optical density of the pigment extract reaches 0.4 at 443 run. Scan a sample to determine absorption maxima

e. Spot five 5,u1 aliquots on a precoated, thin-layer chromatography sheet of silica gel 60 of 0.2 mm thickness. Apply a total of 25 ,u1 per spot, allowing each 5].11 amount to dry before applying the next.

f. Place plate in developing apparatus with anhydrous spectrophotometry grade methanol as the solvent. Allow the solvent front to move approximately 10 cm.

g. Outline the yellow spots with a pencil when the silica gel is still wet. A yellow spot with an average Rf value of 0.45 (range of 0.42 to 0.49) is positive for xanthomonadins.

h. Include a *Flavobacterium* or *Pantoea* species as a negative control.

3. ISOLATION TECHNIQUES USING DIFFERENTIAL AND SEMIS ELECTIVE MEDIA

Plant tissue should be fresh, if possible. although some species can be isolated from dried, pressed material. Wash the material thoroughly in running water and blot dry or surface disinfect. Select a young lesion or the margin of an older lesion and with a sharp sterile scalpel cut out a small piece of tissue. If in doubt as to the lesion being caused by a bacterium. crush the tissue in water on a clean microscope slide (if the tissue is thin enough. mount in a drop of water) and examine under a phase contrast microscope.

The crushed specimen should contain masses of rod-shaped bacteria. If the material is not crushed, look for bacteria streaming from edges of the cut tissue or from the vascular tissue. If masses of bacteria are present, cut another piece of tissue approximately 1 mrn" from beside the original site. crush in a droplet of water on the lid of a sterile disposable Petri dish and streak onto the surface of YDC agar medium using a loop. Incubate the plate at 25-27^0 C for 72 h or longer. Bacteria obtained from dried material may take up to a week to form single colonies.

Plants growing in a moist atmosphere and infected with some xanthomonads, such as X. *translucens* often produce a copious bacterial exudate on the surface of the lesion and this dries to a shiny thin paper-like film. Pure cultures of *Xanthomonas* spp. are usually isolated easily from both the wet exudate and the dry papery film. As for pseudomonads, both differential and selective media should be used for isolating xanthomonads.

A large number of semiselective agar media are available for isolation and identification of xanthomonads. The agar media of choice for isolation and identification are summarized in

Table 2. Cycloheximide is used for soil and seed isolation only and should be prepared as a stock solution in methanol.

a. Recipes for differential media

1) Yeast extract nutrient agar (YNA)

	perL
Yeast extract	5g
Nutrient agar (see a, p. 3)	23 g

Used for general isolation from plant tissues.

2) YDC (see b, p. 4). Before solidifying, mix well on Vortex mixer and set quickly in cold water to avoid settling of $CaC0_3$• Slants are recommended for short-term (3 mo) storage of cultures. This medium is recommended for the evaluation of pigmentation (Plate 4, Fig. 1).

Can be used for general isolation from plant tissues.

3) Yeast extract sucrose peptone agar (YSP) (14)

	perL
Yeast extract	5.0 g
Sucrose	20.0 g
Peptone	10.0 g
Agar	15.0 g

Recommended for isolation of X. *albilineans.*

4) Yeast salts (YS) broth and yeast salts agar (YSA) (14)

	perL
$NH_4H_2P0_4$	0.5 g
K_2HP0_4	0.5 g
$MgS0_4$-$7H_20$	0.2 g
NaCI	5.0 g
Yeast extract	5.0 g

If solid media is required (YSA), add 12.0 g agar. Particularly useful as a storage medium and a basal medium for some cultural tests.

b. Recipes for semiselective media

1) X *campestris* pv. *campestris*

a) SX agar (41)

Table 2. Cycloheximide is used for soil and seed isolation only and should be prepared as a stock solution in methanol.

a. Recipes for differential media

1) Yeast extract nutrient agar (YNA)

	perL
Yeast extract	5g
Nutrient agar (see a, p. 3)	23 g

Used for general isolation from plant tissues.

2) YDC (see b, p. 4). Before solidifying, mix well on Vortex mixer and set quickly in cold water to avoid settling of $CaC0_3$• Slants are recommended for short-term (3 mol storage of cultures. This medium is recommended for the evaluation of pigmentation (Plate 4, Fig. 1).

Can be used for general isolation from plant tissues.

3) Yeast extract sucrose peptone agar (YSP) (14)

	perL
Yeast extract	5.0 g
Sucrose	20.0 g
Peptone	10.0 g
Agar	15.0 g

Recommended for isolation of X. *albilineans.*

4) Yeast salts (YS) broth and yeast salts agar (YSA) (14)

	perL
$NH_4H_2P0_4$	0.5 g
K_2HP0_4	0.5 g
$MgS0_4e7H_20$	0.2 g
NaCI	5.0 g
Yeast extract	5.0 g

If solid media is required (YSA), add 12.0 g agar. Particularly useful as a storage medium and a basal medium for some cultural tests.

b. Recipes for semiselective media

1) X *campestris* pv. *campestris*

a) SX agar (41)

	perL
Starch (soluble-potato)	10.0 g
Beef extract	1.0 g
Ammonium chloride	5.0 g
K_2HPO_4	2.0 g
Methyl violet 2B	1.0 ml *
Methyl green	2.0 ml **
Agar	15.0 g
After autoclaving add:	
Cycloheximide	5.0 ml ***

* 1 % solution in 20% ethanol. Be sure to use methyl violet 2B and not methyl violet B as the latter is more toxic to *X campestris* pv. *campestris*

** 1 % aqueous solution

*** Add 5.0 g to 10.0 ml methanol, bring to 100 ml with sterile water.

SX medium can be used for isolation of *X campestris* and several other starch positive species and pathovars including *X citri* from plant tissues and soil. Also useful for direct plating of seeds contaminated with *X campestris* pv. *campestris* and for differentiation of several xanthomonads (plate 4, Fig.3).

b) 8M agar (9)

	per L
KH_2PO_4	LOg
Na_2HPO_4	2.6 g
NH_4Cl	1.0 g
NaCI	2.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	67.0 mg
Glucose	1.0 g
L-methionine	0.2 g
Starch (soluble potato)	10.0 g
Methyl violet 2B	1.0 ml *
Methyl green	2.0 ml **
Trace element solution	1.0 ml ***
Triphenyltetrazolium chloride	1.0 ml ****
Cycloheximide	50.0 mg
Agar	20.0 g

* 1 % solution in 20% ethanol

** 1 % aqueous solution

** Dissolve the following in 100 ml H_20: EDT A, 250 mg; $FeS0_4 \cdot 7H_20$, 500 mg; $ZnS0_4 \cdot O$, 10 mg; $CuS0_4 \cdot 5H_20$, 10 mg; $MnS0_4 \cdot H_20$, 10 mg; $NaMo0_4 \cdot 2H_20$, 25 mg; $Na_2B_40_7 \cdot 10H_20$, 18 mg; $CoS0_4 \cdot 7H_20$, 10 mg.

* 1 % aqueous solution

****

Especially useful for isolating X. *campestris* pv. *campestris* from soil and debris in soil. Less selective than SX but results in higher recoveries of most strains. Also useful or differentiating various pathovars. (Plate 4, Fig.3)

c) NSCAA (35)

	perL
Nutrient agar (see a, p. 3)	23.0g
Starch (soluble-potato)	15.0 g

After autoclaving add the following to nutrient starch agar:

Cycloheximide	5.0 ml*
Nitrofurantoin	1.0 ml**
Vancomycin	1.0 ml***

* Add 5.0 g to 10.0 ml methanol, bring to 100 ml with water and filter sterilize (0.22 urn membrane).

** Add 50 mg to 5 ml of 50% dimethyl formamide.

** Add 12.5 mg to 25 ml water, and filter as above.

*

Especially useful for isolating X. *campestris* pv. *campestris* from seeds.

d) Fieldhouse Sasser (FS) agar (39, 58)

	per L
Starch (soluble-potato)	10.0 g
Yeast extract	0.1 g
K_2HP0_4	0.8 g
KH_2P0_4	0.8 g
$MgS0_4 \cdot 7H_40$	0.01 g*
Methyl green	1.5 ml**
$KN0_3$	0.5 g
Agar	15.0 g

After autoclaving add:

Cycloheximide	5.0 ml***
Pyridoxine-HCL (1 mg/ml)	1.0 ml
Cephalexin (10 mg/ml)	2.5ml
Gentamycin (2 mg/ml) Trimethoprim	0.2 ml****
(10 mg/ml of ethanol) D-methionine (1	3.0 ml
mg/ml)	3.0ml

* Add first and be sure it is fully dissolved. 1
** % aqueous solution.
** Add 5.0 g to 10.0 ml methanol, bring to 100 ml with water, and ftlter
* sterilize (0.22 um membrane).
**** Gentamycin can be toxic to some xanthomonads and is often eliminated.

Especially useful for isolating X. *campestris* pv. *campestris* from seeds.

2) X. *campestris* pv. *vesicatoria*

a) Tween medium (28) (Plate 4, Fig. 2)

	per L
Peptone	10.0 g
Potassium bromide	10.0 g
$CaC1_2$	0.25 g
Agar	15.0 g

After autoclaving add:

Tween 80	10.0 m1
Cycloheximide	75.0 mg
Cephalexin	25.0mg
5- Fluorouracil	6.0mg
Tobramycin	O.4mg

b) mSQ medium (25, H. Bolkan, personal communication)

	perL
Succinic acid (disodium salt)	10.0 g
Quinic acid	5.0 g
K_2HP0_4	1.5 g
$(NH_4)'ZS04$	1.0 g
Agar	15.0 g
Yeast Extract	0.25 g

pH 7.2-7.5 with 10 N NaOH

After autoclaving add:

Mg $S04^{e}7H_20$ (20%)	7.5 ml
Cephalexin (10% mg/ml water)	3.5 ml
5-fluorouracil (120 mg/lOml 50% methanol)	1.0 ml
Tobramycin (10 mg/100ml water)	2.0 ml
Cycloheximide (250mg/5ml methanol)	3.0 ml

3) Modified Tween medium (28) for X. *campestris pv.juglandis* (Steve Lindow, personal communication)

	per L
Peptone	10.00 g
Potassium bromide	
$CaC1_2$	10.00 g
Boric acid	
Agar	2.0 g
	0.3 g
	15.0 g
After autoclaving add:	
Tween 80	20.0ml
Cycloheximide	1.0 ml * 3.3
Cephalexin	ml ** 660.0 Jll
5-	*** 330.0 Jll
Fluorouracil	****
Tobramycin	

* Add 1.0 g to 10 m150% ethanol.
** Add 100.0 mg to 10 ml water.
*** Add 50.0 mg to 10 ml 50% methanol.
**** Add 10.0 mg to 10 ml water.

A second medium designated MS is available (31).

4) XPS medium of X. *campestris* pv. *pruni (10).*

	per L
Alginic acid	2.0 g
8-azaguanine	0.2 g
Nicotinic acid	2.0 mg
Cysteine	3.0 mg
KH_2P0_4	0.8 g
K_2HP0_4	0.8 g
$MgS0_4$	0.1 g
Agar	15.0 g

After autoclaving add the following filtered stock solutions:

Chlorothalonil (800 mg/10 ml H_20	1.0 ml

Kasugamycin (160 mg/lO ml H:!O	1.0 ml

5) X. *translucens*

a) Kim's medium (22)

	per L
Lactose	10.0 g
D(+) trehalose	4.0 g
Thiobarbituric acid	0.2 g
K:!HP0_4	0.8 g
KH_2P0_4	0.8 g
Yeast extract	30.Omg
NH_4Cl	1.0 g
Agar	15.0 g

Dissolve above on hot plate, and adjust to pH 6.6 with 1 N NaOH.

After autoclaving, cool to 50^0 C and add:

Cycloheximide	100.0 mg
Tobramycin	8.0mg
Ampicillin	1.0 mg

Especially useful for isolating X. *translucens* from soil.

b) XTS agar (40) (Plate 4, Fig. 2)

	per L
Nutrient agar (see a, p. 3)	23.0 g
Glucose	5.0 g

After autoclaving add:

Cycloheximide	2.0 ml*
Gentamycin	0.5 ml**
Cephalexin	1.0 ml***

The following stock solutions should be prepared:

* 1.0 g to 10 ml of 75% ethanol (use only when assaying seeds)

** 50 mg to 5 ml of 75% ethanol (omit if antagonism occurs)

*** 50 mg to 5 ml of 75% ethanol

Especially useful for isolating X. *translucens* from seeds.

6) X. *campestris* pv. *carotae*

a) Modified D-5 (23)

	per L
D-cellobiose	10.0 g*
K_2HPO_4	3.0 g
NaH_2PO_4	1.0 g
NH_4Cl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.3 g
Cycloheximide	0.2 g*
Agar	15.0 g

* Add filter-sterilized (0.22p.m) D-cellobiose and cycloheximide after autoclaving.

Especially useful for isolating X. *campestris* pv. *carotae* from seed and soil.

b) xes Agar (56) (Plate 4, Fig. 2)

	per L
Lactose	10.0 g
D(+) trehalose	4.0 g
2- Thiobarbituric Acid	0.2 g
K_2HPO_4	0.8 g
KH_2PO_4	0.8 g
NH_4Cl	1.0 g
Yeast extract	0.5 g
Agar	15.0 g

Combine all ingredients except agar and antibiotics and adjust to pH 6.6. After autoclaving, cool to 50°C and add the following after filtersterilizing:

Tobramycin	0.4 ml *
Ampicillin (Na salt)	0.5 ml ** 0.5
Vancomycin	ml *** 5.0
Cycloheximide	ml ****

* Add 80 mg to 10 ml water.
** Add 20 mg to 10 ml water.
*** Add 10 mg to 10 ml water.
**** Add 5 g to 10 ml methanol and bring to 100 ml with water.

Especially useful for isolating X. *campestris* pv. *carotae* from seeds. Normally not selective enough for isolation from soils.

7) Esculin-trehalose (ET) medium for X. *campestris* pv. *dieffenbachiae.* *(32)*

(Plate 4, Fig. 4)

	per L
Esculin	1.0 g
Trehalose	0.5 g
$FeCl_3$-$6H_zO$	0.5 g
NaCl $MgS0_4$-$7H_zO$ K_ZHP0_4	5.0 g
Agar	0.2 g
	1.0 g
	15.0 g

The pH is adjusted to 6.8, and the mixture autoclaved immediately. After autoclaving, add the following filtered, sterilized stock solutions:

Cycloheximide	1.5 ml*
Cephalexin	5.0 ml**
Trimethoprine	3.0 ml**
Pyridoxine (Img/ml) D-methionine (Irng/ml) Triphenyl-tetrazoliurn chloride (1 % aqueous)	1.0 ml 3.0 ml 5.0 ml

* 1.0 g to 10 ml of 75% ethanol 100

** mg to 10 ml of 75% ethanol

8) MXP agar for X. *campestris* pv. *phaseoli* (11) (Plate 4, Fig. 2)

	per L
Starch (soluble-potato)	8.0 g
Glucose	1.0 g
Yeast extract	0.7 g
K_ZHP0_4	0.8 g
KH_ZP0_4	0.6 g
Potassium bromide	10.0 g
Methyl violet 2B	30.0 pl*
Methyl green	60.0 pl**
Agar	15.0 g

* 1 % solution in 20% ethanol

** 1 % aqueous solution

After autoclaving add the following filtered, sterilized stock solutions:

Chlorothalonil	1.0 ml *** 10.0
Cephalexin	ml **** 10.0 ml
Kasugamycin	***** 5.0 ml
Gentamycin	******

*** 1.2 ml to 38.8 ml water
**** 0.1 g to 50 ml water ***** 0.1 g to 50 ml water ****** 10 mg to 50 ml water

9) Cefazolin trehalose agar (CTA) medium for X. *campestris* pv. *manihotis* (15) (Plate 4, Fig. 5)

	perL3
K_2HP0_4	.0 g
NaH_2P0_4	1.0g
$MgS0_4$-$7H_2$)	0.3 g
NH_4Cl	1.0g
D (+) -trehalose	9.0 g
Glucose	1.0 g
Yeast extract	1.0 g
Agar	14.0 g

After autoclaving add the following:

Cefazolin, sodium salt	2.5 ml *
Lincomycin, hydrochloride	1.0 ml ** 1.0 ml *** 5.0 ml ****
Phosphomycin, disodium salt	
Cycloheximide	

Useful for isolating X. *campestris* pv. *manihotis* on seeds.

* add 100 mg to 10 ml distilled water and filter
** add 12 mg to 10 ml to 75% methanol
*** add 25 mg to 10 ml distilled water and filter
**** add 5.0 g to 10 ml methanol and bring to 100 ml with distilled water.

10) XAS medium for X. *albilineans* (13)

	per 1
Sucrose	10.0 g
Peptone	5.0 g
K_2HP0_4-$3H_20$	0.5 g
$MgS0_4$-$7H_20$	0.25 g
KBr	5.0mg
Na_2S0_3	50.0 g
Agar	15.0 g

Adjust pH to 6 - 8 and autoclave.
After medium cools down to approximately 50°C add:

Benomyl 50DF	2.0mg
Cycloheximide	10.0 ml *
Kasugamycin	5.0 ml ** 5.0
Novobiocin	ml *** 2.5
Cephalexin	ml ****

Filter sterilize the following stock solutions:

*	1 g cycloheximide to 10 ml methanol, bring to 100 ml with distilled water 0.5 g
**	kasugamycin to 50 ml distilled water
***	0.3 g novobiocin to 50 ml distilled water
****	0.5 g to 50 ml distilled water

11) m-XAS medium for X. *albilineans (54)*

Prepare as for XAS, but add 5 g yeast extract.

This medium is useful for more rapid growth needed for Bio-Pf'R (54).

4. DIFFERENTIA nON OF COMMONLY ISOLATED SPECIES AND PA THOVARS

Most species of X. *campestris* can be differentiated by a combination of several phenotypic tests (Table 3) and growth on semiselective agar media (Table 2 and Plate 4).

Table 2. Media of choice for isolation and differentiation of several xanthomonads.

Xanthomonad	Medium
X. *albilineans*	XAS
X. *campestris* pv. *campestris*	SX, FS*, NseAA*, SM
X. *campestris* pv. *alfalfae*	XTS, xes
X. *campestris* pv. *begoniae* X.	SX, SM, MD-5
campestris pv. *carotae* X.	XCS-. MD-5
campestris pv. *citri*	SX
X. *campestris* pv. *dieffenbachiae*	ET, SX
X. *campestris* pv. *malvacearum* X.	xes
campestris pv. *manihotis*	eTA
X. *campestris* pv. *nigromaculans* X.	SX
campestris pv. *phaseoli*	MXP*
X. *campestris* pv. *vesicatoria*	mSQ, Tween, xes
X. *fragariae*	Tween
X. *campestris* pv. *juglandis*	m Tween

X. *juglandis* pv. *corylina* X.	SX
juglandis pv. *pruni*	XPS
X. *hederae* pv. *pelargonii* X.	XTS, XCS, Tween
hyacinthi	SX
X. *pisi*	SX
X. *translucens* (all pathovars)	XTS

*Most useful for isolating from seeds.

Table 3. Diagnostic tests for differentiation of several *Xanthomonas* spp.

Tests								
Mucoid growth on YDC	+	+		+	+	+	+	+
Growth at 35°C	+		+	+	+	+	+	+
Growth on SX	+						+	
Starch hydrolysis	+	+		+	+		+	+
Esculin hydrolysis	+		+	+	+	+	+	+
Protein digestion	+			+	+	+	+	+
Litmus milk	Alk	Alk	NC	Alk	NC	NC	Alk	Alk
Ice nucleation		+						+
Acid from:								
Arabinose	+							
Utilization of:								
Glycerol	+0		+				+	
Melibiose	V						+0	

• These results are typical for pv. *campestris;* many other pathovars were not tested.

Abbreviations: +, 80% or more strains positive; -, 80% or more strains negative; V, between 21-79% of strains positive; +0 , delayed or weak positive; ND, not determined; Alk, alkaline; NC, no change. Data taken from Vauterin et al, (52) and original data (NWS).

5. DIAGNOSTIC MEDIA AND TESTS

Unless otherwise described, all cultures should be grown at 25-27 °C in 16 mm diameter tubes containing 5 ml (broth) or 8 ml (agar slants) of media previously sterilized by autoclaving at 121°C for 15 min. Broth cultures should be incubated on a rotary or reciprocating shaker.

a. Color and mucoid erowth. The best media for observation of colony color and

mucoid growth are GYCA and YDC (see b, p. 4).

b. Growth at *35*° C. Temperature for growth should be determined in YS broth (see h, p. 47) and the cultures should be incubated in a stirred water bath at 35°C for 10-12 days.

c. Starch hydrolysis. Streak culture onto nutrient starch agar (see c, p. 181).

d. Hydrolysis of esculin. Development of a dark brown color in esculin broth*(YS* broth + ferric ammonium citrate 0.05% (w/v) and esculin 0.1 % (w/v); pH 6.8) after shaker incubation for up to 28 days is regarded as indicating utilization of esculin. Complete hydrolysis can be assumed when no fluorescence is seen with UV light. Alternatively, cells may be spotted on basal ET medium (see 7, p. 186) (32).

e. Protein digestion (liquid or agar) and litmus milk test. For liquid tests, reactions are observed in reconstituted powdered skim milk containing 0.004% bromcresol purple (w/v) and sterilized by steaming for 30 min on three successive days. Prior to steaming, check pH and adjust to 7.0 using 1.0 NaOH, if necessary. Inoculate milk solution in test tubes with a loopful of cells and incubate at 27 ° C, and observe for a clearing reaction (positive digestion of casein). Record results (see Plate 1, Fig. 9) as no change, acid (red) or alkaline (blue).

For the agar plate test (44), reconstituted powdered skim milk, sterilized as above, is mixed at 48 ° C with sterile melted YNA to obtain a 10% v/v concentration and poured over the surface of a thin layer of nutrient agar in Petri plates. The plates should be dried, spot inoculated, and observed for a clear zone around the colonies after 3,5, and 7 days.

f. Ice nucleation (see 1, p. 98).

g. Acid production from arabinose and utilization of carbohydrates (5). (see f, p. 47).

6. PATHOGENICITY TESTS

Plants that are perfectly healthy and growing vigorously should be selected for pathogenicity tests. To prepare the inoculum, cells from YDC (see b, p. 4) slant cultures should be grown in nutrient broth (see a, p. 3) or liquid 523 medium (see 1), p. 27) and adjusted to give 10^6 -10^7 CFU/ml using sterile saline, as described (see 5, p. 226). For most pathogens, leaves should be sprayed with inoculum, using a De Vilbiss atomizer or chromatography sprayer. For some pathogens a 26- gauge needle and 1-3 ml syringe works best. Fill the syringe with inoculum and puncture the leaf holding a finger beneath the leaf. Control plants should be similarly inoculated using sterile diluent only. While the plants are still moist they should be placed in a lighted dew (Percival Manufacturing Co., Boone, Iowa) or mist chamber in which the temperature is controlled to approximately 23-25°C. The chamber should be set so dew occurs only at night and not continually. The plant surfaces should

remain wet continuously without much runoff. If a lighted dew chamber is not available, light must be supplied during daylight hours either naturally or by artificial lights without affecting the temperature. Alternatively the inoculated plant can be covered with a plastic bag in a growth chamber or in a greenhouse at 25-27°C. After 48 h, the plants should be removed from the dew chamber or the bag removed and plants placed on a greenhouse bench for development of symptoms. For vascular infecting pathogens, such as *X campestris* pv. *campestris, X translucens* and *X campestris* pv. *manihotis,* inject the inoculum into a stem, leaf axis, or whorl using a sterile needle and 26 gauge syringe. Place the plants on a greenhouse bench at 27-30°C and observe symptoms 7 days later. For *X oryzae* pv. *oryzicola,* infiltrating leaves with a blunt end 26 gauge syringe works well.

7. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular Techniques

1) PCR primers are available for identification of several xanthomonads (Table 4).

Table 4. PCR Primers for Xanthomonads.

Specificity	Primer Designation	Sequence	Size (bp)	Reference
Nonspecific, many xanthomonads	RST2	(5'AGGCCCTGGAAGGTGCCCTGGA3')	840	26
	RST3	(5'ATCGCACTGCGTACCGCGCGCG3')		
	RS21	(5'GCACGCTCCAGATCAGCATCGAGG3 ')	1075	26
	RS22	(5'GGCATCTGCATGCGTGCTCTCCGA3')		
Nonspecific, certain xanthomonads	RST9	(5'GGCACTATGCAATGACTG3')	355	26
	RSTlO	(5'AATACGCTGGAACTGCTG3')		
X. albilineans	Ala4	(5' CCCGACTGGCTCCACCACTG3')	360	33
	L1	(5' CAAGGCATCCACCGTI')		
	XAFI	(5'CCTGGTGATGACG TGGGTT-3')	600	54
	XARI	(5'CGATCAGCGATGCACGCAGT-3')	440	12
	XaAlb2-f3	(5'CACACACACAATACAGCATTGCGG3')		
	XaAlb2-3	(5'CCCAACTTACTTGAGGCTATGG3')		
	Nested primers			
	XaAlb2-f4	(5'CTTCTGCAGCTTGCTCGTC3')	308	
	XaAlb2-r4	(5'GCTCAGTTACGCTCAGCTAATC3')		
X. campestris pv. *citri*	3	(5'TGGTGTCGTCGCTTGTAT3')	222	1
	2	(5'CACGGGTGCAAAAAATC3')	8	

	4	(5'TGTCGTCGCTIGTAT3') (5 ' GGGTGCGACCGTCAGGA3')	467	1 9
	7 Nested primers			
	94-3 944	(5'CTCGATCACGATGTCCTICTCC3') (5 ı GTGGATGGCATGAGCATGAAG3')	315	
X campestris pv. *manihotis*	XV XX	(5' TICGGCAACGGCAGTGACCACC3 ı › (5' TCAATCGGAGATIACCTGAGCG3 ')	898	53
X campestris pv. *phaseoli*	X4e X4c	(5' -CGCCCGGAAGCACGATCCTCGAAG3) (5'GGCAACACCCGATCCCTAAACAGG3)	730	4
Xi fragariae	XF9 XFll	(5'TGGGCCATGCCGGTGGAACTGTGTGG3') (5' TACCCAGCCGTCGCAGACGACCGG3 ')	537	36
	XF9 XFll	(5' TGGGCCATGCCGGTGGAACTGTGTGG3') (5' TCCCAGCAACCCAGATCCG3')	458	
	241A 24lB	(5'GCCCGACGCGAGTIGAATC3') (5'GCCCGACGCGCTACAGACTC3')	550	34
	245A 245B	(5 ı CGCGTGCCAGTGGAGATCC3') (5' CGCGTGCCAGAACTAGCAG3 ')	300	
	2956A 295B	(5' CGTICCTGGCCGATTAATAG3') (5 ' CGCGTTCCTGCG I I I I I ICG3')	615	
X translucens"	Tl T2	(5'CCGCCATAGGGCGGAGCACCCCGAT3') (5'GCAGGTGCGACGTTTGCAGAGGGATCTTCTGCAAA3')	139	2 9

a Also includes *X campestris* pvs. *arrhenatheri, phlei, poae, graminis*

2) Ribotyping using available peR primers and probes can be very useful for identification of some pathovars of *X campestris (7).*

See Appendix A for details.

b. Serological Techniques

Immunofluorescence tests using polyclonal antibodies are useful for rapid, presumptive identification of *X campestris* pv. *campestris* isolated from crucifer seeds (16,38). Polyclonal antisera are generally specific at the species level, only (40). Genusspecific monoclonal antibodies are available for identification of xanthomonads, and species-specific monoclonal and polyclonal antibodies are available for identification of *X albilineans* (3, 37) and *X oryzae* (5). Identification at the pathovar level is more difficult. Monoclonal antibodies are available for *X campestris* pv. *campestirs* (1) and *X campestris*

pv. *citri* (2), *X campestris* pv. *pelargonii* and *X campestris* pvs. *begoniae (6),*

dieffenbachiae (27), and *vesicatoria* (8). These antibodies react with most, but not all strains. Like polyclonal antibodies, monoclonal antibody techniques should not be used alone in identification ofxanthomonads.

See Appendix B for details.

c. Commercial Automated Techniques

1) ELISA kits for identification of several xanthomonads are available from several commercial companies.

2) Fatty acid analysis is available from MIDI (Newark, DE).

3) Substrate utilization kits are available from Biolog and API.

4) Sequence analysis of 16S rRNA (MicroSeq) is available from Perkin Elmer (New Jersey).

5) A PCR kit is available for *X campestris* pv. *phaseoli* (D-Genos, Angers, France.

See Appendix B and C for details.

8. CULTURE PRESERVATION

Working cultures should be maintained on YDC slants so contamination can be easily determined.

For long term preservation of cultures, store at -85°C as described (see 7, p. 30).

9. LITERATURE CITED

1. Alvarez, A. M., A. A. Benedict, C. Y. Mizumoto, 1. E. Hunter, and D. W. Gabriel. 1994. Serological, pathological and genetic diversity among strains of *Xanthomonas* infecting crucifers. Phytopathology 84: 1449-1457.

2. Alvarez, A. M., A. A. Benedict, C. Y. Mizumoto, L. W. Pollard, and E. L. Civerolo. 1991. Analysis of *Xanthomonas campestris* pv. *citri* and *X campestris* pv. *citrume/o* with monoclonal antibodies. Phytopathology 81:857-865.

3. Alvarez, A. M., S. Schenck, and A. A. Benedict. 1996. Differentiation of *Xanthomonas a/bi/ineans* strains with monoclonal antibody reaction patterns and DNA fingerprints. Plant Pathol. 45:358-366.

4. Audy, P., C. E. Braat, G. Saindon, H. C. Huang, and A. Laroch. 1996. A rapid and sensitive PCR-based assay for concurrent detection of bacteria causing common and halo blights in bean seed. Phytopathology 86:361-366.

5. Benedict, A. A., A. M. Alvarez, 1. Berestecky, W. Imanaka, C. Y. Mizumoto, L. W. Pollard, T. W. New, and C. F. Gonzales. 1989. Monoclonal antibodies specific for *Xanthomonas campestris* pv. *oryzae* and pv. *oryzico/a.* Phytopathology 79:322-328.

6. Benedict, A. A., A. M. Alvarez, and L. W. Pollard. 1990. Pathovar-specific antigens of *Xanthomonas campestris* pv. *begoniae* and *X campestris* pv. *pe/argonii* detected with monoclonal antibodies. Appl. Environ. Micro. BioI. 56:572-574.

7. Berthier, Y., V. Verdier, J. L. Guesdon, D. Chevrier, 1. B. Denis, G. Decoux, and M. Lemattre. 1993. Characterization of *Xanthomonas campestris* pathovars by rRNA gene restriction patterns. Appl. Environ. Microbiol. 59: 851-859.

8. Bouzar, H., 1. B. Jones, R. E. Stall, N. C. Hodge, G. V. Minsavage, A. A. Benedict, and A. M. Alvarez. 1994. Physiological, chemical, serological, and pathogenic analyses of a worldwide collection of *Xanthomonas campestris* pv. *vesicatoria* strains. Phytopathology 84:663-671.

9. Chun, W. C. and A. M. Alvarez. 1983. A starch-methionine medium for isolation of *Xanthomonas campestris* pv. *campestris* from plant debris in soil. Plant Dis. 67:632-635.

10. Civerolo, E. L.) M. Sasser, C. Helkie and D. Burbage. 1982. Selective medium for *Xanthomonas campestris* pv. *pruni.* Plant Dis. 66:39-43.

11. Claflin, L. E.) A. K. Vidaver and M. Sasser. 1985. MXP, a semi-selective medium for *Xanthomonas campestris* pv. *phaseo/i.* Phytopathology 75:730-734.

12. Davis, M. 1., P. Rott, and G. Astua-monge. 1998. Nested, multiplex PCR for detection of both *Clavibacter xyli* subsp. *xyli* and *Xanthomonas albilineans* in sugarcane. 7th Int. Congress Plant Pathology, Edinburgh, Scotland. 3.3.4. (Abstr.).

13. Davis, M. 1., P. Rott, P. Randin, and 1. L. Dean. 1994. Evaluation of selective media and immunoassays for detection of *Xanthomonas albilineans,* causal agent of sugarcane leaf scald disease. Plant Dis. 78:78-82.

14. Dye, D. W. 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N. Z. 1. Sci. 5:393-416.

15. F essehaie, A., K. Wydra, and K. Rudolph. 1999. Development of a new semis elective medium for isolating *Xanthomonas campestris* pv. *manihotis* from plant material and soil. Phytopathology 89: 591-597.

16. Franken, A. A. J. M. 1992. Comparison of immunofluorescence microscopy and dilution-plating for the detection of *Xanthomonas campestris* pv. *campestris.* Neth. 1. Plant Pathol. 98:81-94.

17. Gabriel, D. W., M. T. Kingsley, J. E. Hunter, and T. Gottwald. 1989. Reinstatement of *Xanthomonas citri* (ex Hassee) and *X phaseoli* (ex Smith) to species and reclassification of all *X campestris* pv. *citri* strains. Int. 1. Syst. Bacteriol. 39: 14-22.

18. Hartung, 1. S., 1. F. Daniel, and O. P. Provost. 1993. Detection *ofXanthomonas campestris* pv. *citri* by the polymerase chain reaction method. Appl. Env. Microbiol. 59: 1143-1148.

19. Hartung, 1. S., O. P. Provost, I. Villemot, and A. Alvarez. 1996. Rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and a nested-polymerase chain reaction assay. Phytopathology 86:95-101.

20. hey, M. S. and R. E. Stall. 1982. Value ofxanthomonadins for identification of pigmented *Xanthomonas campestris* pathovars. Pages 85-95. Proc. Fifth Int. Conf. Plant Path. Bacteria. Cali, Columbia.

21. Jeans, A. 1., E. Pittsley, and E. R. Senti. 1961. Polysaccharide B-1459: a new hydrocolloid polyelectrolyte produced from glucose by bacterial fermentation. 1. Appl. Polymer. Sci. 5:519-526.

22. Kim, H. K. 1982. Epidemiological, genetical and physiological studies of the bacterial leaf streak pathogen, *Xanthomonas campestris* pv. *translucens* (1. 1. R.) Dowson, PhD. Dissertation. 94 pp. Montana State University, Bozeman, MT.

23. Kuan, T. L., G. V. Minsavage and R. L. Gabrielson. 1985. Detection of *Xanthomonas campestris* pv. *carotae* in carrot seed. Plant Dis. 69:758-760.

24. Lazo, G. R., R. Roffey and D. W. Gabriel. 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment length polymorphisms. Int. 1. Syst. Bacteriol. 37:214~221.

25. Lee, Y. -A., D. C. Hildebrand, and M. N. Schroth. 1992. Use of quinate metabolism as a phenotypic property to identify members of *Xanthomonas campestris* DNA homology group 6. Phytopathology 82:971-973.

26. Leite, R. P., Jr., G. V. Minsavage, U. Bonas, and R. E. Stall. 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria.* Appl. Env. Microbiol. 60: 1068-1077.

27. Lipp, R. L., A. M. Alvarez, A. A. Benedict, and 1. Berestecky. 1992. Use of monoclonal antibodies and pathogenicity tests to characterize strains of *Xanthomonas campestris* pv. *dieffenbachiae* from aroids. Phytopathology 82:677-682.

28. Me Guire, R. G., 1. B. Jones and M. Sasser. 1986. Tween medium for semiselective isolation of *Xanthomonas campestris* pv. *vesicatoria* from soil and plant material. Plant Dis. 70:887.

29. Maes, M., P. Garbeva, and O. Kamoen. 1996. Recognition and detection in seed ofthe *Xanthomonas* pathogens that cause cereal leaf streak using rDNA spacer sequences and polymerase chain reaction. Phytopathology 86:63-69.

30. Moore, W. E. C. and L. V. H. Moore. 1992. Index of the Bacterial and Yeast Nomenclatural Changes. Published in the International Journal of Systematic Bacteriology Since the 1980 Approved Lists of Bacterial Names. (January 1, 1980 to January 1, 1992) American Society for Microbiology. Washington, D.C.

31. Mulrean, E. N. and M. N. Schroth. 1981. A semiselective agar medium for the isolation of *Xanthomonas campestris* pv. *juglandis* from walnut buds and catkins. Phytopathology 71:336-339.

32. Norman, D. and A. M. Alvarez. 1989. A rapid method for presumptive identification of *Xanthomonas campestris* pv. *dieffenbachiae* and other xanthomonads. Plant Dis. 73: 654658.

33. Pan, Y-b., M. P. Grisham, and D. M. Burner. 1997. A polymerase chain reaction protocol for the detection of *Xanthomonas albilineans*, the causal agent of sugarcane leaf scald disease. Plant Dis. 81: 189-194.

34. Pooler, M. R., D. F. Ritchie, and 1. S. Hartung. 1996. Genetic relationships among strains of *Xanthomonas jragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. Appl. Env. Microbiol. 62: 3121-3127.

35. Randhawa, P. and N. W. Schaad. 1984. Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. Phytopathology 74:268-262.

36. Roberts, P. D., 1. B. Jones, C. K. Chandler, R. E. Stall, and R. D. Berger. 1996. Survival of *Xanthomonas fragariae* on strawberry in summer nurseries in Florida detected by specific primers and nested polymerase chain reaction. Plant Dis. 80:1283-1288.

37. Rott, P., M. 1. Davis, and P. Baudin. 1994. Serological variability *inXanthomonas albilineans* causal agent ofleafscald disease of sugarcane. Plant Path. 43:344-349.

38. Schaad, N. W. 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris.* Phytopathology 68:249-252.

39. Schaad, N. W. 1989. Detection *ofXanthomonas campestris* pv. *campestris* in *Crucifers.* Pages 68-75 in: A. W. Saettler, N. W. Schaad, and D. A. Roth, eds., Detection of Bacteria in Seed and Other Planting Material. APS Press. St. Paul, MN.

40. Schaad, N. W. and R. L. Forster. 1985. A semiselective agar medium for isolating *Xanthomonas campestris* pv. *translucens* from wheat seed. Phytopathology 75:260-263.

41. Schaad, N. W. and W. C. White. 1974. A selective medium for soil isolation and enumeration of *Xanthomonas campestris.* Phytopathology 64:876-889.

42. Schaad, N. W., A . K. Vidaver, G. H. Lacy, K. Rudolph, and 1. B. Jones. 2000. Evaluation of proposed amended names of several pseudomonads and xanthomonads and recommendations. Phytopathology 90:208-213.

43. Skerman, V. B. D., V. McGowan, and P. H. A. Sneath. 1980. Approved List of Bacterial Names. Int. 1. Syst. Bacteriol. 30:225-420.

44. Smith, N. R., R. E. Gordon, and F. E. Clark. 1952. Aerobic spore-forming bacteria. U.S. Department of Agriculture Monograph 16.

45. Sneath, P. H. A. 1992. International Code of Nomenclature of Bacteria and Statutes of the International Committee on Systemic Bacteriology and Statutes the Bacteriology and Applied Microbiology Section of The International Union of Microbiological Societies. Bacteriological Code. 1990 Revision. Amer. Soc. for Microbial., Washington, D. C.

46. Starr, M. P., C. L. Jenkins, L. B. Bussey and A. G. Andrews. 1977. Chemotaxonomic significance of the xanthomonadins, novel brominated arylpolyene pigments produced by bacteria of the genus *Xanthomonas.* Archi. of Microbial. 113: 1-9.

47. Starr, M. P. 1981. The *genusXanthomonas.* Pages 742-763 in: M. P. Starr, H. Stolp, H. G. Truper, A. Balows and H. G. Schlegel, eds., Vol. I The Prokaryotes, Springer-Verlag, Berlin.

48. Swings, 1., M. Van Den Mooter, L. Vauterin, B. Haste, M. Gillis, T. W. Mew, and K. Kersters. 1991. Reclassification of the causal agents of bacterial blight *(Xanthomonas campestris* pv. *oryzicola)* of rice as pathovars of *Xanthomonas oryzae* (ex Ishiyama 1922) sp. nov., nom. rev. Int. 1. Syst. Bacterial. 40:301-311.

49. Swings, 1., P. Devos, M. Vanden Mooter, and 1. Deley. 1983. Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* Hugh (1981) comb. nov. Int. J. Syst. Bacteriol. 33:409-413.

50. Thaveechai, N. and N. W. Schaad. 1986. Serological and electrophoretic analysis of a membrane protein extract of *Xanthomonas campestris* pv. *campestis* from Thailand. Phytopahology 76: 139-147.

51. Vauterin, L., B. Haste, P. Yang, A. Alvarez, K. Kersters, and 1. Swings. 1993. Taxonomy of the genus *Xanthomonas.* Pages 156-191 in: 1. G. Swings and E. L. Civerolo, eds., *Xanthomonas.* Chapman & Hall, London.

52. Vauterin, L., B. Haste, K. Kersters, and 1. Swings. 1995. Reclassification of *Xanthomonas.* Int. 1. Syst. Bacteriol. 45-472-489.

53. Verdier, v., G. Mosquera, and K. Assigbetse. 1998. Detection ofthe cassava bacterial blight pathogen, *Xanthomonas axonopodis* pv. *manihotis,* by polymerase chain reaction. Plant Dis. 82:79-83.

54. Wang, Z. K., J. C. Comstock, E. Hatziloukas, and N. W. Schaad. 1999. Comparison of PCR, BID-PCR, DIA, ELISA and isolation on semiselective medium for detection of *Xanthomonas albi/ineans,* the causal agent ofleaf scald of sugarcane. Plant Path. 48:245252.

55. Willems, A., M. Gillis, K. Kersters, L. Van Den Broeke, and J. Deley. 1987. Transfer of *Xanthomonas ampelina* Panagopoulos 1969 to a new genus, *Xylophilus gen. nov., as Xylophilus ampelinis* (panagopoulos, 1969) comb. nov. Int. J. Syst. Bacteriol. 37:422430.

56. Williford, R. E. and N. W. Schaad. 1984. Agar medium for selective isolation of *Xanthomonas campestris* pv. *carotae* from carrot seeds. Phytopathology 74: 1142 (Abstr.)

57. Young, 1. M., D. W. Dye, J. F. Bradbury, C. G. Panagopoulos and C. F. Robbs. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N. Z. J. Agric. Res. 21:153-177.

58. Yuen, G. Y., A. M. Alvarez, A. A. Benedict, and K. J. Trotter. 1987. Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris.* Phytopathology 77:366-370.

10. CHEMICAL LIST

Chemical	Source

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P. O. Box 14508, 81. Louis, MO 63178.

Alginic acid	
Ampicillin, Na salt	
8- Azaguanine	
Beef extract	Difco
Benomyl 50Dp	
Boric acid	
Brilliant cresyl blue	J.T. Baker
Bromcresol purple	
Cefazolin D-cellobiose	
Cephalexin	
Chlorothalonil (Daconil 7787)	SDS Biotech. Corp., Plainsville, OR
Cycloheximide	
Cysteine	
Dimethylformide	
Esculin	
5-Fluorouracil	
Fosfomycin	
Gentamycin	
L-glutamine	

Glycine	
Kasugamycin	Wako Bioproducts, 1600 Bellwood Road, Richmond, VA 23237
Lactose	
Lincomycin	
Methanol, spectrophotometry grade L-methionine	
Methyl green	Coleman Bell
Methyl violet 2B	Fisher
Methylene green	Allied Chemical
Nicotinic acid	
Nitrofurantoin	
Novobiocin	
Nutrient Agar	Difco
Peptone	Difco
Potassium bromide	
Powdered skim milk	
Pyridoxine - HCL	
Silica gel	E. Mercie, Darmstadt, Germany
Sodium deozycholate	
Starch, soluble potato	J.T. Baker
Thiobarbituric acid	
Tobramycin	
D(+) trehalose	
Trimethoprin	
Triphenyltetrazolium chloride	
Tween 80	
Vancomycin	
Yeast extract	Difco

n, GRAM-NEGATIVE BACTERIA

H. *Xylella fastidiosa*

D. Hopkins

1. INTRODUCTION

Gram-negative, xylem-limited bacteria were first discovered in association with Pierce's disease of grapevine in 1973 and were called rickettsia-like bacteria (10, 18). Previously, Pierce's disease (PD) and phony peach (PP) disease were believed to be caused by xylem-limited viruses. Since then these bacteria have been found to cause many other plant diseases that had no known causal agent. The first of these xylem-limited bacteria was grown in axenic culture in 1978 (3). In a taxonomic study, the name *Xylel/a fastidiosa* was proposed to establish a new genus with one species to include all of the known strains of fastidious, Gram-negative, xylem-limited bacteria (26). Cells of *X fastidiosa* are very narrow and can not be seen by bright field microscopy; they can only be observed by phase contrast or dark field microscopy. Because the cells are small (0.2-0.4 by 1.0-4.0 *,urn)* their size becomes very useful in their identification.

This bacterium causes economic losses in many agriculturally important plants, including grapevine, peach, plum, coffee, and citrus (6, 15). It also causes leaf scorch and declines in many urban shade trees, such as elm, oak, and sycamore (12). Some strains of *X fastidiosa* have very wide host ranges; however, many of the hosts may be symptomless (7). The bacterium is transmitted by sucking insects that feed on xylem sap, such as the sharpshooter leafhoppers (22), but is not transmitted mechanically from plant to plant.

Although *X fastidiosa* strains are normally not host specific, they are cell or tissue specific. In the plant, they are restricted to xylem vessels or intercellular spaces of the xylem. Bacteria also tend to accumulate in specific plant parts and the pattern of bacterial distribution often is related to the type symptoms produced in that host. Plant symptoms induced by *X fastidiosa* include leaf scorch, leaf chlorosis, stunting, dieback, and general decline.

2. ISOLATION TECHNIQUES USING DIFFERENTIAL MEDIA

X jastidiosa strains are difficult to isolate and grow in axenic culture. They do not grow on most common bacterial media, but require specialized media, such as PD2 (4), PW (2), CS20 (1), or BCYE (27, Table 1). Growth is slow on these specialized media with doubling times of9 h to 2.0 days (26). This makes contamination by faster growing bacteria a serious problem in the isolation of *X jastidiosa* from plant tissue. An important factor in the successful isolation is the selection of the host tissue for isolation that contains the largest populations of the bacterium. Microscopic examination for the presence of bacteria is a direct way of determining the tissue to sample. Lacking this direct observation, the symptoms observed in the plant host provides clues to the tissue location of the bacterium (23). In the leaf scorch diseases, the leaf veins and petioles are good sources of the bacterium. In diseases with die-back and general decline with no obvious

leaf scorch, such as occurs in infected live oak, the trunk of the tree or branches in the sector with symptoms are generally the best tissues to sample. In diseases with stunting as the primary symptom, such as phony peach, the roots may provide the best sample.

Blotting expressed plant sap directly onto media is a most effective technique for the isolation of *X fastidiosa* from leaf veins, petioles, small twig segments, and small roots. The plant sections (0.5 to 7 cm in length) are first surface sterilized by soaking in alcohol and flaming or by soaking in 1 % sodium hypochlorite for 3-5 min and rinsing in sterile water. Surfacesterilized segments (0.5-1.0 cm) are aseptically cut from the center of the samples and squeezed with forceps or pliers. The sap that exudes from the section is blotted directly onto medium, or placed onto medium with a sterile capillary Pasteur pipet. PW, CS20, or BCYE agar media should be used, since PP group strains will not grow on PD2 (Table 1).

A couple of techniques can be utilized to obtain *X fastidiosa* cells from larger branches or roots for isolation. Stem or root sections are surface sterilized, as described above. The outer bark is removed and each section is cut into fine chips in 1 m1 of supplemented PW broth medium (24). After vigorous agitation, drops of the suspension are placed onto supplemented PW agar. Alternatively, *X fastidiosa* can be extracted from larger stem and root segments by vacuum infiltration with a phosphate buffer (8, 17). A vacuum is applied to the basal end ofthe segment to pull buffer through the section. The vacuum extracts are then concentrated by centrifugation at 4,500 g for 15 min and resuspended in 0.5 ml of buffer. Drops of the concentrated extract are placed on PW agar medium. Since contamination is a serious problem with both of these techniques, several duplicate tubes and plates should be used.

X fastidiosa also can be isolated from vector insects (13). Sharpshooter leafhoppers are surface sterilized by successive l-rnin immersions in 90% ethanol and 2% sodium hypochlorite, followed by three rinses in sterile water. The sharpshooter head is severed from the body and homogenized for 2 min in 2 ml of sterile phosphate-buffered saline using a homogenizer. Drops of the homogenized tissue samples are placed on PW agar medium.

After plant or insect extract is placed on media, plates are observed regularly (every 3-4 days) for colony development for a month using a binocular microscope. Any bacterial colony visible to the unaided eye within 2 days is discarded as a contaminant. Colonies visible after 2 days are transferred to nutrient glucose agar (NGA) and PW medium or the medium used in the isolation. Colonies not growing on NGA, but growing on the other media are subjected to ELISA or PCR assays to confirm that they are *X fastidiosa* (9,20,25). Pathogenicity tests can be useful for identifying Pierce's disease strains of the bacterium, since symptoms develop in 3-6 wk after inoculation. However, with some of the tree hosts, symptoms may take 18-24 months to develop.

3. DIFFERENTIA nON OF COMMONLY ISOLATED PATHOTYPES

Although all strains of *X fastidiosa* have been classified into a single species, there definitely are different pathotypes and possibly different subspecies. Most of the pathotypes have not been characterized and compared with each other very well. Strains can be separated into two distinct groups (i.e., PD and PP groups) based on nutritional fastidiousness (16), enzymelinked immunosorbent assay (ELISA) (5), and the polymerase chain reaction (PCR) (20, Table 1). Those strains in the PD group are most easily differentiated from the PD group by their growth on PD2 agar (Table 1). Most, ifnot all, strains in the Pierce's disease group produce symptoms in *Vitis vinifera* grapevines. Pathogenicity to grape can be evaluated in a 2-3 month test (16). In the phony peach group, there appears to be several different pathotypes that have not been characterized. Pathogenicity tests with some of these tree strains require 18-24 months to complete.

The citrus variegated chlorosis pathotype can be quickly identified using PCR. A set of PCR primers (CYC-1 and 272-2-int) was developed that are specific for strains of *X fastidiosa* that cause citrus variegated chlorosis (CYC) and/or coffee leaf scorch (6,21).

Table 1. Differentiation of Pierce's disease (PD) group strains of *Xylella fastidiosa* from phony peach (PP) group strains

Identification test	PD group strains"	PP group strains"
Growth on:		
Nutrient glucose agar		
PD2 agar	+	
PW, BCYE, and CS-20 agar	+	+
Intensity of ELISA reaction with antisera to:"		
Pierce's disease strain	+++	+
Phony peach strain	+	+++
Digestion ofPCR amplification product by *RsaI*[d]		+

"Xylella fastidiosa strains that produce symptoms on *Vitis vinifera* grapevines and grow on PD2 agar.
bDiverse group of *X fastidiosa* strains that will not produce symptoms on grapevine and will not grow on PD2 agar, probably several different, uncharacterized pathotypes.
'Relative intensity of the absorbance from the enzyme-linked immunosorbent assays of antisera with strains from the two groups.

'The *RsaI* restriction enzyme digests the polymerase chain reaction product (primers RST3I/RST33) from the phony peach group strains into two fragments, but does not digest the product from Pierce's disease strains (20).

4. DIAGNOSTIC MEDIA AND TESTS

3. Diagnostic media for *X fastidiosa*

Specialized media are required for the cultivation of *X fastidiosa.* While there are no diagnostic media that definitively identify a bacterial colony as *X fastidiosa,* there are media that provide presumptive identification; however, confirmation requires the use of other methods, such as light microscopy, ELISA, immunofluorescence, or PCR. Bacterial colonies obtained on PD2, BCYE, CS20, or PW medium that will not grow on NGA, or grow very slowly on NGA., should be tested for confirmation as *X fastidiosa.* Growth, or the lack of growth, on PD2 medium is an indication of the pathotype group of the strain (Table 1).

1) PD2 medium (4)

	perL
Soytone	2.0 g
Tryptone	4.0 g
Trisodium citrate	1.0 g
Disodiurn succinate	1.0g
Hemin chloride	10.0 mg
Potato starch, soluble	2.0 g
$MgS0_4 \cdot 7H_20$ K_2HP0_4	0.4 g
KH_2P0_4	1.5 g
Agar	1.0 g
	15.0 g

2) PW medium (2)

	perL
Soytone	4.0 g
Tryptone	1.0 g
Hemin chloride	10.0 mg
$MgS0_4 \cdot 7H_20$	0.4 g
K_2HP0_4	1.2 g
KH_2P0_4 Phenol	1.0 g
red L-	20.0 mg
glutamine	4.0 g
Bovine serum albumin fraction V	6.0 g
Agar	12.0 g

Stock solutions of glutamine and BSA are filter-sterilized (0.2 urn filter) and added aseptically after the basal medium is autoclaved.

3) BCYE medium (27)

	perL
Yeast extract	10.0 g
Activated charcoal	2.0 g
L-cysteine HCI	0.4 g
Ferric pyrophosphate, soluble	0.25 g
ACES buffer	10.0 g
Agar	17.0 g

L-cysteine HCI and ferric pyrophosphate are dissolved, filter sterilized (0.2 urn filter), and added to the autoc1aved basal medium, pH 6.9.

4) CS-20 medium (1)

	perL 2.0
Soytone	g 2.0 g
Tryptone	5.0 mg
Hemin chloride	0.4 g
$MgS0_4 \cdot 7H_20$	0.85 g
$(NH4)2^{HP0}4$	1.0 g
KH_2P0_4 L-	6.0 g
glutamine	1.0 g
Dextrose	2.0 g
Potato starch, soluble	1.0 g
L-histidine nci Phenol	10.0 mg
red	12.0 g
Agar	

Add each ingredient separately and allow to dissolve. After autoclaving, shake gently to distribute starch. The pH should be 6.6 to 6.7 without adjustment.

5) Supplemented PW medium (24)

	perL4
Soytone	.0 g
Tryptone	LOg
Hemin chloride	10.0
$MgS0_4 \cdot 7H_20$	mg
K_2HP0_4	0.4 g
KH_2P0_4	1.2 g
$(NH4)2^{HP}04$	1.0 g
Phenol red L-	0.8 g
glutamine L-	20.0mg
histidine Hel	4.0 g
	LOg

Bovine serum albumin fraction V	6.0 g
Potato starch, soluble	2.0 g
Cycloheximide	25.0 mg
Agar	12.0 g

6) Nutrient glucose agar (NGA)

	perL
Beef extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g

b. Light microscopy

When symptoms such as leaf scorch or dieback suggest that the causal agent could be *X jastidiosa,* there are several microscopic diagnostic tests that can be attempted. The oldest and simplest techniques involve direct observation with the light microscope, either of plant extracts or of symptomatic plant tissue.

1) KOH extraction method (8)

a) Suspect root and twig samples, 6 em long and 0.5 cm in diameter, are selected for testing.

b) One ml of 0.1 M KOH is drawn through the samples by vacuum infiltration.

c) A drop of the extract is dried on a microscope slide and examined at 800 X by phase contrast or dark field microscopy for the presence of small rod-shaped bacterial cells typical of *X jastidiosa.*

Xylem extracts for light microscopy can also be obtained from leaf petioles by squeezing segments (0.5-1.0 em) with forceps or pliers and blotting the exuded sap directly onto a microscope slide. A syringe method also can be used to obtain extracts from leaf petioles (6). With this method, leaf petioles are excised and fitted to a 2-4 cm long, small diameter (2-3 nun) plastic tube attached to a syringe. Sterilized water is then forced through the xylem of the petiole by applying manual pressure to the syringe, producing drops of extract for microscopic examination.

In plant xylem tissue, *X jastidiosa* is difficult to distinguish from the matrix material in which it is commonly embedded. Hematoxylin and Giemsa stains can be used to differentiate the bacteria from background matrix material in the xylem. In one procedure (14), stem, petiole, or leafvein sections (2- to 3-mm long) were fixed in

formalin-alcohol-acetic acid, and embedded in paraffin. Sections 15 *J.lm* thick were cut with a microtome, stained with Harris' hematoxylin and orange G. This stains the bacterium dark blue against an orange-stained background. With Giemsa stain, the bacterial cells are a dark blue against a light blue background.

c. Electron microscopy

Leaf petioles and veins from symptomatic leaves as well as small stems can be excised, fixed and observed with both transmission electron microscopy and scanning electron microscopy using standard techniques (see b, p. 11). Positive identification of bacteria observed by transmission electron microscopy can be obtained by immunogold labeling utilizing *X fastidiosa-specific* antiserum (11).

5. PATHOGENICITY TESTS

Since *X fastidiosa* colonizes the xylem, inoculation techniques must deliver the inoculum directly into the xylem vessels. The simplest techniques involve puncturing the stem with a syringe needle or pin. The inoculum should be a visibly turbid suspension (1\;00 = 0.2) containing 10^8 to 10^9 CFU/ml in a buffer, such as phosphate buffered saline (20 mM sodium phosphate, 0.85% NaCl, pH 6.8). With the pin-prick method, a drop of the inoculum is placed on two ofthe lower internodes of the host plant. A needle is used to prick the stem several times through the drops until the inoculum is pulled into the xylem vessels of the stem by the negative pressure in the vessels. With the syringe needle method, the stem is punctured with the needle and the inoculum is forced into the wound by applying pressure with the syringe. To insure that sufficient inoculum is taken in by the xylem in larger plants, a flap of stem tissue may be raised by cutting tangentially upward with a razor blade, to expose the wood. The inoculum then is applied to the sliced branch either by placing the flap of tissue into a tube filled with inoculum or by placing drops of bacterial suspension underneath the flap with a pipette.

With some tree strains of *X fastidiosa* (such as phony peach), it may be necessary to apply inoculum to the roots. With one method, the roots are washed, pruned 50%, and immersed in the inoculum. The stem is cut 15 cm above the soil line and vacuum is applied to the cut stub to pull at least 1 ml of inoculum into the cut roots of the plant. With another method (1), roots are washed and one large root of each plant is severed. The severed root is connected immediately by a short piece of tubing to a 10-ml pipette containing 10 ml of the bacterial suspension. Plants are repotted with the pipette reservoirs connected to the roots. Most of the volume should be absorbed within 3 days.

With all procedures, control plants are inoculated with buffer only. Symptoms produced by *X fastidiosa* strains in various hosts require from 3 weeks after inoculation (Pierce's disease of grapevine) to 18 - 24 months (e.g. phony peach) for symptom development.

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

3. Molecular techniques

A sensitive and specific PCR protocol for the detection of *X fastidiosa* in plant samples is available (20). Plant extracts are prepared either by forcing buffer through petioles, stems, or roots or by chopping or grinding plant tissue in extraction buffer. The pH 7.0 sample buffer consists of disodium succinate, 1.0 gIliter; trisodium citrate, 1.0 glliter; K_2HP0_4, 1.5 gIliter; KH_2P0_4, 1.0 gIliter; 0.02 M sodium ascorbate, and 5% acidwashed insoluble polyvinylpyrrolidone (PVPP; Sigma Chemical Company, S1, Louis, MO). Amplification of DNA by PCR is inhibited in undiluted plant extracts unless the sodium ascorbate and acid-washed PVPP are added to the extraction buffer. DNA is extracted from aliquots of the plant extracts by the cetyltrimethylammoniumbromide (CTAB) procedure.

The following PCR primers amplify DNA of all *X fastidiosa* strains:

RST31 (5'-GCGTT AATTTTCGAAGTGATTCGATTGC-3 ')
RST33 (5'-CACCATTCGTATCCCGGTG-3').

The amplification product (733 bp) is observed by agarose gel electrophoresis and visualized with ethidium bromide. Restriction endonuclease digestion of the amplification product with *Rsal* are used to differentiate two pathotype groups of *X fastidiosa* strains, the Pierce's disease group and the group causing other diseases, usually called the phony peach group (Table 1).

Other PCR protocols are available for citrus variegated chlorosis (CYC) and coffee leaf scorch (CLS) strains (21) using the following two PCR primers:

With all *X fastidiosa* strains

272-1-int (5'-CTGCACTTACCCAATGCATCG-3')
272-2-int (5'-GCCGCTTCGGAGAGCATTCCT -3')

The primers yield a product of700 bp. It should be noted that they yield the same size product with *Xanthomonas campestris* and *Erwinia amylovora.*

CYC specific primers (21)
CYC-l (5'-AGATGAAAACAATCATGCAAA-3')
272-2-int (5'-GCCGCTTCGGAGAGCATTCCT -3')

These primers yield a product of 500 bp.

See Appendix A for details.

b. Serological techniques

1) Immunofluorescence can be used for rapid presumptive identification of strains of the phony peach group of *Xyella fastidiosa* (9). In a few cases, regular direct- or indirect-immunofluorescence can be used to detect *X fastidiosa* in plant extracts. However, the *X fastidiosa* concentration is usually too low and the plant debris background too high for accurate diagnosis.

2) Membrane entrapment immunofluorescence of plant extracts (6)

a) Plant extracts are prepared by forcing buffer through petioles or stems or by chopping or grinding plant tissue in extraction buffer.

b) The plant extract is centrifuged for 5 min at 1000 g to remove large debris.

c) The supernatant is transferred to a syringe and pushed through a multiple holder adapter that contains as. O-pm membrane for trapping cellular debris in the first holder and a 0.2-pm membrane for trapping bacteria in the second holder.

d) Direct or indirect antibody labeling can be used to detect *X fasttdiosa.* With the indirect method, the 0.2-pm membrane is removed from the second holder and incubated for 1 h in *X jastidiosa-specific* immunoglobulin in Tris-BSA-gelatin buffer (20 mM Tris, 0.9% NaCl, pH 8.2 containing 0.1 % bovine serum albumin and 1% gelatin).

e) After a buffer wash, the membrane is incubated in tetramethyl-rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG for 1 h.

f) After rinsing, the membrane is mounted and viewed with an epifluorescence microscope.

3) Dot immunobinding assay (DmA) (19)

a) Plant extracts are prepared by various techniques, as described for MEIF assay, in phosphate buffered saline (0.01 M $KP0_4$, 0.15 M NaCI, 0.02% sodium azide, pH 7.4) with 2 % polyvinyl pyrrolidone (PVP-40).

b) The plant extract is centrifuged for 5 min at 1000 g to remove large debris.

c) A 2 .ul aliquot is spotted on a nitrocellulose membrane, allowed to dry, and blocked for 1 h in buffer (10.0 mM Tris, 0.15 M NaCl, 0.1 % Tween 20, pH 8.0) containing 1.0 % bovine serum albumin and 2.0% Triton x-ioo.

d) After rinsing, the membrane is incubated overnight in IgG (1 ,ug/ml) specific for *X fastidiosa* in conjugate buffer (PBS plus 2 % PVP-40 and 0.5 % BSA).

e) After rinsing in PBS plus 0.1 % Tween 20, the membrane is incubated for 2-4 h in goat anti-rabbit IgG with alkaline phosphatase in conjugate buffer, as recommended by the supplier.

t) After rinsing, substrate is added as recommended by the supplier.

4) Enzyme-linked immunosorbent assay (ELISA)

ELISA is the most commonly-used diagnostic test for *X fastidiosa* in suspected hosts, especially in landscape trees with leaf scorch symptoms.

See Appendix B for details.

c. Commercial techniques

The isolation of *X fastidiosa* from trees is usually difficult and time-consuming. It is most convenient and reliable to use a commercially-available ELISA test kit, such as the "Pathoscreen-Xf" kit by Agdia, Inc (25). Generally, the assay can be performed according to the manufacturer's instructions, but, with some plants, the background absorbance readings can be high and make interpretation of the results very difficult. This can result in reduced sensitivity and false positive readings. Cross-absorption of antisera with extracts from plants free of *X fastidiosa* can reduce these false positive readings (13).

See Appendix C for details.

7. CULTURE PRESERVATION

Long-term culturing of *X fastidiosa* strains by serial transfer often results in a loss of virulence. Virulence of strains must be tested frequently. Virulent strains can be maintained through storage of cultures in glycerol at -70°C or lyophilized stock cultures.

8. LITERATURE CITED

1. Chang, C. 1. and 1. T . Walker. 1988. Bacterial leaf scorch of northern red oak: Isolation,

cultivation, and pathogenicity ofaxylem-limited bacterium. Plant Dis. 72:730-733.

2. Davis, M. 1., W. 1. French, and N. W. Schaad. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. CUff. Microbiol. 6:309-314.

3. Davis, M. 1., A. H. Purcell, and S. V. Thomson. 1978. Pierce's disease of grapevines: isolation of the causal bacterium. Science 199:75-77.

4. Davis, M. 1., A. H. Purcell, and S. V. Thomson. 1980. Isolation medium for Pierce's disease bacterium. Phytopathology 70:425-429.

5. Davis, M. 1., B. C. Raju, R. H. Brlansky, R. F. Lee, L. W. Timmer, R. C. Norris, and R. E. McCoy. 1983. Periwinkle wilt bacterium: Axenic culture, pathogenicity, and relationships to other gram-negative, xylem-inhabiting bacteria. Phytopathology 73: 15101515.

6. De Lima, 1. E. 0., V. S. Miranda, 1. S. Hartung, R. H. Brlansky, A. Couthino, S. R. Roberto, and E. F. Carlos. 1998. Coffee leaf scorch bacterium: Axenic culture, pathogenicity, and comparison *withXylellajastidiosa* of citrus. Plant Dis. 82:94-97.

7. Freitag, 1. H. 1951. Host range of Pierce's disease virus of grapes as determined by insect transmission. Phytopathology 41:920-934.

8. French, W. 1., R. G. Christie, and D. L. Stassi. 1977. Recovery of rickettsial ike bacteria by vacuum infiltration of peach tissues affected with phony disease. Phytopathology 67:945-948.

9. French, W. 1., D. L. Stassi, and N. W. Schaad. 1978. The use of immunofluorescence for the identification of phony peach bacterium. Phytopathology 68: 1106-1108.

10. Goheen, A. C., G. Nyland, and S. K. Lowe. 1973. Association ofa rickettsia-like organism with Pierce's disease of grapevines and alfalfa dwarf and heat therapy of the disease in grapevines. Phytopathology 63:341-345.

11. Hartung, 1. S., 1. Beretta, R. H. Brlansky, 1. Spisso, and R. F. Lee. 1994. Citrus variegated chlorosis bacterium: Axenic culture, pathogenicity, and serological relationships with other strains *ofXylellajastidiosa.* Phytopathology 84:591-597.

12. Hearon, S. S., 1. L. Sherald, and S. 1. Kostka. 1980. Association of xylem-limited bacteria with elm, sycamore, and oak leaf scorch. Can. 1. Bot. 58: 1986-1993.

13. Hill, B. L. and A. H. Purcell. 1995. Acquisition and retention of *Xylellajastidiosa* by an efficient vector, *Graphocephala atropunctata.* Phytopathology 85:209-212.

14. Hopkins, D. L. 1981. Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. Phytopathology 71:415-418.

15. Hopkins, D. L. 1989. *Xylella fastidiosa:* Xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.

16. Hopkins, D. L. and W. C. Adlerz. 1988. Natural hosts of *Xylella fastidiosa* in Florida. Plant Dis. 72:429-431.

17. Hopkins, D. L., F. W. Bistline, L. W. Russo, and C. M. Thompson. 1991. Seasonal fluctuation in the occurrence of *Xyle//a fastidiosa* in root and stem extracts from citrus with blight. Plant Dis. 75: 145-147.

18. Hopkins, D. L. and H. H. Mollenhauer. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. Science 179:298-300.

19. Lee, R. F., M. 1. G. Beretta, K. S. Derrick, and M. E. Hooker. 1992. Development of a serological assay for citrus variegated chlorosis - a new disease of citrus in Brazil. Proc. Fla. State Hortic. Soc. 105:32-34.

20.Minsavage, G. V., C. M. Thompson, D. L. Hopkins, R. M. V. B. C. Leite, and R. E. Stall. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84:456-461.

21. Pooler, M. R. and 1. S. Hartung. 1995. Specific PCR detection and identification of *Xylella fastidiosa* strains causing citrus variegated chlorosis. Curro Microbiol. 31:377-381.

22. Purcell, A. H. 1990. Homopteran transmission of xylem-inhabiting bacteria. In *Advances in Disease Vector Research,* ed. K. F. Harris, 5:243-266. New York: SpringerVerlag.

23. Purcell, H. A. and D. L. Hopkins. 1996. Fastidious xylem-limited bacterial plant pathogens. Annu. Rev. Phytopathol. 34: 131-151.

24. Sherald,1. L., S. S. Hearon, S. 1. Kostka, and D. L. Morgan. 1983. Sycamore leaf scorch: Culture and pathogenicity of fastidious xylem-limited bacteria from scorch-affected trees. Plant Dis. 67:849-852.

25. Sherald, J. L. and J. D. Lei. 1991. Evaluation ofa rapid ELISA test kit for detection of *Xylella fastidiosa* in landscape trees. Plant Dis. 75:200-203.

26. Wells, 1. M., B. C. Raju, H- Y. Hung, W. G. Weisburg, L. Mandelco-Paul, and D. 1.

Brenner. 1987. *Xylella fastidiosa* gen. Nov.: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int. 1. Syst. Bacteriol. 37: 136-143.

27. Wells, 1. M., B. C. Raju, G. Nyland, and S. K. Lowe. 1981. Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. Appl. Environ. Microbiol. 42:357-363.

5. CHEMICAL LIST

Chemical	Source
Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O.Box 14508, St. Louis, MO 63178.	
ACES buffer	
Activated Charcoal	Norit SG
Bovine Serum Albumin fraction V	
CTAB	
Gelatin	
Hemin Chloride Pathoscreen-Xf kit	
Polyvinylpyrrolidone (PVPP)	Agdia, Inc.
Sodium azide	
Soy Peptone	
Soytone	Scott Laboratories
Tryptone	Difco orBBL
	Difco orBBL

II. GRAM-NEGA TIVE BACTERIA

I. *Rhizomonas suberifaciens*

L. E. Datnoff and R. T. Nagata

1. INTRODUCTION

Rhizomonas suberifaciens and an unnamed *Rhizomonas* species cause corky root of lettuce (4, 7, 9). It has been reported from various lettuce production areas of North America (Canada, California, Florida, New York, and Wisconsin), Australia, New Zealand, and Western Europe (Italy, Netherlands). Yield losses from reduced head size have been reported between 30 to 70% from severely infested fields in Florida and California (1,2,3).

Symptoms of corky root initially appear as yellow lesions or bands on tap and lateral roots (2, 6, 9). As the disease progresses, the roots become dark greenish-brown, corked, and brittle (Plate 5, Fig. 1). The taproot can become shortened or decayed, and lateral roots are sparse or nonexistent. Internal yellowing or browning within the tap or lateral root also may occur at this time.

Rhizomonas suberifaciens is a Gram-negative bacterium that belongs to the rRNA superfamily IV (7, 8). It has small, motile rods with one lateral, subpolar, or polar flagellum. Most colonies are typically nonpigmented (creamy-white), umbonate, firm, and wrinkled (Plate 5, Fig. 2). The bacterium grows at temperatures between 20 to 33°C with optimal growth occurring at 29 to 33 °C (4), and will not grow at a pH of 5.1 or below. It is aerobic, metabolizes glucose oxidatively, oxidase positive, weakly catalase positive, reduces nitrate to nitrite and ammonia but not to nitrogen gas. The bacterium is nitrogenase and arginine dihydrolase negative, ethanol is not converted to acetic acid, does not fluoresce on KB and utilizes very few carbon sources. The whole-cell fatty acid composition is unique, especially the presence of relatively large amounts of2-tetradecanol (l4:0-20H). The guanine-plus-cytosine content of the DNA is 59 mol%.

2. ISOLA nON TECHNIQUE USING SEMISELECTIVE MEDIUM

a. Baiting technique for isolation.

Recovery of the bacterium from infected tissue is very difficult. Therefore isolations must be made from recently infected tissue. Isolations from soil are made using a 2- to 3-week-old susceptible lettuce cultivar such as Salinas or Shawnee. Soil suspensions are made by mixing 50 g of soil in 75 ml of distilled water plus 3 drops of Tween 20. The suspensions are stirred for 10 to 15 min and filtered through six layers of cheesecloth. Suspensions (5 ml) are dispensed at the stem base ofthe 2- to 3-week-old susceptible plant. After 3 to 4 weeks, plants are uprooted and isolations are made from yellow or slightly corked areas on the roots. Symptomatic roots are washed gently in

running distilled water to remove adhering soil particles and placed in 20 ml sterile distilled water in large test tubes, 2 x 20 cm. The roots are sonicated for 20 min in an ultrasonic cleaner (B-12, Branson Cleaning Equipment Co., Shelton, CT) and vortexed for 30 sec; placed back into the sonicator for 10 min and vortexed for 45 sec. Then 10 ml of extract from the root surface is filtered through a *0.65-,um* membrane filter (Micronsep, Honeoye Falls, NY) and 0.03 ml of filtrate and of diluted filtrate (a serial dilution consisting of 10^{-1} , 10^{-2} ,10^{-3}) is plated onto S-medium. The roots are then soaked in 0.5% sodium hypochlorite for 1 min, rinsed in 10 ml of sterile water and comminuted in a mortar. The root extract is filtered as previously described, and 30 ,ul of diluted filtrate (10^{-1}, 10^{-2},10^{-3}) are plated onto the S-medium and incubated at 29°C. After seven days of growth, colonies of *R suberifaciens* are only 1 mm in diameter. After 20 days the colonies will be 3 mm in diameter.

b. S- medium (see **h,** p. 5).

3. DIFFERENTIATION OF COMMONLY ISOLATED SPECIES.

R. suberifaciens and the other unnamed *Rhizomonas* species produce acid from glucose, maltose, mannose, and salicin, whereas the latter unnamed species also produce acid from myo-inositol and cellobiose.

4. DIAGNOSTIC MEDIA AND TESTS.

a. Flagella (see a, p. 10)

b. Colony morphology. Grow the bacterium on S-medium for three weeks at 29°C. Colonies are creamy in appearance and are wrinkled with undulated margins (Plate 5, Fig. 2).

c. Oxidase reaction (see 7, p. 10)

d. Nitrate reduction to ammonia (see b, p. 46)

e. Acid is produced from the following carbohydrates (see f, p. 47): cellobiose, glucose, maltose, myo-inositol, and salicin, depending upon the species.

5. PATHOGENICITY TESTS

Susceptible lettuce cultivar such as 'Salinas' or 'Shawnee' are grown for 2 to 3 weeks in a standard potting mix and then inoculated by pouring a 5 ml bacterial suspension of *R. suberifaciens* (1×10^7 CFU/ml) around the stem base of the lettuce seedling (see 5, p. 226). It is important to keep control plants at least 50 to 100 em away from the inoculated plants since gnats or aerosols may transfer inoculum. The suspension of *R. suberifaciens* is prepared by culturing

the bacterium in S-broth (S-medium without the agar) for 4 days. Continuous culturing can lead to a loss in pathogenicity. After, centrifuging the bacterial suspension at 7500 rpm for 20 min, the pellet is resuspended, centrifuged, washed, centrifuged, and resuspended in sterile distilled water. Bacterial concentrations can be determined using a standard counting chamber or plating onto Smedium. Approximately three to four weeks after inoculation at 20 ° C, lettuce plants can be uprooted and visually rated for corky root development (5,9). The ratings include a symptom intensity from 1 to 9, where 1 = no discoloration of the taproot and 9 = dark brown discoloration and/or partial disintegration of the taproot, longitudinal cracks penetrating well into the cortex, and a percentage root infection from 0 to 11, where 0 = no disease and 11 == 90 to 100% of the root surface infected.

6. CULTURE PRESERVATION

Cultures are stored at -80°C (see 7, p. 30).

7. LITERATURE CITED

1. Datnoff, L. E. and R. T. Nagata. 1992. Relationship between corky root disease and yield of crisphead lettuce. J. Am. Soc. Hort. Soc. 117: 54-58.

2. Datno:ff, L. E. and R. T. Nagata. 1990. Isolation *ofRhizomonas suberifaciens* in Florida. Plant Dis. 74:394.

3. 0' Brien, R. D. and van A. H. C. Bruggen. 1992. Yield losses to iceberg lettuce due to corky root caused by *Rhizomonas suberifaciens*. Phytopathology 82: 154-159.

4. O'Brien, R. D. and A. H. C. van Bruggen. 1993. Effect of temperature on corky root disease oflettuce and growth of the pathogen *Rhizomonas suberifaciens*. Can. J. Plant Path. 15:85-90.

5. Nagata, R. T. and L. E. Datnoff 1991. Cultural control oflettuce corky root by the use of transplants. Proc. Fla. State Hort. Soc. 104:265-266.

6. van Bruggen, A. H. c., P. R. Brown, and K. N. Jochimsen. 1989. Corky root oflettuce caused by strains of a gram-negative bacterium from muck soils of Florida, New York, and Wisconsin. Appl. and Environ. Microbiol. 55:2635-2640.

7. van Bruggen, A. H. c. P. R. Brown, and K. N. Jochimsen. 1990. *Rhizomonas suberifaciens* gen.nov., sp. nov., the causal agent of corky root oflettuce. Int. J. Syst. Bacteriol. 40:175-188.

8. van Bruggen, A. H. c., K. N. Jochimsen, E. M. Steinberger, P. Segers, and M. Gillis. 1993. Classification of *Rhizomonas suberifaciens,* an unnamed *Rhizomonas* species, and

Sphingomonasspp. inrRNAsuperfamilyIV. Int. 1. Syst. Bacteriol. 43:1-7.

9. van Bruggen, A. H. C., R. G. Grogan, C. P. Bogdanoff, and C. M. Waters. 1988. Corky root oflettuce in California caused by a Gram-negative bacterium. Phytopathology 78: 1139-1145.

8. CHEMICAL LIST

Chemical

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.

Agar Noble Casein
hydrolysate
Cellobiose
Glucose
K2HP043H20
KN03
Maltose
Mannose Myo-
inositol $Mg^{S0}4$
?H20 Ca(N03h
4H20 Salicin
Streptomycin sulfate
Tween 20

ill. Gram-Positive Bacteria

A. Coryneform Plant Pathogens

M. J. Davis and A. K. Vidaver

1. INTRODUCTION

The term coryneform (28,30) refers to a diverse group of plelomorphic bacteria from various habitats that include some plant pathogens. In complex media, these bacteria usually occur as irregular shaped rods that vary considerably in size and shape, and include straight, bent, curved, wedge-shaped and club-shaped forms. Angular cell arrangements, frequently Vformations, are usually present. Mycelia are usually not formed but rudimentary branching may occur. In stationary phase cultures, cells are often less irregular in shape and shorter, and coccoid cells may occur in various proportions. The cells may be motile or non-motile. Endospores are not formed. These bacteria are Gram-positive, but may decolorize easily and exhibit uneven staining. They are not acid-fast. The phytopathogenic coryneform bacteria generally fit this description and in addition, but not exclusively, are all strict aerobes, catalase-positive, oxidasenegative, and often contain metachromatic granules.

Until recently, the phytopathogenic coryneform bacteria were all classified in the genus *Corynebacterium,* primarily based on their morphological and staining characteristics. Classification ofthese bacteria in the genus *Corynebacterium* is now deemed generally inappropriate on the basis of physiological, numerical taxonomic and chemotaxonomic studies (7). Furthermore, insufficient differences were noted between some of these pathogens to warrant ranking them as separate species (2, 20). Proposals to reclassify the phytopathogenic coryneform bacteria in other described genera and two new genera have been published (3, 4, 11, 24, 40, 51). We have chosen to use the nomenclatural combinations in Table 1. Prior combinations with *Corynebacterium* as the designated genus are also valid for the pathogens presently classified in the genera *Arthrobacter, Clavibacter, and Rhodococcus* (2, 43). The genus *Rathayibacter* was proposed for some species in the genus *Clavibacter* (40,51), but we have chosen not to use these new combinations in part, because a clear concept of the separation between species and genera has not yet been reached. A proposal has been published that, in effect, would reclassify all the subspecies of *Corynebacterium flaccumfaciens* in the genus *Curto bacterium* as pathovars of *Curtobacteriumflaccumfaciens* (3). Although we generally agree with the placement of these pathogens in the genus *Curtobacterium,* we would prefer that their subspecies rank be retained, and therefore, we have chosen not to use this proposed classification. We will refer to coryneform species as listed in Table 1.

The plant pathogenic coryneform bacteria cause a variety of diseases with symptoms that include galls, gurnmosis, and wilts (8, 47, 48). In nature, all of these pathogens, except for *Rhodococcus jascians,* and *Clavibacter michiganensis* subsp. *michiganensis,* have been found associated with only a single genus of host plant. *Clavibacter michiganensis* subsp. *michiganensis* also is limited in host-specificity in that it only infects solanaceous hosts, principally

tomato and pepper. The phytopathogenic coryneform bacteria are relatively easy to identify when isolated from young lesions of a known host with characteristic symptoms because of their cell morphology and Gram-stain characteristic. *Clavibacter michiganensis* subsp. *sepedonicus* is often difficult to isolate from potato tubers because of the presence of other microorganisms. However, a semiselective medium (NCP-88, see 5), p. 222) has been used with relative success (17). Secondary identifying traits are colony morphology, pigmentation on complex media, and the presence or absence of motility. Colony formation requires 3-21 days, depending on the pathogen. The fastest growing pathogens are C. *flaccumfaciens;* the slowest are the C. *xyli* subspecies.

Table 1. Species and diseases caused by coryneform plant pathogens.

Species	Disease	Reference
Arthrobacter ilicis	Blight of American holly	
Clavibacter iranicum	Inflorescence gummosis of grains	
Clavibacter michiganensis		
subsp. *insidiosus*	Alfalfa wilt	
subsp. *michaganensis*	Tomato canker	
subsp. *nebraskensis*	Blight and wilt of com (Goss's wilt)	
subsp. *sepedonicus*	Wilt and tuber rot of potato (ring rot) Mosaic-	
subsp. *tessellarius*	like syndrome on wheat Inflorescence	
Clavibacter rathayi	gummosis of grains Inflorescence gummosis of	
Clavibacter tritici	grains (wheat) Inflorescence gummosis of	
Clavibacter toxicus	grains (rye grass)	
Clavibacter xyli		
subsp. *cynodontis* subsp.	Bermudagrass stunting	
xyli Curtobacterium	Ratoon stunting disease of Sugarcane	
flaccumfaciens		
pv. *betae*		
pv. *flaccumfaciens*	Vascular wilt and leaf spot of red beet Vascular wilt of beans *(Phaseolus* species and *Glycine max)*	
pv.oortii	Vascular disease and leaf and bulb spot of tulips	
pv. *poinsettiae*	Leaf spot and stem canker of poinsettia	
Rhodococcus fascians	Leafy gall and fasciation of numerous plant species	

Gram stains (Plate 1, Fig. 1) should be performed on young cultures using both a positive and a negative control (see b, p. 7). Some species stain unevenly, depending on culture age, growth medium, and strain. To determine cell morphology, young cultures (24 - 72 hrs old) should be observed. Colony morphology ranges from small (approx. 1-3 mm, except for those of C. *xyli* which are 0.1-1 mm), circular and convex to large (approx. 5-8 mm), irregular, and fluidal. Pigmentation is usually in shades of yellow to orange on nutrient rich media, with non-pigmented species in the minority. Intracellular granular pigmentation by indigoidine is usually produced by

C *michiganensis* subsp. *insidiosus,* and extracellular purple pigment is produced by some strains *ofCj1accumjaciens subsp.j1accumjaciens.* Motility is predominant among strains of
Curto bacterium and *Arthrobacter* species. Young cultures should be observed; a droplet put on a clean slide, with coverslip, is generally adequate for observation with phase-contrast microscopy. F or most accurate results, the preparation should be examined immediately. Alternatively, hanging drop preparations may be examined if there is doubt about motility. In this procedure, a drop of culture is put on a coverslip; a depression slide is gently put over the drop, and the slide and coverslip are quickly turned over together so as to retain the droplet in place. Further details on motility procedures and precautions can be obtained in reference 21.

It is strongly recommended that known coryneform bacteria be used for comparative studies with unknowns, preferably using type cultures.

A nutrient-broth yeast extract medium (NBY) is recommended as a medium of choice for growth, pigmentation, and colony differentiation (Plate 5, Fig. 3), except for C *xyli,* which will grow only on special media (SC medium, see b, p. 225). Other satisfactory general media can be used (37).

2. ISOLATION TECHNIQUES USING SEMISELECTIVE MEDIA

a. Plant Material

Material should be washed with tap water and gently blotted dry with paper toweling. Surface sterilization is usually not necessary and may be detrimental in isolating *R. jascians.* Tissues from young lesions, freshly infected material, or the margins of decayed areas should be sought. Direct puncture from fresh tissue by use of a sharp, sterilized dissecting needle onto agar should be successful, as should be streaking or plating of comminuted tissues after serial dilution. It may be necessary to inoculate peas or other hosts with crude material to enrich for *R. jascians,* with subsequent isolation from the newly infected host. A selective medium has not been developed for C *xyli* subsp. *xyli;* therefore, surface sterilization of plant material and precautions to avoid other microorganisms are necessary when isolating this pathogen on SC medium (10).

b. Soil

Aqueous suspensions (0.1 % w/v) of soil samples should be diluted in 10-fold series to 10^{-5} and 0.1 ml from the last three dilutions spread over the surface of solid medium with a sterile L-shaped glass rod. Selective media may be useful or even necessary. Medium D2 (29) can be used for isolation of some coryneform bacteria. Medium CNS (25) allows better selection and faster growth of some coryneform bacteria. Furthermore, the color of the colonies on CNS (Plate 5, Fig. 4) is similar to the color of the colonies on NBY agar (Plate 5, Fig. 3). Medium SCMS can be used to isolate C *xyli* subsp. *cynodontis* (9) and SCM agar is very helpful for isolating C *michiganensis* subsp.

michiganensis (21) (Plate 5, Fig. 5). A modified SCM is best for tomato seeds (50) (Plate 5, Fig. 5).

c. Recipes for selective media.

1) Medium eNS (25) (Plate 5, Fig. 4)

	g/500ml4
Nutrient broth, Bacto	.0 g
Yeast extract, Bacto	1.0 g
K_2HP0_4	1.0 g
KH_2P0_4	0.25 g
LiCI	5.0 g3
Agar	7.5 g

Autoclave together (after autoclaving the pH is 6.9), cool to about 50°C, then add the following ingredients:

Cycloheximide	2.0 ml * 1.25
Nalidixic acid	ml ** 1.6 ml"
Polymyxin p sulfate" (8000 USP units/mg)	***
Daconi12787-F (530 mg chlorothaloni1/ml)	30.0 ml **** 2.5
Glucose	g
Magnesium sulfate, anhydrous	62.0 mg

Swirl flask to mix well and pour.

"The LiCI is transiently toxic to freshly isolated C. *michiganensis* subsp. *nebraskensis*, hence it can either be omitted from the medium or plating of plant specimens (in buffer containing sodium) can be delayed for 1-2 hours (44).

bPolymyxin p sulfate preparations can vary, hence the medium should be pretested to determine recovery effectiveness.

* 1 g/lOO ml deionized distilled (dd) water. Store at 4 DC. 0.1 g,

** in 7 ml dd water pJus 3 ml 1 N NaOH. Store at 4 D C. 1 gt

*** 100-ml dd water. 'Store at 4 DC.

**** 1.2 ml/58.8 ml or 0.2 ml/9.8 ml dd water. Store at room temperature. (Active ingredient is tetrachloroisophthalonitrile.)

2) D2 Medium (29)

	~h
Glucose	10.0 g
Casein hydrolysate	4.0 g
Yeast extract ~Cl	2.0 g
$MgS0_4'7H_ZO$	1.0 g
LiCl	O.Jg
Tris [tris (hydroxymethyl) amino methane]	5.0 g
Agar	1.2 g
	15.0 g

Adjust to 7.8 with HCl and autoclave. The medium is cooled to about 50^0 C and polymyxin sulfate and sodium azide are added.

Polymyxin B sulfate"	0.4 mg
Sodium azide	2.0mg

'Add a total of300 USP units.
This medium should be freshly prepared since azide and polymyxin break down with time.

3) SCMS Medium (9)

Add the following to 1 liter of SC agar (see b, p. 225):

	~h
Cycloheximide	50.0 mg
Hymexazol	35.0 mg
Colistan methane sulfonate (1190 units/mg)	10.0 mg
Polymyxin B sulfate (7400 USP units/mg)	50.0 mg

All ingredients, except SC agar, are dissolved in 10 ml of water, filter sterilized using a 0.22 ,um-pore-size membrane filter, and added to autoclaved SC agar cooled to about 50° C.

4) eMS (21) (Plate 5, Fig. 5)

	Q§:h
Sucrose Yeast	10.0 g
extract	0.1 g
K_ZHP0_4	2.0 g
KH_ZP0_4	0.5 g
$MgS0_4 \cdot 7H_Z0$	0.25 g
Boric acid	1.5 g

After autoclaving add:

100 mg nicotinic acid (dissolved in 20 ml sterile distilled water) 30 mg nalidixic acid (sodium salt, dissolved in 1 ml 0.1 m NaOH)
10 mg potassium tellurite (1 ml of 1% chapman tellurite solution from Difco) 200 mg cycloheximide (dissolved in 1 ml absolute methanol)

Useful for isolation of C *michiganensis* subsp. *michiganensis* from soil and tomato seeds.

5) m SCM (50) (Plate 5, Fig. 5).

Use mannose in place of sucrose and eliminate potassium tellurite.

Some strains of C *michiganensis* subsp. *michiganensis* grow poorly in presence of potassium tellurite. Best for isolating from tomato seeds.

6) NCP-88 (17)

	per L
Nutrient agar	23.0 g
Yeast extract	2.0 g
K_2HP0_4	2.0 g
KH_2^P04	0.5 g
$MgS0_4 \cdot 7H_20$	5.0 g
D-Mannitol	5.0 g

After autoclaving, add:
300 Ill of polymyxin B sulfate (7,900 units/mg, 10 mg/ml stock) 800 Ill of nalidixic acid, Na-salt (10 mg/ml in 10 mM NaOH)
2 ml of cycloheximide (100 mg/ml in 75% ethanol)

Useful for isolation of C *michiganensis* subsp. *sepedonicus* from potato tubers.

3. DIFFERENTIATION OF COMMONLY ISOLA TED SPECIES AND SUB-SPECIES

Phytopathogenic coryneform bacteria can be differentiated on the basis of the characteristics given in Table 2. With experience, most species and subspecies can be differentiated by colony characteristics on NBY or SC agar alone. Colonies are yellow or yellow-orange, except those ofC *michiganensis* subsp. *sepedonicus* and C. *xyli* subsp. *xyli,* which are white. Definitive identification may need to be confirmed by pathogenicity and/or serological tests.

Table 2. Differential characteristics of phytopathogenic coryneform bacteria.

Pathogen	Pigment[d]	Growth		Acid Production[e]		Utilization[b]		Hydrolysis[c]
			TTC		Inulin	Acetate	Formate	
Arthrobacter ilicis	Y		+		-	+	+	
Clavibacter iranicum	Y		ND		+			
Clavibacter michiganensis								
subsp. *insidiosus*	Y/B		+					+ + + +
subsp. *michiganensis*	Y/V_1		+			+		
subsp. *nebraskensis*	O/V_2							
subsp. *sepedonicus*	W	-						
subsp. *tessellarius*	O	+						+ + + +
Clavibacter rathayi	Y	V	ND			+		
Clavibacter tritici	Y	+	ND					
Clavibacter toxicus	Y	ND	ND					
Clavibacter xyli								
subsp. *cynodontis*								
subsp. *xyli*								
Curtobacterium flaccumfaciens								
pv. *betae*	Y			+		+ +		0+ + +
pv. *flaccumfaciens*	Y/O/P			+				
pv. *oortii*	Y			+		+ +		
pv. *poinsettiae*	O			+		+ +	+	
Rhodococcus fascians	O							-

Abbreviations: +, 80% or greater positive; -, 80% or greater negative; V, depending on the strain; $+^D$, delayed positive; ND, not determined.

[a]RSD broth (see d, p. 225) with yeast extract reduced to 0.1 g/L, and the test compound (0.5% w/v) replacing glucose.

[b]RSD broth as above, except the test compound at 0.1% w/v and bovine serum albumin omitted from the medium when testing *Arthrobacter* and *Corynebacterium* species.

[c]SC medium (see b, p. 225) supplemented with 1% (w/v) casein or 0.1% (w/v) esculin and 0.05% (w/v) ferric citrate. Clearing of the medium with casein or the development of a brown color in the medium with esculin indicates hydrolysis.

[d]NBY agar (see c, p. 4) used for all pathogens, except SC agar used for *C. xyli* subspecies. Y, yellow; B, blue; O, orange; W, white or colorless; P, purple; V_1, various pigments (occasional variants are pink, red, orange, and white or colorless); V_2, occasional variants are yellow; ND, not done. Colors refer to those of non-diffusable pigments, except as noted. The blue pigment is intracellular indigoidine granules sometimes produced in addition to yellow pigment. The purple is extracellular and occasionally found.

4. DIAGNOSTIC MEDIA AND TESTS

a. NBY medium (see c, p. 4)

b. SC medium (10)

	~~
Phytone peptone or soytone	8.0 g
Bovine hemin chloride	15.0 ml *
$KH_zP0_4 \cdot 3H_zO$	1.5g
K_zHP0_4	0.5 g
$MgS0_4 \cdot 7H_zO$	0.2
Com meal agar	g
Glucose	17.0 g
L-cysteine (free base)	1.0 ml **
Bovine serum albumin fraction V	10.0 ml ***
	10.0 ml ****

Ingredients are added and dissolved in water in the order given. The medium components, except for glucose, cysteine, and bovine serum albumin, are autoclaved. Glucose, cysteine, and bovine serum albumin stock solutions are filter sterilized and added to the rest of the medium cooled to about 50° C. The pH should be 6.6-6.7 without adjustment.

*	0.1% w/v in 0.5 NNaOH
**	50% w/v aqueous solution.
***	10% aqueous solution. 20%
****	aqueous solution.

c. TTC medium (31)

	per~
Glucose	5.0 g
Peptone	10.0
Casamino acids (casein hydrolysate)	g 1.0
Agar	g
	17.0 g

Autoclave in 200-ml portions and add 1.0 ml ofa 1% aqueous solution of2,3,5 triphenyl tetrazolium chloride (autoc1aved separately) to each portion.

d. RSD broth medium (8)

	per~
Yeast extract	1.0
$(NH4)zHP0_4$	g
Bovine hemin chloride	1.0 g 15.0
L-cysteine (free base)	ml * 1.0 g

Bromthymol blue	1.0 ml **
$MgS0_4$"$7H_20$ KCI	0.2 g
Glucose	0.2 g
Bovine serum albumin fraction V	2.0 g
	10.0 ml ***

Ingredients are added and dissolved in water in the order given. All ingredients, except for bovine serum albumin, are mixed and the pH adjusted to 7.2 with 1.0 N NaOH or HCI before autoclaving. The bovine serum albumin stock solution is filter sterilized and added to the rest of the medium at room temperature. The final pH should be 6.6-6.7.

* 0.1% w/vin 0.5 NNaOH 0.8%
** w/v aqueous solution. 20%
*** aqueous solution.

Other media have been evaluated for specificity of isolation, rapidity of growth, colony morphology and pigmentation. These media are not included here because of one or more deficiencies such as excess foaming, variation due to differences in fresh potatoes, decreased pigmentation, or limited use to date.

e. Gram reaction. (see 1, p. 7)

f. Motility. (see d, p. 46) (21)

5. PATHOGENICITY TESTS

The following procedures can be used for most plant pathogenic bacteria. Variations in temperature, liquid media, diluents, and agar media can be used equally well for certain species. Using a sterile loop, remove a mass of cells of the bacterium from a fresh NBY agar slant culture. Transfer the cell mass to a 125 or 250 ml Erlenmeyer flask containing 50 ml ofliquid NBY (see c, p. 4), medium 523 (see 1), p. 27), or nutrient broth (see a, p. 3), and shake on a rotary shaker at 24-28° C. After 15 hours (overnight), remove 1.0 m1 of the suspension and add it to a fresh flask of broth medium as before and place on a rotary shaker. Incubate for 4-6 hours until the culture reaches a turbidity reading ofO.080.1 optical units at 640 nm with a spectrophotometer or 50 Klett units with a KlettSummerson photoelectric colorimeter (green filter). If the culture grows beyond the desired cell density, adjust the concentration by dilution with the appropriate medium. Determine culture purity and the actual number of colony forming units (CFU) by pipetting aliquots of ten-fold dilutions in buffer, such as phosphate buffered (0.01 M, pH 7.1) saline (0.85% NaCl), onto the surface of duplicate plates of 523 or NBY agar. A dilution should be chosen which gives 50-200 CFU/plate. The inoculum should be spread evenly of an Lshaped glass rod (a turntable may be helpful), and the plates incubated 3-5 days at 24-28°C prior to counting. It is very important to treat each inoculum preparation the same way,

because the viable cell concentration of cultures with similar absorbencies are not necessarily the same. Qualitative tests may be performed by using 4-6 day old agar cultures suspended in buffer as above.

Inoculum containing either of the C. *xyli* subspecies can be prepared by growing heavily inoculated cultures on SC medium at *28°* C for 2-3 weeks, removing the growth with a spatula, and suspending it in phosphate buffer (0.01 M, pH 6.9). The concentration of cells in the inoculum should be at least 10^8 cells/ml (A_{56o} = 0.1). The CFU should be determined by plating serial dilutions of the suspension on SC medium.

a. Inoculation

For most coryneform plant pathogens, dilute the freshly prepared broth culture with water, buffer, or saline to 10^5 to 10^6 CFU/ml based on spectrophotometer or colorimeter reading. If many inoculations are to be made, it is good practice to keep the culture on ice until ready to use. Such cultures can be used up to 4-6 hours. The inoculum is then introduced into the plant using toothpicks, scalpels, needles, pressure injection devices, or knives. Control plants should be treated similarly but with diluent only. One inoculation method that works well with vascular pathogens is to wound an axillary bud by inserting a dissecting needle approximately 4 mm deep, and then place 5-10,u1 of bacterial suspension on the wound. The suspension is usually taken into the transpiration stream in a few minutes. It should also be noted that eggplant is a preferred host for testing C. *michiganensis* subsp. *sepedonicus* and nematodes are necessary vectors for normal transmission of and infection by C. *trifid* and C. *rathayi*, as well as production of "cauliflower" syndrome of strawberry by *R. fascians.*

Sugarcane and Bermudagrass can be inoculated as single-node cuttings with either of the C. *xyli* subspecies by immersing cut surfaces of freshly prepared cuttings into inoculum before planting.

6. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

a. Molecular techniques

For rapid identification of the individual pathogens, a number of polymerase chain reaction (PCR) assays have been developed including a fluorogenic 5' nuclease PCR assay using a real-time TaqMan detection system (perkin Elmer) (41) (Table 3).

See Appendix A for details.

Table 3. peR primers for the detection and identification of coryneform phytobacteria

Specificity	Primer Designation	Sequence	Size (bp)	Reference
Species	CMR16Fl	(S'GTGATGTCAGAGCITGCTCTGGCGGAT3')	1,500·	(32)
michiganensis	CMR16R1	(S'GTACGGCTACCTTGITACGACITAGT3')		
	CMRI6F2	(S'CCCCGACTCTGGGATAACTGCTAJ')	1,300·	
	CMR16R2	(5'CGGTTAGGCCACTGGCTTCGGGTGTTACCGA3')		
subsp.	CMM5	(S'GCGAATAAGCCCATATCAAJ')	614[11]	(19)
michiginensis	CMM6	(5'CGTCAGGAGGTCGCTAATA3')		
sepedonicus	Splf	(S'CCTTGTGGGGTGGGAAAAJ')	215	(33)
	Sp5r	(S'TGTGATCCACCGGGTAAAJ')		
sepedonicus	CMS6	(S'CGCTCTCCCTCACCAGACTC3')	258	(42)
	CMS7	(S'TCCCGTGCTTGCCTGCGTTG3')		
sepedonicus	CmsSOF	(S'GAGCGCGATAGAAGAGGAACTC3')	224	(36)
	CmsSOR	(5'CCTGAGCAACGACAAGAAAAATAG3')		
	Cms72F	(S' AGTTCGAGTTGATAGCAATCCGC3')	247	
	Cms72R	(5'GTGTCTCGGAITCACGATCACC3')		
	Cms85F	(S' AAGATCAGAAGCGACCCGCC3')	232	
	Cms85R	(5'TCGCACAGCCAAA TCCAGC3')		
sepedonicus	A47A	(S'CACCCCTCGACTGCGAGAAACG3')	670	(23)
	A47B	(5'TCCTCCGAGACTTTCGGGACGC3')		
sepedonicus	CSRSC	(S'GGCCATGACGTTGGTGACAC3')	1054<	(44)
sepedonicus	CmsSO-2F	(5'GGCAGAGCATCGCTYCAGTACC3')	NA	(41)
	Cms133-R	(S'CGGAGCGCGATAGAAGAGGAJ')		
	TaqMan Probe	(S'-FAM-AAGGAAGTCGTCGGATGAAGATGGG-TAMRA-3')		
subsp. *xyli*	CxxITSf#5	(5'TCAACGCAGAGATTGTCCAJ')	305	(22)
	CxxITSr#5	(5'GTACGGGCGGTACCTTTTC3')		
cynodontislxyli	CxFOR	(5' AAGGAGCATCTGGCACCCT3')	446	
	CxxREV	(S' AGGATTCGGTTCTCATCTCA3')		
	CxFOR	(S' AAGGAGCATCTGGCACCCT3')	278	
	CxcREV	(S'GAA TCGATCGGCGTCTCCTC3')		
subsp, *xyli*	Cn!	(S'CCGAAGTGAGCAGATTGACC3')	438	(39)
	Crn	(5' ACCCTGTGTTGTTTTCAACG3')		
xyli	RSD33	(5'CTGGCACCCTGTGITGTTITC3')	265	(12)
	RSD292	(5'TTCGGTTCTCATCTCAGCGT3')		
	RST60	(S'TCAACGCAGAGAITGTCCAG3')	229	(12)
	RSTS9	(S'CGTCTTGAAGACACAGCGATGAG3')		
Ri fasciens	JRERIGHT	(S'CGGGATCCATATCGAACCGCCCTC3')	225	(47)
	JRELEFf	(5'GGGAAITCCGACCGTATCCAGTGT3')		

• Estimated value.

[b] Avirulent strains are negative.

[c] Gives same 1054 bp product with C. *michiganensis* subsp. *insidious.*

NA, not applicable

b. Serological techniques

Immunodiagnosis of coryneform plant pathogens has been found to be especially useful for those pathogens that are difficult to isolate in culture or cause latent infections, especially when spread of the pathogen by vegetative propagation is a major concern. In this respect, serological procedures are commonly used for detection ofC *xyli* subsp. *xyli* in sugarcane stalks (6, 26) and C *michiganensis* subsp. *sepedonicus* in potato tubers (16, 15,33). Immunodiagnosis is also useful to detect coryneform plant pathogens in true seed, such as C *michiganensis* subsp. *insidiosus* in alfalfa seed (38) and *Ci flaccumfaciens pv.f1accumfaciens* in mungbean seeds (18). Various serological methods have been used including agglutination, latex agglutination, ELISA fluorescent antibody staining, dot-blot, and tissue-blot. Immunofluorescence assays have frequently been found to be the most reliable, in part due to the combining of visual and immunochemical recognition factors. An evaporative-binding ELISA (5) or modified stalk imprint immunoassay (tissue-blot assay) are used most often for detection ofC *xyli* subsp. *xyli* in multiple sugarcane stalk samples (13).

See Appendix B for details.

b. Commercial Techniques

1) Fatty acid methyl ester analysis (FAME)

Commercial analysis of cellular fatty acids can provide a rapid presumptive identification of plant-pathogenic coryneform bacteria (27).

2) Diolog

Commercially available kits such as the API ZYM test strips provide standardized and rapid performance of many physiological tests, however, they have not been found generally useful for differentiating coryneform phytobacteria (14).

See Appendix C for details.

7. CULTURE PRESERVATION

Young cultures streaked on slants ofNB Y agar or SC agar can be stored at 4°C for several weeks. Strains can be stored at -80^{D}C in broth medium with 10% (v/v) glycerol. For long term storage, freeze drying of cells from young cultures that have been suspended in sterile, 10% skim milk is recommended.

1. LITERATURE CITED

1. Baer, D. and N. C. Gudmestad. 1993. Serological detection ofnonmucoid strains of *Clavibacter michiganensis* subsp. *sepedonicus* in potato. Phytopathology 83: 157-163.

2. Carlson, R. R. and A. K. Vidaver. 1982. Taxonomy of *Corynebacterium* plant pathogens, including a new pathogen of wheat, based on polyacrylamide gel electrophoresis of cellular proteins. Int. J. Syst. Bacteriol. 32:315-26.

3. Collins, M. D. and D. Jones. 1983. Reclassification *ofCorynebacteriumjlaccumjaciens, Corynebacterium betae, Corynebacterium oortii* and *Corynebacterium poinsettiae* in the genus *Curtobacterium,* as *Curtobacterium flaccumfaciens* comb. nov. J. Gen. Microbiol. 129:3545-348.

4. Collins, M. D., D. Jones, and R. M. Kroppenstedt. 1981. Reclassification of *Corynebacterium ilicis* (Mandel, Guba and Litsky) in the genus *Arthrobacter,* as *Arthrobacter ilicis* comb. nov. Zentralbl. Bakteriol. Parasienkd, Infektionskr. Hyg. Abt. 1 Orig. Reihe C. 2:318-323.

5. Croft, B. J., A. D. Greet, T. M. Leaman, and D. S. Teakle. 1994. RSD diagnosis and varietal resistance screening in sugarcane using the EB-EIA technique. Proc. 1994 Conf Australian Soc. Sugar Cane Technol. 14:143-151.

6. Davis, M. J. 1985. Direct-count techniques for enumerating *Clavibacter xyli* subsp. *xyli* which causes ratoon stunting disease of sugarcane. Phytopathology 75: 1226-1231.

7. Davis, M. J. 1986. Taxonomy of plant pathogenic coryneform bacteria. Ann. Rev. Phytopathol. 24: 115-140.

8. Davis, M. J. 1991. Fastidious bacteria of plant vascular tissue and their invertebrate vectors. *In:* The Prokaryotes, A Handbook on the Biology of Bacteria, Ecophysiology, Isolation, Identification, Applications, Vol. 4 (A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer, eds.). pp.4030-4049. Springer-Verlag, New York, NY.

9. Davis, M. J. and B. J. Augustin. 1984. Occurrence of the bacterium that causes Bermudagrass stunting disease. Plant Dis. 68: 1095-1097.

10. Davis, M. J., A. G. Gillaspie, Jr., R. W. Harris, and R. H. Lawson. 1980. Ratoon stunting disease of sugarcane: Isolation of the causal bacterium. Science 210: 1365-67.

11. Davis, M. J., A. G. Gillaspie, Jr., A. K. Vidaver, and R. W. Harris. 1984. Clavibacter: A new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp.nov., subsp. nov. *and Clavibacter xyli* subsp. *cynodontis* subsp. nov.,

pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. Int. 1. Syst. Bacteriol. 34:107-17.

12. Davis, M. 1., P. Rott, and G. Astua-Monge. 1998. Nested, multiplex PCR for detection of both *Clavibacter xyli* subsp. *xyli* and *Xanthomonas albilineans* in sugarcane. Offered Papers, vol 3. International Congress of Plant Pathology, August 9-16, 1998. Edinburgh, Scotland. Paper 3.3.4.

13. Davis, M. 1.,1. L. Dean, 1. D. Miller, and 1. M. Shine Jr. 1994. A method to screen for resistance to ratoon stunting disease of sugarcane. Sugar Cane (6):9-16.

14. De Bruyne, E., 1. Swings, and K. Kersters. 1992. Enzymatic relatedness amongst phytopathogenic coryneform bacteria and its potential use for their identification. System. Appl. Microbiol. 15:393-401.

15. De Boer, S. H., T. L. Haan, and 1. Mawhinney. 1989. Predictive value of post harvest serological tests for bacterial ring rot of potato. Can. 1. Plant Pathology 11:317-321.

16. De Boer, S. H., D. E. Stead, A. S. Alivizatos, 1. D. Janse. J, Van Vaerenbergh, T. L. De Haan, and 1. Mawhinney. 1994. Evaluation of serological tests for detection of *Clavibacter michiganensis* subsp. *sepedonicus* in composite potato stem and tuber samples. Plant Disease 78:725-729.

17. de la Cruz, A. R., M. V. Wiese, and N. W. Schaad. 1992. A semiselective agar medium for isolation of *Clavibacter michiganensis* subsp. *sepedonicus* from potato tissues. Plant Dis. 76:830-834.

18. Diatloff, A., W. C. Wong, and B. A. Wood. 1993. Non-destructive methods of detecting *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in mungbean seeds. Let. Appl. Microbiol. 16:269-273.

19. Dreier, 1., A. Bermpohl, and R. Eichenlaub. 1995. Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis.* Phytopathology 85: 464-68.

20. Dye, D. W. and W. 1. Kemp. 1977. A taxonomic study of plant pathogenic *Corynebacterium* species. N. Z. 1. Agric. Res. 20:563-82.

21. Fatmi, M. and N. W. Schaad. 1988. Semiselective agar media for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology. 78: 121-126.

22. Fegan, M., B. 1. Croft, D. S. Teakle, A. C. Hayward, and G. R. Smith. 1998. Sensitive

and specific detection of *Clavibacter xyli* subsp. *xyli,* causal agent of ratoon stunting disease of sugarcane, with a polymerasechain reaction-based assay. Plant PathoL 4:495504.

23. Firrao, G. and R. Locci. 1994. Identification of *Clavibacter michiganensis* subsp. *sepedonicus* using polymerase chain reaction. Can. 1. Microbiol. 40: 148-151.

24. Goodfellow, M. 1984. Reclassification *ofCorynebacteriumjascians* (Tilford) Dowson in the genus *Rhodococcus* as *Rhodococcus fascians* comb. nov. Syst. Appl. Microbiol. 5:225-29.

25. Gross, D. and A. K. Vidaver. 1978. A selective medium for isolation of *Corynebacterium nebraskense* from soil and plant parts. Phytopathology 69:82-87.

26. Harrison, N. A. and M. 1. Davis. 1988. Colonization of vascular tissues by *Clavibacter xyli* subsp. *xyli* in stalks of sugarcane cultivars differing in susceptibility to ratoon stunting disease. Phytopathology 78:722-727.

27. Henningson, P. 1. and N. C. Gudmestad. 1991. Fatty acid analysis of phytopathogenic coryneform bacteria. J. Gen. Microbiol. 137:427-440.

28. Jensen, H. L. 1952. The coryneform bacteria. Ann. Rev. Microbiol. 6:77-90.

29. Kado, C. 1. and M. G. Heskett. 1970. Selective media for isolation *ofAgrobacterium, Corynebacterium, Erwinia, Pseudomonas, and Xanthomonas.* Phytopathology 60:969976.

30. Keddie, R. M. 1978. What do we mean by coryneform bacteria? Pages 1-12. *In:* 1. J. Bousfield, A. G. Callely (eds.). Coryneform Bacteria. Academic Press, London.

31. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on tetrazolium medium. Phytopathology 44:693-695.

32. Lee, 1. M., 1. M. Bartoszyk, D. E. Gundersen, B. Mogen, and R. E. Davis. 1997. Nested PCR for ultrasensitive detection of the potato ring rot bacterium, *Clavibacter michiganensis* subsp. *sepedonicus.* App. and Environ. Microbiol. 7:2625-30.

33. Li, Xiang and S. H. de Boer. 1995. Selection of polymerase chain reaction primers from an RNA intergenicspacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus.* Phytopathology 85:837-42.

34. u, x., S. H. De Boer, and L. 1. Ward. 1997. Improved microscopic identification of Clavibacter Michiganensis subsp. sepedonicus cells by combining in situ hybridization with immunofluorescence. Let. Appl. Microbiol. 26:431-434.

35. Meynell, G. G., and E. Meynell. 1970. Theory and practice in experimental bacteriology. 2nd ed. Cambridge University Press, London.

36. Mills, D., B. Russell, and J. W. Hanus. 1997. Specific detection *ofClavibacter michiganensis* subsp. *sepedonicus* by amplification of three unique DNA sequences isolated by subtraction hybridization. Phytopathology 87:853-861.

37. Moffet, M. L., P. C. Fahy, and D. Cartwright. 1983. *Corynebacterium.* Pages 45-65. *In:* P. C. Fahy and G. J. Persley (eds.). Plant Bacterial Diseases. A Diagnostic Guide. Academic Press, Sydney.

38. Nemeth, J. A. and N. Kovacs. 1997. Determination *ofClavibacter michiganensis* subsp. *insidiosus* infection in lucerne seeds by immunofluorescence staining and direct isolation using selective medium. *In:* Diagnosis and identification of Plant Pathogens: Proceedings of the 4th International Symposium of the European Foundation for Plant Pathology, September 9-12, 1996. Bonn, Germany. Kluwer Academic, Netherlands. p. 395-397.

39. Pan, Y. B., M. P. Grisham, D. M. Burner, K. E. Damann, Jr., and Q. Wei. 1998. A polymerase chain reaction protocol for the detection of *Clavibacter xyli* subsp. *xyli*, the causal bacterium of sugarcane ratoon stunting disease. Plant Dis. 3:285-290.

40. Sasaki, J., M. Chijimatsu, and K. Suzuki. 1998. Taxonomic significance of 2,4-diaminobutyric acid isomers in the cell wall peptidoglycan of actinomycetes and reclassification of *Clavibacter toxicus* as *Rathayibacter toxicus* comb. nov. Int. J. Syst. Bacteriol. 48:403-410.

41. Schaad, N. W., Y. Berthier-Schaad, A. Sechler, and D. Knorr. 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluroescence detection system. Plant Disease. In press.

42. Schneider, J. B., J. Zhao, and C. S. Orser. 1993. Detection *ofClavibacter michiganensis* subsp. *sepedonicus* by DNA amplification. FEMS Microbiol. Lett. 109:207-212.

43. Skerman, V. B. D., V. Mcgowan, and P. H. A. Sneath. 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.

44. Slack, S. A., J. L. Drennan, A. A. G. Westra, N. C. Gudmestad, and A. E. Oleson. 1996. Comparison ofPCR, ELISA, and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. Plant Dis. 80:519-24.

45. Smidt, M. L., and A. K. Vidaver. 1984. Inhibition of *Corynebacterium michiganense* subsp. *nebraskense* by certain lots of polymyxin used in the selective medium. Plant Dis. 68:536.

46. Society of American Bacteriologists. 1957. Manual of Microbiological Methods. H.1. Conn,ed. Mc~avv-flill,~

47. Stange, R. R. Jr., D. Jeffares, C. Young, D. B. Scott, 1. R. Eason, and P. E. Jameson. 1996. PCR amplification of the fas-1 gene for the detection of virulent strains of *Rhodoeoecus fascians.* Plant Path. 45: 407 -417.

48. Vidaver, A. K. 1982. The plant pathogenic corynebacteria. Ann. Rev. Microbiol. 36:495-517.

49. Vidaver, A. K., and M. P.Starr. 1979. Phytopathogenic coryneform and related bacteria. Pages 1879-1887. *In:* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel, (eds.). The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria. Springer-verlag, Berlin, Heidelberg, New York.

50. Waters, C. M. and H. A. Bolkan. 1992. An improved semi-selective medium for detecting *Clavibacter miehiganensis* subsp. *michiganensis* in tomato seeds. Phytopathology 82: 1072. (Abstract)

51. Zgurskaya, H. 1., L. 1. Evtushenko, V. N. Akimov, and L. V. Kalakoutskii. 1993. *Rathayibaeter* gen. nov., including the species *Rathayibaeter tritici* comb. nov., and *Rathayibaeter iranicus* comb. nov., and six strains from annual grasses. Int. 1. Syst. Bacteriol. 43: 143-149.

9. CHEMICALS AND MATERIALS

Chemicals	Source
Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178.	
Ammonium Chloride	Fisher
Ammonium phosphate	
Bromthymol blue	
Bovine serum albumin	
Bovine serum albumin fraction V (no, 4503)	
Casein	
Casarnino acids (casein hydrolysate)	
Colistan methane sulfonate	
Com meal agar	BBL
Cycloheximide	
L-cysteine (free base)	

Daconi12787-F (tetrachloroisophthalonitrile)	Fermenta Plant Protection Co., P. O. Box 8000 Mentor,OH 44061
Esculin	
Ferric citrate	Fisher
Hemin chloride (bovine)	
Hymexazol	Tachigaren, Sanko co., Ltd., Japan
Lithium chloride	
Magnesium sulfate, heptahydrate	Fisher
Nalidixic acid	
Pepton, Phytone or Soytone	BBL or Difco, respectively
Polymyxin B sulfate	Cal Biochem
Potassium phosphate, monobasic	Fisher
Potassium phosphate, dibasic, trihydrate	Fisher
Sodium azide	
Sodium chloride	Fisher
Sodium hydroxide	Fisher
2-3-5 triphenyl tetrazolium chloride	Difco
Tris	
Yeast Extract	BBL or Difco

ID. GRAM-POSITIVE BACTERIA

B. *Streptomyces*

R. Loria, C. A. Clark, R. A. Bukhalid, and B. A. Fry

1. INTRODUCTION

Streptomyces are novel plant pathogens in that they are filamentous prokaryotes. Unlike other spore-producing bacteria, *Streptomyces* species produce spores that are the primary method of dispersal. Spore chains are formed through fragmentation of aerial hyphae that are produced on the substrate mycelium. The shape of the spore chains, which may be straight, branched, spiral or wavy, is an important taxonomic characteristic (33). *Streptomyces* are easily differentiated from fungi by their smaller hyphae and spores.

Only a very small number of the more than 400 described *Streptomyces* species are known to be plant pathogens and isolations from tissue and soil often yield a high proportion of saprophytic strains. Plant pathogenic *Streptomyces* species cause diseases of underground structures of diverse plant species; information on pathogen biology and disease management has been reviewed by Loria et al. (23). S. *scabies* (19), a cause of common scab of potato and similar diseases of tap root crops (radish, rutabaga, turnip, parsnip and carrot) was the first of these species described and is the best studied of the pathogenic species. S. *acidiscabies* (18) and S. *turgidiscabies* (26) produce symptoms like those of S. *scabies* on potato and other tap root crops. However, these species have a much more limited geographic distribution than S. *scabies.* A diverse group of *Streptomyces* species have been reported to cause russet (1,9) and netted scab and fibrous root rot on potato (3), though much less is known about these pathogens than about the three species mentioned previously. S. *ipomoeae* (31) causes *Streptomyces* soil rot (pox) of sweet potato which is characterized by necrosis of fibrous roots and cankers on storage roots at any stage of their development. This pathogen has not been reported to cause disease on other crop plants.

Thaxtomins, a family of phytotoxins, appear to playa major role in most diseases caused by *Streptomyces* species. Several studies have demonstrated that production of thaxtomins by *Streptomyces* strains is predictive of plant pathogenicity, except for those strains causing superficial diseases of potato (russet or netted symptoms). Evidence for involvement of thaxtomins in pathogenicity of *Streptomyces* spp. has been reviewed (4, 23).

2. ISOLA TION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

Members of the genus *Streptomyces* are filamentous, a characteristic useful in identifying colonies on solid media; Vegetative filaments are approximately 1 urn in diameter. Initially, colonies consist of substrate mycelium, and are smooth (not shiny) and firm to rubbery in texture. On most media, the colonies produce fuzzy, white aerial mycelium several days after plating. Subsequently, aerial mycelium will fragment into pigmented spore chains with a characteristic

morphology (spiral, rectiflexuous, straight, etc.), producing a colony with a powdery appearance and a characteristic color. Pigment production in the medium surrounding the colonies is a useful taxonomic criterion for some species, but is media specific.

a. **Plant** material

Potato tuber or tap root crops. Water agar is the medium of choice for isolation of *Streptomyces* from lesions on potato tubers and tap root crops. Most pathogenic species grow and sporulate on water agar and observation of the filamentous nature of the nonsporulating colonies is easiest on this relatively clear medium. In some cases, the presence of fungal or bacterial contaminants may require the use of a more selective medium; many bacterial and fungal contaminants can be eliminated by use of media, such as NPPC water agar (see p. 238). Light gray or white sporulation is occasionally visible in lesions on tubers freshly collected from the field. If present, spores and mycelium may be taken from the surface of scab lesions, suspended in a drop of sterile water, and streaked for isolation directly onto water agar or NPPC water agar. Tubers which do not have visible mycelium should be surface disinfected in 1.5% NaDCI for I min and rinsed with sterile H_20. After cutting away the brown-black lesion, a piece of the tissue is removed from beneath (this may be straw-colored) and macerated in a small volume of sterile water with a sterile mortar and pestle, and suspended in 5 ml sterile H_20. Using a sterile pasteur pipette, a drop of this suspension is spread by streaking onto water agar or NPPC water agar. Alternatively, the whole tissue piece can be placed in a tube of sterile water and immersed in a hot water bath for 30 min at 60°C (28), then placed onto water agar or NPPC water agar.

Sweet potato storage roots. Lesions on sweet potato roots are washed in distilled water. Small pieces (I mm-) of necrotic tissue are removed and crushed in 0.2 ml of sterile, distilled water containing 0.85% NaCI and 2mM mannitol. The suspension is streaked onto S. *ipomoeae* isolation medium (SlIM) and plates are incubated for 3-5 days at 32-36°C in the dark (27). It takes about 5-7 days for blue/green aerial mycelia to develop with S. *ipomoeae* on SGM at 32°C.

b. Soil

Isolating plant pathogenic *Streptomyces* spp. from natural soil is technically very difficult. Pathogenic species are similar in appearance to many saprophytic species and usually represent only a small proportion «1%) of the streptomycetes in natural soil. S. *scabies* has been isolated from natural soil using a procedure described by Keinath and Loria (12). Air dried soil (10 g) is added to 100 ml of sterile distilled water, and agitated on a shaker for 10 min. Five successive tenfold dilutions of the soil suspension are made using sterile water and 0.1 ml of the three most dilute suspensions are spread uniformly onto NPPC water agar plates and incubated at 28-30°C for 7-10 days. The number of bacterial contaminants on isolation plates can be reduced by airdrying soil before preparing the soil suspension.

c. Recipes for semiselective media

1) Nystatin, polymyxin, penicillin, cycloheximide (NPPC) water agar (34)

	perL
Antibiotic stock solution	10.0 ml
Agar	20.0 g

Prepare the antibiotic stock solution by adding the following to a bottle of distilled water (100 ml) which has been autoclaved and cooled (34): Nystatin, 500 mg; Polymixin B sulfate, 50 mg; Sodium Penicillin-G, 10 mg; Cycloheximide, 500 mg. This stock solution may be filter-sterilized and stored for up to 4 weeks at 5°C or frozen at -20°C. Add the antibiotic solution to agar which has been autoclaved for 20 min at 15 Ibs pressure, and cooled to 45-55°C.

2) *Streptomyces ipomoeae* isolation medium (SUM) (27)

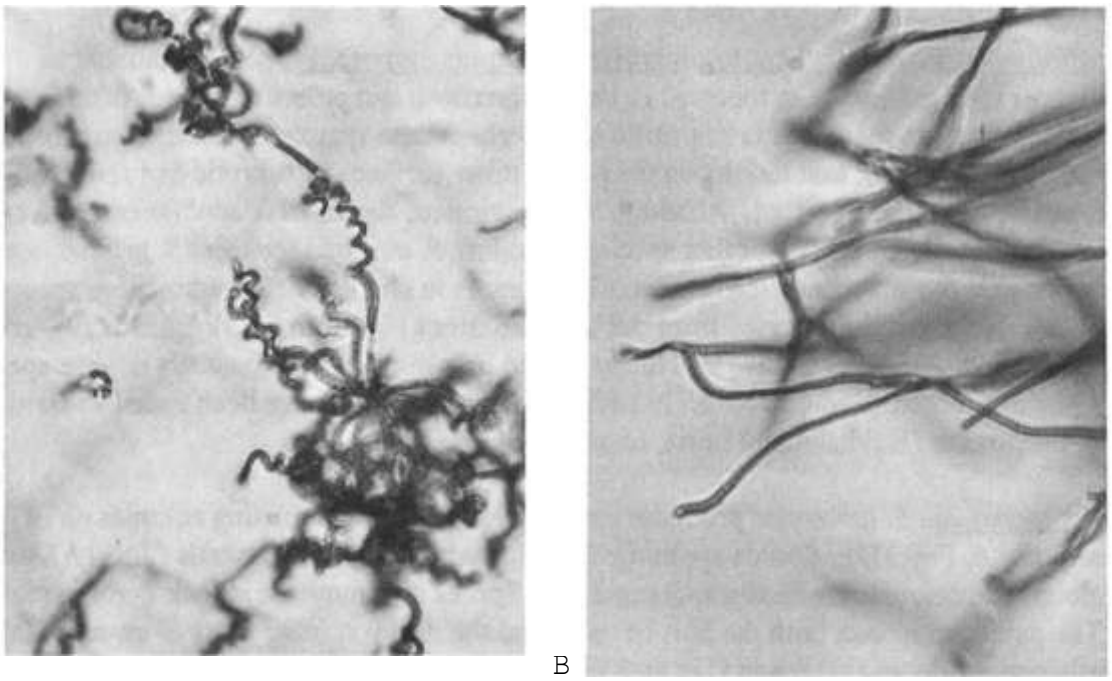
	perL
K_2HPO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	5.0 g
$CaCO_3$	2.0 g
Sodium propionate	1.0 g
Yeast extract	0.05 g
Agar	18.0 g
$CoCl_2$	110.0 mg

Autoclave the medium and cool to approximately 52°C. Add the following filter-sterilized ingredients per liter: raffinose, 1.0 g; cycloheximide, 100 mg; nalidixic acid (dissolved in 0.1 N NaOH), 150 mg; and sodium azide (caution - toxic), 25 mg.

3. DIFFERENTIA nON OF COMMONLY ISOLA TED SPECIES

S. *scabies*. Using morphological and physiological characteristics, S. *scabies* forms a relatively homogeneous group that is characterized by the ash gray color of sporulating colonies on YME medium (plate 6, Fig. 1), smooth spores borne in spiral chains (Fig. lA), production of melanin (Plate 6, Fig. 2A), and utilization of all of the sugars (Table 1) used for characterization of *Streptomyces* species as recommended by the International Streptomyces Project (ISP) (33). Spores are smooth and their color in mass is gray. S. *scabies* is the most important plant pathogen in the genus *Streptomyces* worldwide. This pathogen can cause superficial, erumpent or pitted lesion types on potato tubers, but erumpent lesions are the most common and most lesions have a raised, rough, corky appearance. This pathogen also incites scab of root crops such as radish, turnip and carrot in some countries. Interestingly, S. *scabies* infects peanut, resulting in a disease called "pod wart of peanut" in South Africa (7,8) and Israel (16) that is characterized by

raised, necrotic lesions on peanut hulls (pericarp tissue). S. *scabies* produces thaxtomin A and several other thaxtomins in host tissue and on oatmeal media (13, 14,22).



A B

Fig. 1. Spore chains of plant pathogenic *Streptomyces* spp. A) corkscrew spiral spore chains of *Streptomyces scabies.* B) rectiflexous (flexuous) spore chains of S. *acidiscabies.* S. *ipomoeae* and S. *turgidiscabies* spore chains are similar in appearance to those of S. *scabies* and S. *acidiscabies,* respectively. Spore chains develop from aerial hyphae that arise from substrate mycelium.

S. *acidiscabies.* This pathogen causes symptoms on tubers and other crops that are identical to those produced by S. *scabies* (25). S. *acidiscabies* (18) is characterized by white to pink sporulating colonies on TIv1E medium (Plate 6, Fig. IB), spores borne in flexuous chains (Fig. IB) and a red/yellow, pH sensitive diffusable pigment (plate 6, Fig. 2B) on modified salts starch agar (MSSA) (see 3), p. 242) (Table 1). Spores are smooth and their color in mass-is red. S. *acidiscabies* is unable to utilize raffinose as a sole carbon source, though it uses all of the other ISP recommended sugars. It can grow at a pH of 4.0 in culture, compared to a minimum pH of 5.0 for S. *scabies.* It causes "acid scab," a term describing the occurrence of the disease in low-pH soils. S. *acidiscabies* has only been confirmed to occur in the northeastern United States. This species also produces thaxtomin A and several other thaxtomins in host tissue and on oatmeal media.

S. *turgidiscabies.* This species has been described from the island of Hokkaido in Japan (26), but not elsewhere. It has a wide host range, affecting potato and many commercially grown tap root crops; symptoms are indistinguishable from those of S. *scabies.* S. *turgidiscabies* has gray sporulating colonies (plate 6, Fig. 1 C), flexuous spore chains (Fig. IB), lacks melanin

production and uses all of the ISP recommended sugars (Table 1). Spores are smooth and their color in mass is gray. S. *turgidiscabies* produces thaxtomin A and several other thaxtomins in host tissue and on oatmeal media (4).

Streptomyces spp. that cause superficial symptoms on potato tubers. A number of *Streptomyces* species have been reported to cause superficial symptoms on potato tubers, variously described as russet, netted or turtle back scab. These symptoms differ from those produced by S. *scabies* in that lesions on the potato tuber surface are necrotic and rough in texture, but never raised or pitted. Although weakly virulent strains of S. *scabies* can cause superficial lesions on tubers (23), other species, including S. *aureofaciens* and S. *griseus*, are often responsible for superficial scab lesions. Differences in characteristics among the groups of russet scab-causing strains reported from the United States (10), Europe (3), Canada (9), and Japan (29) indicate that this disease is actually caused by a number of genetically diverse species, making positive identification difficult. In addition, the strains that have been tested so far do not produce thaxtomins (Bukhalid and Loria, unpublished data).

S. *ipomoeae*. S. *ipomoeae* produces smooth bluish green sporulating colonies on SGM medium (Plate 6, Fig. ID). Spores are borne in chains with open loops, spirals (Fig. lA), or in spiral globose structures resembling sporangia (6). Spores are smooth and their color in mass is blue. The pathogen infects both the fibrous roots and the fleshy storage roots of sweet potato. This pathogen produces thaxtomin C in host tissue but not in media (15); other described thaxtomins are not produced by this species.

4. DIAGNOSTIC MEDIA AND TESTS

a. Morphological and physiological characterization.

The identification of species within the genus *Streptomyces* is difficult because of the complex characterization procedures (33) and uncertainty of the taxonomy of this group. The procedures for characterization described here are short cuts which allow the pathologist to select strains for pathogenicity testing. The most useful characteristics are summarized in Table 1. See Shirling and Gottlieb (33) for a complete description of the procedures for characterization of *Streptomyces* spp. recommended by the International Streptomyces Project.

Select colonies with a powdery texture from isolation plates and streak onto appropriate growth media (Table 1). Make three serial transfers from single colonies to obtain pure cultures. Streak onto slants and incubate for two weeks to get mature (sporulating) stock cultures. Streak from stock cultures onto plates of media in a crosshatched pattern for morphological observations (Table 1) and incubate for 10-14 days . .

Make aerial mass color and reverse color observations on appropriate

media. Observe spore chains by direct microscopic examination of the culture surface of opened dishes of the crosshatched culture. A minimum of 10 microscopic fields should be observed at 200-400 X. Strains with gray sporulating colonies and spiral spore chains should be grown on agar to determine if diffusable brown to black melanoid pigments (Plate 6, Fig. 2A) are produced. Strains with white to pink colonies and flexuous spore chains should be grown on MSSA to look for production ofa diffusable red pigment (plate 6, Fig. 2B) which is pH sensitive.

Table 1. Selected morphological and physiological characteristics and growth conditions for well-characterized pathogenic *Spreptomyces* spp.

Species (reference)	Spore chain type (medium)!	Aerial color of mature colony (medium)	Pigment production (medium)	ISP sugar utilization patterns"	Growth temperature and medium
S. *scabies (19)*	Spiral (YME)	Gray(YME)	Brown (PYI)	All sugars	28-30°C (YME)
S. *acidiscabies (18)*	Rectiflexous (YME)	White/pink (YME)	Red/yellow (MSSA)	All except raffmose	25-28°C (YME)
S. *turgidiscabies (26)*	Rectiflexous (YME)	Gray(YME)	None	All sugars	25-28°C (YME)
S.*ipomoeae* (30)	Spiral (SGM)	Blue/green (SGM)	None)	Not galacturonic acid	30-32°C (SGM)

1 Growth media: YME=Yeast malt extract, PYI=Peptone-yeast extract iron agar, MSSA=Modified salt starch agar, *SGM=Streptomyces* growth medium.

2 Sugars include: D-Glucose (positive control), L-arabinose, sucrose, D-xylose, I-inositol, D-mannitol, D-fructose, raffmose, cellulose, galacturonic acid; ISP-Intemational *Streptomyces* Project.

b. Thaxtomin production, extraction and identification

Detailed methods used for production and purification of thaxtomin A are described in Babcock et al. (2), Loria et al. (22), and King and Lawrence (13). These methods are appropriate for use with strains obtained from potato tubers (S. *scabies,* S. *acidiscabies,* and S. *turgidiscabiesi.* Since S. *ipomoeae* does not produce thaxtomins in media, strains must be grown on potato tuber slices and thaxtomin must be extracted from the infected tissue as described by Lawrence et al. (15, 20).

Oatmeal broth (OMB, see 5, p. 243) is inoculated with a concentrated spore suspension or macerated agar culture of test strains. The cultures are incubated at 28°C on a shaker at 180 to 200 rpm for 4-7 days. Thaxtomins are usually evident after 4 days as a yellow diffusable pigment. However, there are also saprophytic strains that produce yellow diffusable pigments that are not members of the thaxtomin family and are not indicative of pathogenicity. Cultures

are filtered to remove mycelium and the filtrate is extracted twice with one and a half volume of ethyl acetate, and dehydrated by the addition of anhydrous sodium sulfate, The ethyl acetate is then evaporated to dryness *"in vacuo"* at 25°C, and the yellow residue is taken up in 1 to 2 ml methanol. Thaxtomin A is detected by reverse phase thin layer chromatography on Whatman (Maidstone, England) LKC-18F plates (20 x 20 em, 250 zzm) using an acetone and water (3 :2) solvent system. Thaxtomins are apparent as deep yellow bands and can be identified based on Rfvalues (14, 20), by their ability to cause necrosis of potato tuber tissue (22) or by hypertrophy and cell death of seedlings (21) (plate 6, Fig. 3).

a. Diagnostic media

1) Yeast malt extract agar (YME) (33)

	perL4,
Yeast extract	0 g
Malt extract	10,0 g
Dextrose	4,0 g
Agar	20.0 g

Adjust to pH 7.0-7.2 with NaOH before adding agar. The medium is dissolved in water and autoclaved for 15 min at 15 lb pressure. Commercially available as ISP Medium 2.

2) Peptone-yeast extract iron (PYI) agar (33)

	perL
Peptone Iron Agar (Difco)	36.0 g
Yeast extract	1,0 g

Combine all ingredients, adjust the pH to 7.0-7.2 with NaOH or HCI if necessary.

3) Modified salts starch agar (MSSA) (18)

	perL
Soluble starch	10.0 g
NaNG)	1.0 g
MgCO)	1.0 g
K_2HP0_4	0.3 g
NaCI	0,5 g
Agar	15,0 g

Glycerol may be substituted for starch.

4) *Streptomyces* growth medium (SGM) (6)

	perL
Mannitol	20.0 g
K_ZHP0_4	0.2 g
$MgS0_4$-$7H_zO$	0.2 g
NaCI	5.0 g
$CaC0_3$	2.0 g
Sodium propionate	1.0 g
Yeast extract	1.0 g
coci,	0.11 mg
Agar	18.0 g

5) Oatmeal agar (OMA) and oatmeal broth (OMB)

Oatmeal media are prepared by boiling rolled oats (20 gil) in distilled water for 20 min., straining through layers of cheesecloth, bringing up the volume to 1 liter and adjusting to pH 7.0-7.2 with 1 M NaOH. OMA contains 1.5% agar.

5. PATHOGENICITY TESTS

a. Potato tuber assay *(S. scabies, S. acidiscabies,* and *S. turgidiscabies).* Many methods for testing pathogenicity of S. *scabies* on potato tubers have been devised. They all involve producing spores on a suitable growth medium, such as YME agar, and planting pathogen-free seed tubers or stem cuttings (22, 24) of potato in an appropriate plant growth medium, which has been infested with the pathogen. Soil moisture is kept as low as possible during tuberization to encourage infection. Labruyere (17) describes a simple procedure for evaluating the pathogenicity of S. *scabies* strains that can also be used for S. *acidiscabies* and S. *turgidiscabies:*

1. Add spores of test strains, grown on YME agar plates for 14 days, to sterile 'Perlite' (used in soil mix) that has been saturated with a nutrient solution (per liter: 20.0 g sucrose, 1.2 g L-asparagine, 0.6 g $KzHPO_4$, 10.0 g yeast extract) and placed in Petri plates (15 em diameter). Incubate plates at 28°C for 10-14 days then mix one plate of the Perlite (rich in mycelium and spores) into steam-sterilized potting mix (a mixture of sand, peat, and composted manure) at a ratio of one plate per pot of mix. Sand may be used in place of potting soil.

2. The potting mix or sand is put into polyethylene bags and placed into clay pots (12.5 em diameter x 15.0 em height). Holes punched in the bottoms of bags allow for drainage and bags are partially filled with infested potting mix or sand. Roll back the bag, allowing about 10 cm to protrude above the level of pots to prevent cross contamination during irrigation.

3. Seed tubers which are free of scab lesions should be surface disinfected with 0.5% NaOCl for 10 min then planted (one per pot) 5 ern below the surface of the potting mix or sand. Plants in the potting mix should be irrigated with tap water and those in sand should be irrigated with Hoagland's solution (11). Once tuber initiation begins (tuber diameter is twice that of the stolon), irrigation is kept to a minimum to favor infection. Tubers can be viewed through the bag and disease development can be monitored during the test. After about 10 weeks, tubers may be harvested and evaluated for the presence of scab lesions.

b. Seedling assays *(S. scabies,* S. *turgidiscabies,* and S. *acidiscabies).* Radish seeds are surface disinfected with 0.5% NaOCI and 0.1 % Tween 20 for 3 min, rinsed twice in sterile distilled water and allowed to germinate by incubating on water agar or moist filter paper overnight at room temperature. Germinated seeds with protruding radicals are selected for uniformity and individually placed in glass culture tubes (25 mm diameter) containing 10 ml of 1 % water agar or 0.5% gelrite (plate 6, Fig. 3A). Seeds are then inoculated with 0.1-0.5 ml of undiluted *Streptomyces* cultures grown for 4-6 days in OMB as described in section 4B (no filtration is necessary). Seedlings are grown under a 12 h light cycle for 6-10 days at room temperature. Pathogenic strains cause brown to black lesions and stunting of the seedling (plate 6, Fig. 3B); radial swelling and cell hypertrophy are sometimes visible.

c. Sweetpotato assays *(S. ipomoeae).* Isolates can be quickly screened for pathogenicity on storage roots and fibrous roots using plugs from 5-7 day-old SGM cultures for inoculum. Storage roots are washed, peeled, and 1-2 em trimmed from each end. The storage roots or sweetpotato vine segments are then surface disinfected for 10 min in 0.5% NaOCl.

The storage roots are cut with a flamed knife into approximately l-cm-thick slices which are placed in sterile Petri dish moist chambers. A plug of agar is transferred from the culture and placed mycelium side down on the cut surface of the storage root slice. The slice is incubated at 32°C for 5-10 days after which the callus which develops on the cut surface is gently scraped away with a knife or steel wool. Pathogenic isolates cause a dark brown to black necrosis that extends into the storage root tissue. Nonpathogenic isolates may grow superficially on the surface or sometimes cause superficial light brown discoloration, but do not induce necrosis extending into the slice.

Vine segments are eut into one-node pieces which are placed on water agar and allowed to develop roots in the dark for 2-3 days prior to inoculation. A plug from the culture is placed on the tip of the root and after 3-5 days at 32°C, a characteristic necrotic lesion develops that progresses along the root. Nonpathogenic isolates either induce no reaction or at most slight superficial browning at the site of inoculation.

Pathogenicity can be further assessed on plants grown in the greenhouse or on

storage root slices using the methods developed by Moyer et al. (27). For these inoculations, inoculum can be produced on a medium (VMS) of 4 parts sterile vermiculite to 3 parts SGM (v/v) modified by omitting agar and NaCI and reducing mannitol to 5.0 gIL. A spore suspension from SGM plates is aseptically mixed into the VMS and incubated for 2-3 wk at 32 "C. The resulting inoculum can be mixed in soil at varying ratios for greenhouse tests or used without dilution for storage root slice assays.

6. MOLECULAR TECHNIQUES

Much less is known about the molecular genetics of pathogenicity in *Streptomyces* spp. than for other bacterial pathogens. However, molecular analyses have shown that at least one gene, called *nec1,* is present in most strains of S. *scabies,* S. *acidiscabies,* and S. *turgidiscabies* (4,5); this gene has not been found in nonpathogenic *Streptomyces* or S. *ipomoeae.* Specific primers (forward (Nt) 5'-ATGAGCGCGAACGGAAGCCCCGGA-3' and reverse (Nr) 5' -GCAGGTCGTCACGAAGGATCG-3') for *nec1* have been described and amplification is possible using PCR. Though a very small proportion of S. *scabies* strains lack *nec1,* its amplification from S. *scabies,* S. *acidiscabies,* and S. *turgidiscabies* is a reliable indicator of pathogenicity.

Streptomyces cultures are grown in 5 ml CRM (glucose 10 gil, sucrose 103 gil, $MgCl_z$. $6H_zO$ 10.12 gil, tryptone 15 gil, yeast extract 5 gil; autoclave at 15 psi for 15 min) (32) in test tubes for 24-48 h at 30°C. DNA is extracted by pelleting and resuspending mycelium in sterile distilled water (200-500 zzl), followed by boiling, and recovery of DNA by centrifugation as previously described (4). Amplification is carried out using 10 j.l-l of DNA, 0.1 *J,IM* of each primer, 100 *J,IM* of each dNTP, Ix *Taq* buffer, 2.5 mM MgCI" and 1 unit of *Taq* polymerase (perkin Elmer) in a 100 *J,ll* reaction under the following conditions: DNA denaturing at 95 DC for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min for 30 cycles. The amplified DNA is then run on 1% agarose gels to determine if a 0.7 kb amplification product is present.

See Appendix A for details.

7. CULTURE PRESERVATION

Cultures are best stored as concentrated spore suspensions, as follows: spores are harvested from sporulating plates ofYME or OMA by adding 1-2 ml sterile distilled water to each plate, loosening spores with an inoculating loop, concentrating spores by centrifugation, then resuspending them in 20% glycerol. Long term storage should be in sterile cryo vials at $\_80^0$ C or -135°C. To store for 2 - 3 months, -20^DC is adequate.

Streptomyces spp. also can be conveniently stored for many years on silica gel crystals (30). Screw-cap tubes are filled halfway with silica gel crystals (grade 40,6-12 mesh, Aldrich Chemical Company) and the loosely capped tubes are sterilized in dry heat at 180°C for 1.5 h. Skim milk (7%) is pasteurized at 11 psi for 13 min and 15% glycerol is autoclaved separately at 121 ° C for

20 min. Skim milk and glycerol are combined in equal proportions and used to make a dense spore suspension from cultures. The spore suspension is transferred to the silica gel crystals at a rate of approximately 0.2 ml of suspension to 3 g crystals, the tube is capped, vigorously vortexed and transferred to an ice bath to dissipate the heat generated. The tubes can be stored at 4 ° C, or -20°C. To revive the culture, a few crystals can be shaken onto an appropriate medium and the storage tube returned to the freezer for future use. Cultures of S. *ipomoeae* have been preserved for longer than 10 yr at -20°C by this method.

8. LITERATURE CITED

1. Archuleta, 1. G. and G. D. Easton. 1981. The cause of deep-pitted scab of potatoes. Am. Potato J. 58:385-392.

2. Babcock, M. 1., E. C. Eckwall, and 1. L. Schottel. 1993. Production and regulation of potato-scab-inducing phytotoxins by *Streptomyces scabies.* 1. Gen. Microbiol. 139: 1579-1586.

3. Bang, H. 1995. Netted scab: Cause, biology and control. Vaxtskyddsnotiser 59:99-106.

4. Bukhalid, R. A., S. Y. Chung, and R. Loria. 1998. *necl,* a gene conferring a necrogenic phenotype, is conserved in plant pathogenic *Streptomyces* species and linked to a transposase pseudogene. Mol. Plant-Microbe Interact. 11:960-967.

5. Bukhalid, R. B. and R. Loria. 1997. Cloning and expression of a gene from *Streptomyces scabies* encoding a putative pathogenicity factor. 1. Bacteriol. 179: 7776-7783.

6. Clark, C. A. and A. Lawrence. 1981. Morphology of spore-bearing structures in *Streptomyces ipomoea.* Can. 1. Microbiol. 27:575-579.

7. De Klerk, A., A. McLeod, R. Faurie, S. W. J., and F. D. N. Denner. 1996. Identification and toxin production of *Streptomyces* isolates responsible for common scab in the northern Cape Province of South Africa. Proc. Eur. Assoc. Potato Res., Veldhoven, The Netherlands.

8. De Klerk, A., A. McLeod, R. Faurie, and P. S. Van Wyk. 1997. Net blotch and necrotic warts caused by *Streptomyces scabies* on pods of peanut *(Arachis hypogaea).* Plant Dis. 81:958.

9. Faucher, E., B. Otrysko, E. Paradis, N. C. Hodge, R. E. Stall, and C. Beaulieu. 1993. Characterization of streptomycetes causing russet scab in Quebec. Plant Dis. 77: 1217-1220.

10. Harrison, M. D. 1962. Potato russet scab, its cause and factors affecting its development.

Am. Potato 1. 39:368-387.

11. Hoagland, D. R. and D. I. Arnon. 1950. The water culture method of growing plants without soil. Circular 347. California Agriculture Experiment Station.

12. Keinath, A. P. and R. Loria. 1989. Population dynamics of *Streptomyces scabies* and other actinomycetes as related to common scab of potato. Phytopathology 79:681-687.

13. King, R. R. and C. H. Lawrence. 1996. Characterization of new thaxtomin A analogues generated *in vitro* by *Streptomyces scabies.* 1. Agric. Food Chern. 44: 1108-1110.

14. King, R. R., C. H. Lawrence, and L. A. Calhoun. 1992. Chemistry ofphytotoxins associated with *Streptomyces scabies,* the causal organism of potato common scab. 1. Agric. Food Chern. 40:834-837.

15. King, R. R., C. H. Lawrence, L. A. Calhoun, and 1. B. Ristaino. 1994. Isolation and characterization of thaxtomin-type phytotoxins associated with *Streptomyces ipomoeae.* 1. Agric. Food Chem. 42:1791-1794.

16. Kritzman, G., C. A. Shani, B. Kirshner, Y. Riven, Z. Bar, 1. Katan, and A. Grinstein. 1996. Pod wart disease of peanuts. Phytoparasitica 24:293-304.

17. Labruyere, R. E. 1971. Common scab and its control in seed potato crops. Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands.

18. Lambert, D. H. and R. Loria. 1989. *Streptomyces acidiscabies* sp. nov. Int. 1. Syst. Bacteriol. 39:393-396.

19. Lambert, D. H. and R. Loria. 1989. *Streptomyces scabies* sp. nov., nom. rev. Int. 1. Syst. Bacteriol. 39:387-392.

20. Lawrence, C. H., M. C. Clark, and R. R. King. 1990. Induction of common scab symptoms in aseptically cultured potato tubers by the vivotoxin, thaxtomin. Phytopathology 80:606-608.

21. Leiner, R. H., B. A. Fry, D. E. Carling, and R. Loria. 1996. Probable involvement of thaxtomin A in pathogenicity of *Streptomyces scabies* on seedlings. Phytopathology 86:709-713.

22. Loria, R., R. A. Bukhalid, R. A. Creath, R. H. Leiner, M. Olivier, and 1. C. Steffens. 1995. Differential production ofthaxtomins by pathogenic *Streptomyces* species *in vitro.* Phytopathology 85:537-541.

23. Loria, R., R. A. Bukhalid, B. A. Fry, and R. R. King. 1997. Plant pathogenicity in the genus *Streptomyces.* Plant Dis. 81:836-846.

24. Loria, R. and B. A. Kempter. 1986. Relative resistance of potato tubers produced from stem cuttings and seed-piece-propagated plants to *Streptomyces scabies.* Plant Dis. 70: 1146-1148.

25. Manzer, F. E., G. A. McIntyre, and D. C. Merriam. 1977. A new potato scab problem in Maine. University of Maine at Orono: Life Sciences and Agriculture Experiment Station Technical Bulletin 85:3-24.

26. Miyajima, K., F. Tanaka, T. Takeuchi, and S. Kuninaga. 1998. *Streptomyces turgidiscabies* sp. nov. Int. 1. of Syst. Bacteriol. 48:495-502.

27. Moyer, 1. W., C. L. Campbell, E. Echandi, and W. W. Collins. 1984. Improved methodology for evaluating resistance in sweet potato to *Streptomyces ipomoea.* Phytopathology 74:494-497.

28. O'Brien, 1. G., R. A. Blanchette, and 1. B. Sutherland. 1984. Assessment of *Streptomyces* spp. from elms for biological control of Dutch elm disease. Plant Dis. 68: 104-106.

29. Oniki, M., T. Suzui, T. Araki, R. 1. Sonoda, T. Chiba, and T. Takeda. 1986. Causal agent of russet scab of potato. Bull. Natl. Inst. Agro. Environ. Sci. 2:56-59.

30. Perkins, D. D. 1962. Preservation of *Neurospora* stock cultures with anhydrous silica gel. Can. 1. Microbiol. 8:591-518.

31. Person, L. H. and W. 1. Martin. 1940. Soil rot of sweet potatoes in Louisiana. Phytopathology 30:913-926.

32. Pigac, J. and H. Schrempf. 1995. A simple and rapid method of transformation of *Streptomyces rimosus* R6 and other streptomycetes by electroporation. Appl. and Environ. Microbiol. 61:352-356.

33. Shirling, E. B. and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. Int. 1. Syst. Bacteriol. 16:313-340.

34. Williams, S. T. and F. L. Davies, 1965. Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. 1. Gen. Microbiol. 38:251-262.

9. **CHEMICAL LIST**

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O. Box 14508, S10 Louis, MO 63178.

L-asparagine	
L-arabinose	
Dipotassium phosphate ($K2^{HPO}$ 4)	
Calcium carbonate	
Cellulose	
Dextrose	Difco
Colbalt chloride	ICN Biochemicals and Reagents
Cycloheximide	
D-fructose	
Gelrite	
Glucose (dextrose)	
Hoagland's NO.2 Basal salt mixture	
I-inositol	
ISPmedium2	Difco
D-sylose	
Malt extract	Difco
D-mannitol	
Nystatin (mycostatin)	
Penicillin-G, sodium	
Penicillin	
Peptone iron agar, dehydrated	Difco
Perlite	
Polymixin B sulfate	
Raffinose	
Rhannose	
Sodium propionate	
Sodium azide	
Soluble starch	
Tween 20	
Yeast extract	Difco

ill. GRAM-POSITIVE BACTERIA

C. *Bacillus*

W. Chun and A. K. Vidaver

1. INTRODUCTION

Plant-associated bacilli are recognized either as plant pathogens, saprophytes, or as biological control agents. There are only three known phytopathogenic bacilli. *Bacillus megaterium* pv. *cerealis* incites white blotch of wheat (12) and *B. circulans* causes a disease in tissue cultures of date palm seedlings and a discoloration in heart tissue of mature plants (14, 15, *32). Bacillus polymyxa* has been reported as the causal agent of tomato seedling blight (5).

Other members of this endospore-forming group of bacteria have been found associated with plant tissues or as endophytes. *Bacillus megaterium* and *B. cereus* are found in ovules and seeds (1.8), in healthy potato tubers (11, 17), and as strong colonizers of soybean roots (16). *Bacillus pumilus* has been observed in the vascular stele of pea and tomato roots (1,2). Inoculated *B. subtilis* can also colonize the xylem of maple (9, 10).

Certain species of *Bacillus* can impart beneficial effects on plants (4, 19, 30) as well as serve as insect vector control agents (7, 20). Numerous *Bacillus* species have been used as antifungal biological control agents. *Bacillus subtilis* strains suppress rhizoctonia seedling disease of peas (3) and summer patch symptoms in Kentucky bluegrass (28). It has also been used experimentally as a post-harvest treatment for peach brown rot (21-23). *Bacillus cereus* UW85 treatment significantly increased yields of soybeans that were tolerant or resistant to *Phytophthora sojae* (19) and suppressed oomycetous seedling diseases of cucumber (26) and tomato (26). Agents can be applied as seed treatments (3, 19,29), or in furrows (19). Some *Bacillus* species have a broad range of biological control activity and may be useful under reduced tillage (13).

Bacillus spp. are rod-shaped, catalase-positive, non-acid-fast, endospore-forming, aerobic or facultative anaerobic bacteria and normally stain Gram positive. Variable characters include, motility, oxidase reaction, and the method by which carbohydrate is utilized. The bacilli can be divided into the following three groups based on endospore morphology (32).

Group I:	Spores oval or cylindrical; central; subterminal or terminal. Bacillary body swollen slightly or not swollen at all.
Group II:	Spores oval, rarely cylindrical; subterminal or terminal. Bacillary body noticeably swollen.
Group ill:	Spores spherical; subterminal or terminal. Bacillary body swollen.

The Gram reaction for bacilli may vary with culture age, medium, and strain. It is very important that cells in the early log stage of growth be used for the Gram stain. For example, of the 163 strains of group II *Bacillus,* 46% are Gram-variable after 6 hours of incubation and 200.10

are Gram-negative after 48 h (6). *Bacillus circulans*, isolated from date palms, is Gram-negative with typical Gram-negative cell membranes (15). One cannot therefore depend entirely on the Gram stain for identification of *Bacillus* spp. but should include the observation of endospore production and if possible, thin section electron microscopy for determination of cell wall structure.

2. ISOLATION TECHNIQUES USING DIFFERENTIAL MEDIA

a. Plant Material

Plant material should be washed thoroughly with tap water and air-dried. When lesions or discoloration in the plant tissues are evident, small pieces (about 3 mm x 3 mm x 6 mm) can be excised using sterile scalpels and forceps and ground with a glass rod in a small volume of water. When no visible discoloration is observed, several pieces of plant tissue can be ground in sterile water. A loopful of the ground suspension can then be streaked on the appropriate medium. Since a large population of *Bacillus* may be needed in order to induce symptoms, a nutrient glucose medium should be used to speed growth of such slow growing species as *B. circulans.*

b. Seeds

Seeds can be surface sterilized for 5-10 min in a 1: 10 dilution of a commercial bleach such as Clorox" (5.25% active sodium hypochlorite). Adequate washing with distilled water should be performed to remove all traces of the sodium hypochlorite. Seeds can then be placed on the agar medium of Mundt and Hinkle (18). Seeds with a thick coat should be punctured with a dissecting needle before being placed on the medium. The plates or slants are incubated at 24 D C and observed daily for broken seed coats and growth of bacteria. An alternative protocol is to grind a sample of 109 of seeds. Mix the ground seed in 95 ml of 1;2 strength nutrient-glucose broth. Stir for 3 hours on a magnetic stirrer and place 1.0,0.5, and 0.1 g samples of the sediment into separate tubes of Mundt and Hinkle semi-solid slants. Incubate at 24 DC and streak on agar plates to obtain single colonies.

c. Recipes for differential media

1) Mundt and Hinkle medium (18)

To 1000 ml Difco nutrient broth, add:	perL
Yeast extract	3.0 g
Dextrose	5.0 g
Cycloheximide	2.0 g
Agar (slants)	5.0 g
Agar (plates)	17.0 g

2) Casein dextrose (CD) agar

	per L 10.
Casein acid hydrolysate	0 g 5.0
Yeast extract	g 10.0
Dextrose (50% sterile stock solution)	rn1 4.0
K_2HP0_4	g 17.0 g
Agar	

The 50% stock solution of dextrose is sterilized separately and added aseptically to the basal medium.

3. DIFFERENTIATION OF COMMONLY ISOLATED SPECIES

Species of *Bacillus* can be differentiated on the basis of physiological tests (Table 1).

Table 1. Characteristics used in the identification of *Bacillus* species. [1]

	Plant Pathogens		Other Species									
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+
Motility'	+	0	+	+	+	+	+	+	~	~	~	~
Spore position												
Terminal	+						+	+	+			+
Central	+	+		+	+	+	+	+	+		+	
Subterminal	+						+	+	+			
Swelling of bacillary body	+						V	+		+	+	+
Growth at 45°C	V			V	+	+	+	V	V	V		V
Growth at pH 5.7	V	+	+	+	+	+				V		+
Growth in 7"10 NaCI	V		+	V	+	+						
Utilization of citrate		+	V	+		+		+	V		V	
Anaerobic growth in glucose broth	V		+	+	+			+	+		+	+
Acid from:												
Arabinose	+	NO				+	+	V		V	+	+
Mannitol	+	+				+	+	V		V	+	+
Xylose	+	+				+	+	V		V		+
Voges-Proskaur test			+	+	+	+	V	+				
Starch hydrolysis	+	+	+	+	+	+	+	+				0

+,80% or more strains positive; +0,80% or more strains delayed positive; V, between 21-70% of strains positive; -, 80% or more strains negative; NO, not determined.

ı Adapted from Cowan (6) and Hosford for *Bacillus megaterium* pv. *cerealis* (12) and Leary and Chun for *B. circulans (15)* 2 All species may produce non-motile cells.

4. DIAGNOSTIC MEDIA AND TESTS

a. Gram Reaction

b. Gram stain (see b, p. 7) (Plate 1, Fig. 1)

c. KOH Test (see a, p. 7)

b. Motility

c. Method 1: Examine young broth cultures under phase contrast light microscopy for motility.

d. Method 2: Stab inoculate motility medium (8) tubes to a depth of 5 rnrn. Motile organisms migrate through the medium making it turbid.

Motility medium of Edwards and Bruner (8)

	perL
Gelatin	80.0 g
Peptone	10.0 g
Beef extract	3.0 g
NaCI	5.0 g
Agar	4.0 g

Distribute into tubes (10 ml) and autoclave at 121°C for 15 min.

c. Spore shape, position and swelling of bacillary body

1) Light Microscopy. Resuspend several bacterial colonies from a CD (see 2), p. 252) plate in a drop *ofHzO* and examine with a phase microscope. Spores may also be observed by staining. Resuspend bacteria in a drop of water on a slide and air dry. Flood with 5% malachite green and stain for 10 minutes. Wash under running water. Counter stain with 0.5% aqueous safranin for 15 s. Rinse with water and blot dry. Bacterial bodies will stain red and the spores will stain green.

2) Electron Microscopy (see b. p. 11). For electron microscopic examination of thin sections of the bacterium, scrape cells ofa 5-day-old culture from the surface of a CD agar plate and suspend in 1 ml of physiological saline. Pellet the cells in a bench-top centrifuge and fix the pellet with 25% glutaraldyhyde in 0.1 M phosphate buffer (pH 7.2). Rinse the pellet several times in the same buffer and then embed in 2% agar. Post-fix in 1% osmium tetroxide in 0.1 M phosphate buffer, followed by several rinses in the same buffer. Dehydrate in an acetone series, and embed in epoxy resin (32). The block containing the fixed pellet should then be trimmed and sectioned with a glass knife on an ultramicrotome. Collect thin sections (600 - 800 *J.lm)* on 150 mesh formvar-coated copper grids and post-stain for 15 minutes with 1 % aqueous uranyl acetate and 5 min with lead citrate.

The post-stained ultrathin sections can then be viewed with a transmission electron microscope.

Whole cells are prepared for viewing with the electron microscope by placing a drop of the pellet on the surface ofa 100 mesh thick-bar forrnvarcoated and carbon-coated copper grid. Allow the cells to dry, and then unidireetionally shadow cast with 13 mm ofa 0.02 mils wire of 80% platinum and 20% palladium coiled around a 0.025 mils tungsten filament wire oriented at a 1 - 3°5 slope to the specimen in a high vacuum evaporator. The grids can then be viewed under the same conditions as those described for use with the thin sections. Endospores are visible as dark bodies (plate 6, Fig. 4).

d. Growth at 45^0 C and 65^0 C.

Inoculate liquid CD medium and adjust pH to 5.7. Incubate at the desired temperature. Turbid cultures within 5 days are recorded as positive.

e. Growth in 7% NaCl (6).

Inoculate liquid CD or nutrient broth (see a, p. 3) plus 7% NaCl and observe daily for growth. If growth does not occur in 7% NaCl, the maximum NaCI concentration at which growth will occur can be determined by repeating the test with a range ofNaCI concentrations from 1 % - 6%.

f. Utilization of citrate (24).

Stab the butt and streak the surface of a slant of Simmon's citrate agar. Blue color and growth indicate citrate utilization. Original green color indicates citrate was not utilized.

Simmon's citrate agar (24)

	per L
$MgS0_4 \cdot 7H_20$	0.2 g
$NH_4H_2{}^P04$	l.0g
K~04	2.0 g
Sodium citrate	2.0 g
NaCl Bromthyrnol	5.0 g
blue Agar	80.0 mg
	15.0 g

Adjust to pH 6.8 - 6.9 before autoclaving. Melt agar, dispense into tubes and autoclave at 121°C for 15 minutes.

g. Anaerobic growth in glucose broth (6).

Inoculate tubes of glucose broth and incubate in an anaerobic jar. Alternatively, overlay the broth with sterile mineral oil and incubate at 24°C.

Glucose broth

	perL
Nutrient broth (Difco)	g 50.0
20% dextrose stock'	ml 950.0
H_2O	ml

'Autoclaved separately or filter sterilize throuzh a *0.2j.lm* filter. Add the appropriate amount of dextrose stock to autoclaved nutrient broth tubes.

h. Acid and gas production from carbohydrates: dextrose, arabinose, mannitol, and xylose. Stab inoculate duplicate tubes of Hugh and Leifson's OF medium (see 3, p. 9) containing the appropriate carbohydrate. Seal one tube with sterile mineral oil to a depth of 1 em. Incubateat 28 - 30°C and examine daily for 14 days. Note acid production (yellow), gas production (helpful in identifying *Bacillus* spp.), and motility (turbid medium).

Reaction Table:

Reaction	Open Tube	Sealed tube
Fermentation	Yellow	Yellow
No action	Blue or green	Green

Pockets of gas in the medium indicate gas production.

i. Starch hydrolysis (6).

Inoculate starch agar plates and incubate plates for 5 days. Flood plates with Lugol's iodine. Clear, colorless zones indicate starch hydrolysis. Note: some *Bacillus* spp. produce restricted zones so colonies should be scraped away for easier reading of results.

Lugol's iodine

	g/100 ml
Iodine	5.0 g
Potassium iodide (KI)	10.0 g

Dissolve iodine and KI in 10 ml of H_2O. Adjust to volume with distilled water. For use, dilute 1/5 with distilled water.

Starch agar

	perL
Nutrient agar Soluble	8.0 g
potato starch Distilled	10.0
water	g 1.0
	L

5. PATHOGENICITY TESTS

The pathogenicity of *Bacillus* spp. to plants appears limited. Therefore, very few specific methods have been developed and published. What is most important to consider is that if a *Bacillus* species is consistently isolated from diseased plant tissues and that particular *Bacillus* is the only organism consistently isolated, then it may be the pathogen. Thereafter, inoculation procedures must be used to satisfy Koch's postulates.

F or wheat, the inoculum can be applied by dipping or spraying leaves with a bacterial suspension (12). Placing the plants in a vacuum chamber evacuated to 15 mm Hg three successive times will aid infiltration (31). Subsequent placement of the inoculated plants in a mist chamber for 1 to 2 days may also be helpful.

The disease of date palms caused by *B. circulans* is a seedling disease. For such diseases, the inoculum should be prepared as a suspension of -1 0^4 - 10^6 bacteria/ml and the seeds soaked in the suspension for 1 hour. Seeds should then be planted in appropriate containers and observed for percentage germination, total wet weight, length of seedlings, and subsequent appearance of symptoms (see p. 226 for details).

6. COMMERCIAL AUTOMATED TECHNIQUES

The Biolog GP Microplatel'" provides standardized and rapid performance of many physiological tests. Particularly useful are the Biolog MT plates which allow design of custom carbohydrate utilization tests. In most instances, members of this group are readily identified by the Microlog IT software (Biolog, Inc.).

For details see Appendix C.

7. CULTURE PRESERVATION

Standard methods employed by the American Type culture collection are applicable to this genus.

8. LITERATURE CITED

1. Benhamou, N., 1. W. Kloepper, A. Quadt-Hallman, and S. Tuzun. 1996. Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. Plant Physiol. 112:919-929.

2. Benhamou, N., 1. W. Kloepper, and S. Tuzun. 1998. Induction of resistance against Fusarium wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. Planta 204: 153-168.

3. Bochow, H. and K. Gantcheva. 1995. Soil introductions of *Bacillus subtilis* as biocontrol agent and its population and activity dynamic. Acta Hortic. 382: 194-172.

4. Broadbent, P., K. F. Baker, N. Franks, and 1. Holland. 1977. Effect of *Bacillus* spp. on increased growth of seedlings in steamed and in nontreated soil. Phytopathology 67: 1027-1034.

5. Caruso, F. L., M. G. Zuck, and A. E. Bessette. 1984. Bacterial seedling blight of tomato caused by *Bacillus polymyxa.* Plant Dis. 68:617-620.

6. Cowan, S. T. 1974. Cowan and Steel's Manual for the Identification of Medical Bacteria. Second ed., S. T. Cowan, ed. Cambridge University Press, Great Britain.

7. Drobniewski, F. A. 1994. The safety of *Bacillus* species as insect vector control agents. 1. Appl. Bacteriol. 76:101-109.

8. Edwards, P. R. and D. W. Bruner. 1942. Serological identification of *Salmonella* cultures. Circ. Ky. Exp. Sta.

9. Hall, T. J. 1986. Effects of xylem-colonizing *Bacillus* spp. on *Verticillium* wilt in maples. Plant Dis. 70:521-524.

10. Hall, T. J. and W. E. Davis. 1990. Survival of *Bacillus subtilis* in silver and sugar maple seedlings over a two-year period. Plant Dis. 74:608-609.

11. Hollis, 1. P. 1951. Bacteria in healthy potato tissue. Phytopathology 41:350-366.

12. Hosford, R. M. 1982. White blotch incited in wheat by *Bacillus megaterium* pv. *cerealis.* Phytopathology 72: 1453-1459.

13. Kim, D. S., R. J. Cook, and D. M. Weller. 1997. *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology 87:551-558.

14. Leary, J. V. and W. Chun. 1985. Pathogenicity of *Bacillus circulans* to date palm *(Phoenix dactylifera)* seedlings. Phytopathology 75: 1289 (Abstr.).

15. Leary, 1. V. and W. W. C. Chun. 1989. Pathogenicity of *Bacillus circulans* to seedlings of date palm *(Phoenix dactylifera).* Plant Dis. 73:353-354.

16. Liu, Z. L. and 1. B. Sinclair. 1992. Population dynamics of *Bacillus megaterium* strain B153-2-2 in the rhizosphere of soybean. Phytopathology 82:1297-1301.

17. Lutman, B. F. and H. E. Wheeler. 1948. *Bacillus megaterium* DeBary from the interior of healthy potato tissue. 1. Wash. Acad. Sci. (D.C.) 38:336-340.

18. Mundt, 1. O. and N. F. Hinkle. 1976. Bacteria within ovules and seeds. Appl. Environ. Microbiol. 32:694-698.

19. Osburn, R. M., 1. L. Milner, E. S. Oplinger, R. S. Smith, and 1. Handelsman. 1995. Effect of *Bacillus cereus* UW85 on the yield of soybean at two field sites in Wisconsin. Plant Dis. 79:551-556.

20. Priest, F. G., M. Aquino-de-Muro, and D. A Kaji. 1994. Systematics of insect pathogenic bacilli: uses in strain identification and isolation of novel pathogens. FEMSSymp. 75:275-295.

21. Pulsey, P. L., M. W. Hotchkiss, and H. T. Dulmage. 1988. Pilot tests for commercial production and application of *Bacillus subtilis* (B-3) for postharvest control of peach brown rot. Plant Dis. 72:622-626.

22. Pusey, P., L., Lawrence, and C. L Wilson. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis.* Plant Dis. 68:753-756.

23. Pusey, P. L., C. L. Wilson, and M. W. Hotchkiss. 1986. Compatibility of *Bacillus subtilis* for postharvest control of peach brown rot with commercial fruit waxes, dichloran, and cold-storage conditions. Plant Dis. 70:587-590.

24. Simmons, J. S. 1926. A culture medium for differentiating organisms of typhoid-colon aeorgenes groups and for isolation of certain fungi. J. Inf Dis. 39:209-214.

25. Smith, K. P., J. Handelsman, and R. M. Goodman. 1997. Modeling dose-response relationships in biological control: partitioning host responses to the pathogen and biocontrol agent. Phytopathology 87:720-729.

26. Smith, K. P., M. J. Havey, and 1. Handelsman. 1993. Suppression of cottony leak of cucumber with *Bacillus cereus* strain UW85. Plant Dis. 77:347-342.

27. Suslow, T. V., M. N. Schroth, and M. Isaka, 1982. Application ofa rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology 72:917-918.

28. Thompson, D. C., B. B. Clarke, and D. Y. Kobayashi. 1996. Evaluation of bacterial antagonists for reduction of summer patch symptoms in Kentucky bluegrass. Plant Dis. 80:856-862.

29. Turner, 1. T. and P. A. Backman. 1991. Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis,* Plant Dis. 75:347-353.

30. Wei, G., 1. W. Kloepper, and S. Tuzun. 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. Phytopathology 86:221-224.

31. Willis, 1. W., E. Allingham, and 1. V. Leary. 1982. Identification ofa unique bacterium isolated from date palm tissue culture. Phytopathology 72:990 (Abstr.).

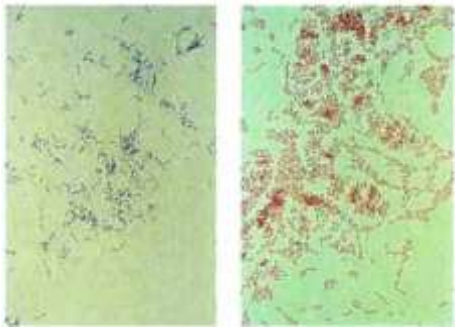
32. Wolf, 1. and A. N. Barker. 1968. The genus *Bacillus:* Aids to the identification ofits species. Pages 93-109 in: B. M. Gibbs and D. A. Shapton, eds., Identification Methods for Microbiologists, Part B. Academic Press, New York.

9. CHEMICAL LIST

Unless stated otherwise, all chemicals in this list were obtained from Sigma Chemical Co., P.O. Box 14508, S1, Louis, MO 63178

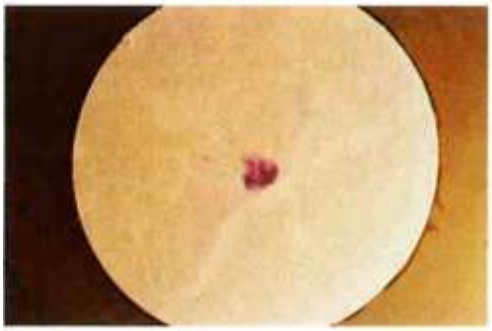
Acetone	Fisher Scientific
Agar, Bacto	Difco Laboratories
Ammonium phosphate, dibasic $NH_4H_2P0_4$	
Arabinose	
Beef extract	Difco
Bromthymol blue	
Casein acid hydrolysate	
Cycloheximide	
Gelatin	Difco
Glucose (dextrose)	Fisher Scientific
Glutaraldehyde	
Formvar	
Iodine	Mallinckrodt
Lead acetate	
Magnesium sulfate	

Malachite green	
Mannitol	Fisher Scientific
Nutrient broth	Difco
Osmium tetroxide	Ted Pella, Inc.
Peptone (Baeto)	Difeo
Potassium hydroxide	Mallinckrodt
Potassium iodide	
Potassium phosphate, dibasic	
Potato starch	Fisher Scientific
Sodium chloride	
Sodium citrate	
Safranin	
Yeast extraet	Difeo



Positive Negative

Fig. 1. Gram stain



Positive

Fig. 2. Oxidase test

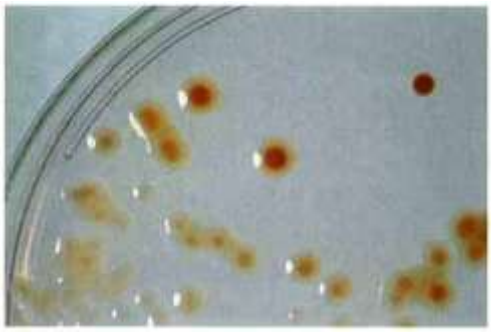


Fig. 3. Range of colony types of *Agrobacterium tumefaciens* on Brisbane and Kerr medium 1 A

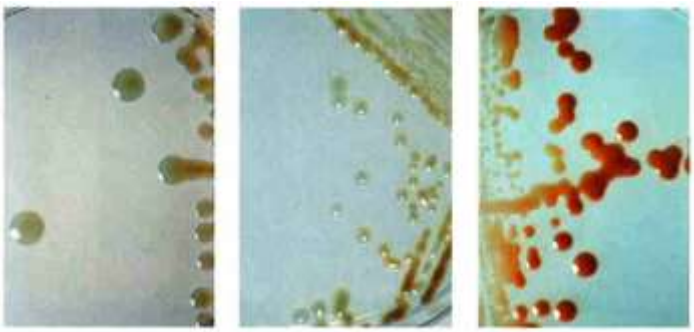


Fig. 4. Range of colony types of *Agrobacterium rhizogenes* on Brisbane and Kerr medium 2E

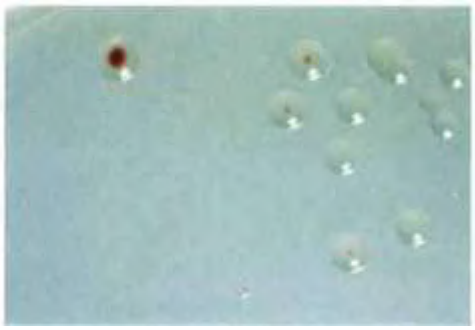


Fig. 5. Colonies of *Agrobacterium vitls* on Roy and Sasser medium

Positive Negative

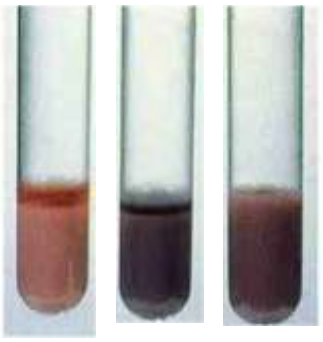
Fig. 6. 3-Ketolactose test

Negative Positive Negative

Fig. 7. Ferric ammonium citrate

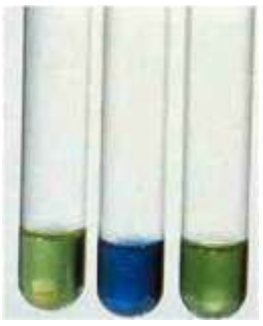
Positive Negative

Fig. 8. Citrate utilization



Acid Alkaline Uninoculated

Fig. 9. Action on litmus milk

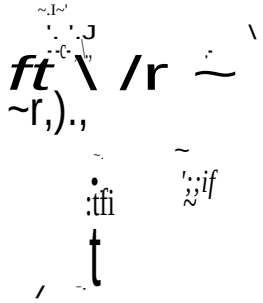


Negative Positive Negative

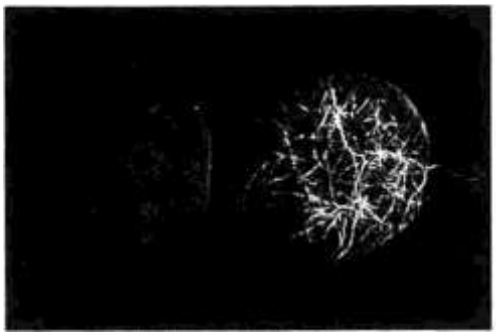
Fig. 10. Alkali from malonic acid

Photo credits: Fig. 1, S. Poe. Figs. 2-10, L. M. Moore.

4)



A.
Tomato



B. Carrot root disk

Fig. 1. Pathogenicity tests for *Agrobacterium tumefaciens* (A) and A. *rhizogenes* (B)

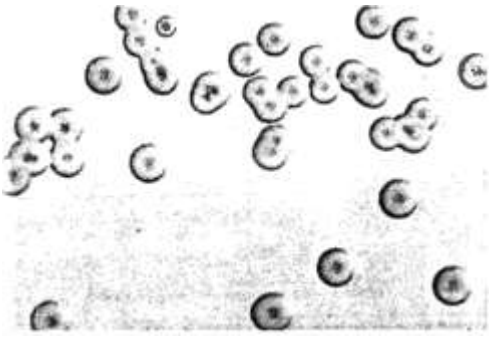


Fig. 2. Colonies of *Erwinia amylovora* on CCT medium

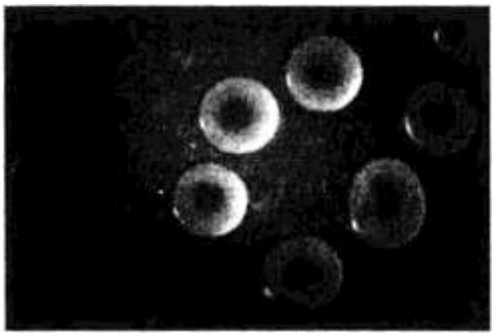


Fig. 3. Colonies of *Erwinia amylovora* on MS medium

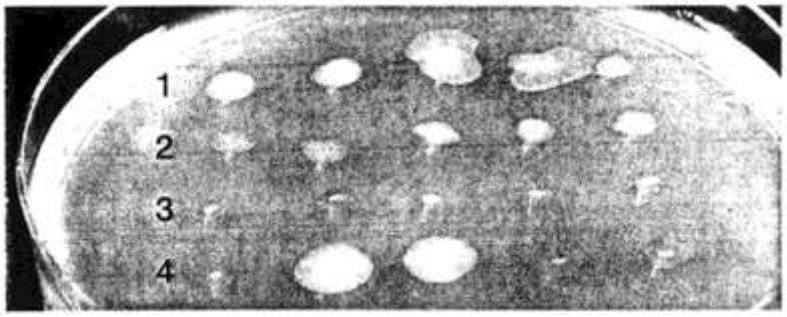
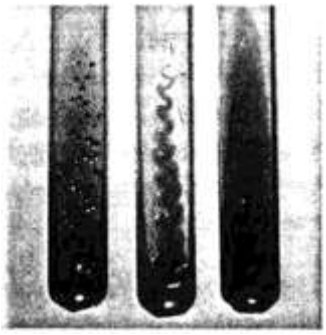


Fig. 4. Colonies of *Erwinia amylovora* (rows 1 and 2), *Pantoea herbicola* (row 3, two colonies on the left), *E. carotovora* subsp. *atroseptica* (row 3, three colonies on the right), *Agrobacterium tumefaciens* (row 4, three colonies on the left), and *Pseudomonas syringae* pv. *syringae* (row 4, two colonies on the right) on MM2Cu medium after 4 days at 28 C

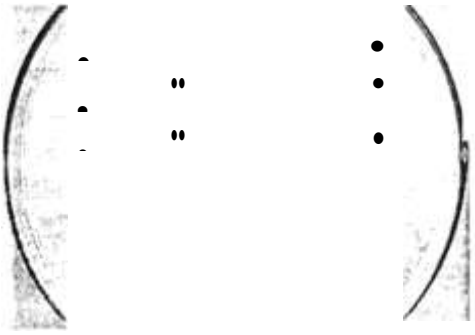
Pseudomonas spp. (top) *Erwinia camtovora* (bottom)

Fig. 5. Colonies of soft rotting bacteria on CVP medium



Negative Positive Uninoculated

Fig. 6. Acid production from carbohydrates



Positive (lop) Negative (bottom)

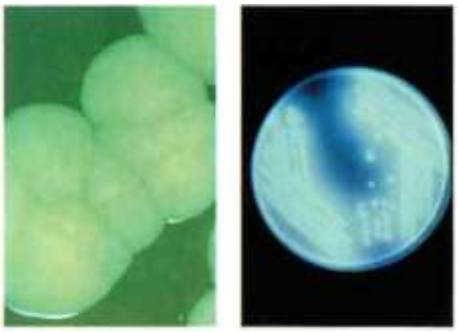
Fig. 7. Utilization of keto-methyl glucoside

Photo credits: Fig. 1, L. M. Moore. Fig. 2, C. A. Ishimaru. Fig. 3, M. N. Schroth. Fig. 4, K. Geider. Figs. 5 and 7, A. Kelman. Fig. 6, D. W. Dye.

PLATE 2

Erwinia carotovora subsp. *atroseptica* | *Pseudomonas marginalls*

Fig. 1. Potato soft rot



Visible light | UV light

Fig. 2. Colonies of *Pseudomonas syringae* on King et al.'s medium B

Negallve | Positive

Fig. 3. Arginine dihydrolase test

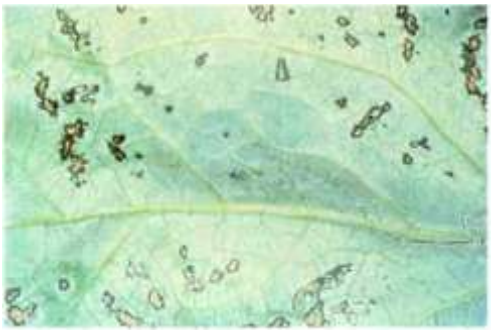
Fig. 4. Tobacco hypersensitive test

Distortion reaction

Fig. 5. Kidney bean inoculated with *Pseudomonas syringae* pv. *pisi*

Angular lesions

Fig. 6. Kidney bean inoculated with *Pseudomonas syringae* pv. *tabaci*



Water-soaked lesions

Fig. 7. Lima bean inoculated with *Pseudomonas syringae* pv. *phaseolicola*

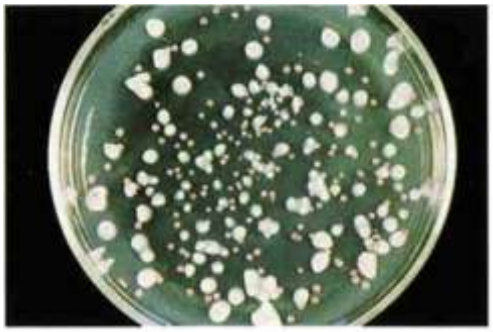
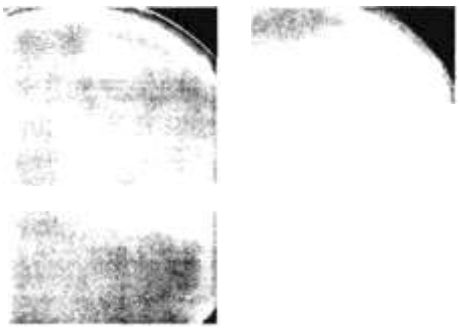


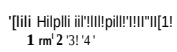
Fig. 8. Colonies of virulent strains (white colonies) and avirulent strains (red colonies) of *Ralstonia solanacearum* on TIC medium

Photo credits: Fig. 1, A. Kelman. Fig. 2, N. W. Schaad and D. C. Sands. Fig. 3, L. M. Moore. Figs. 4-7, D. C. Sands. Fig. 8, A. Kelman.

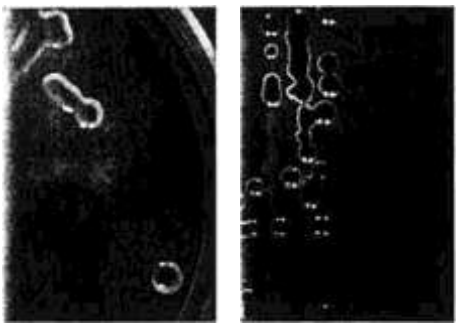


pv. *campesrris* pv. *cirri* type A

Fig. 1. Colonies of *Xanthomonas campestris* on YDC agar

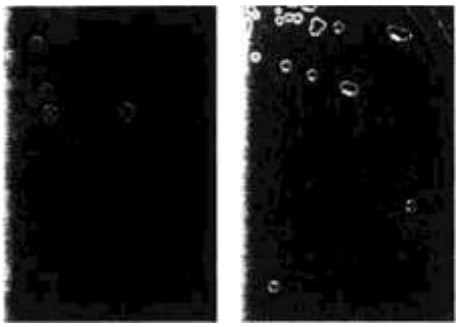


Scale for Fig. 1



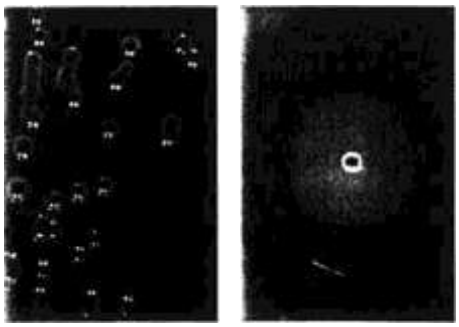
SX SM

pv. *cempesttis*



SX S M

pv. *begoniae*

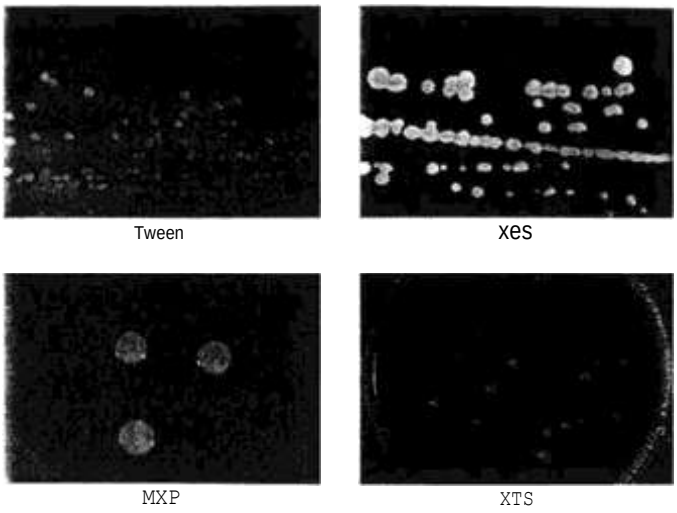


S X SX

pv. *glyciniae* pv. *dieffenbachiae*

Fig. 3. Colonies of *Xanthomonas campestris* on SX and SM agars

Scale for Fig. 3 (cm)



Tween xes

MXP XTS

Fig. 2. Colonies of *Xanthomonas campestris* pvs. *vesicatoria, carotae,* and *vignicola* and *X. translucens* on Tween, XCS, MXP, and XTS, respectively

Scale for Fig. 2 (cm)

Fig. 4. Colonies of *Xanthomonas campestris* pv. *dieffenbachiae* on ET medium (also useful for esculin test)

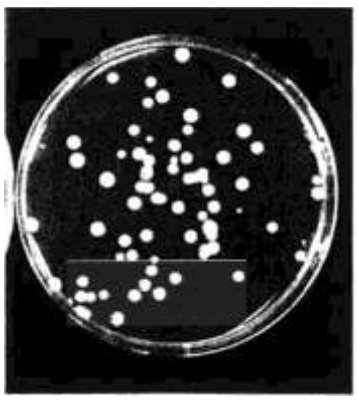


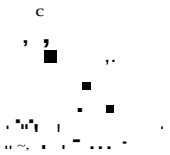
Fig. 5. Colonies of *Xanthomonas campestris* pv. *manihotis* on CTA medium

Photo credits: Fig. 1, N. W. Schaad. Fig. 2, J. Olsen and N. W. Schaad. Fig. 3, J. Olsen. Fig. 4, A. M. Alvarez. Fig. 5, A. Fessehaie.

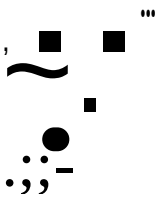
Fig. 1. Lettuce with symptoms of corky root

Fig. 2. Colonies of *Rhizomonas suberifaciens* on S-medium

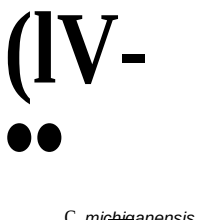
C. *flaccumfaciens* pv. *flaccumfaciens*



R. fascians



C. *mlchiganensis* subsp. *Insidiosus*



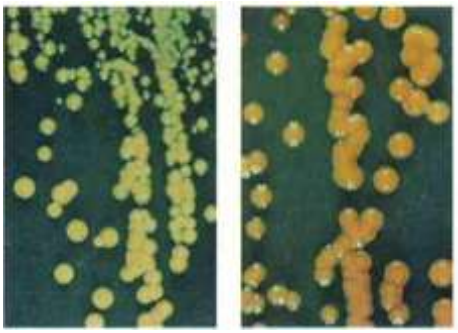
C. *michiganensis* subsp. *michiganensis*

C. *michiganensis* subsp. *nebraskensis*

C. *michiganensis* subsp. *sepedon~us*

C. *michiganensis* subsp. *tessellarius*

Fig. 3. Colonies of *Curtobacterium flaccumfaciens, Rhodococcus fascians,* and several *Clavibacter michiganensis* subspecies on NBY medium



subsp. *nebraskensis* subsp. *tessellarius*

Fig. 4. Colonies of *Clavibacter michiganensis* subspecies on CNS medium

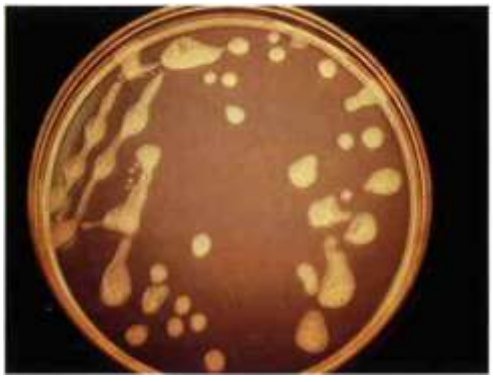


Fig. 5. Colonies of *Clavibacter michiganensis* subsp. *michiganensis* growing on SCM (top) and m SCM (bottom)

Photo credits: Figs. 1 and 2, L. E. Datnoff. Figs. 3 and 4, R. R. Carlson. Fig. 5, M. Fatmi (top) and H. A. Bolkan and C. M. Waters (bottom).

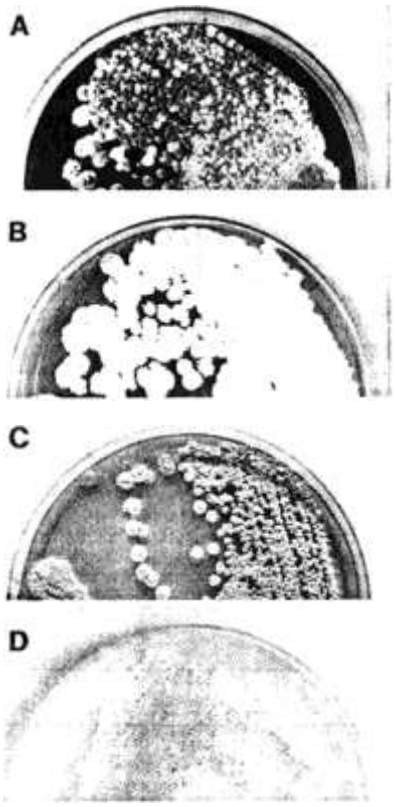


Fig. 1. Sporulating colonies of (A) *Streptomyces scabies.* (B) S. *acidiscabies.* and (C) S. *turgidiscabies* on YME (ISP2) medium and (0) S. *ipomoeae* on SGM

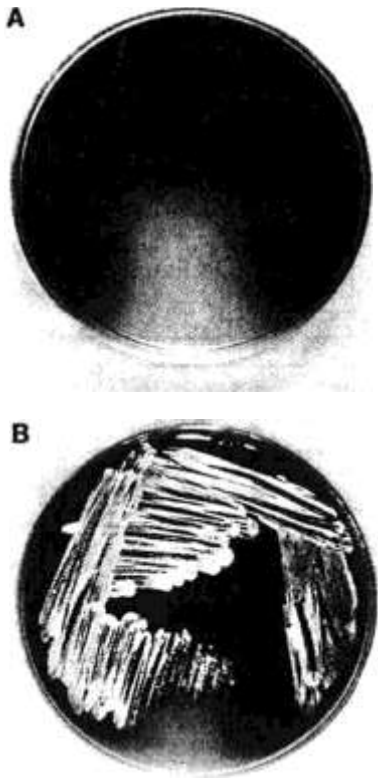


Fig. 2. Pigment production by pathogenic *Streptomyces* spp.: (A) S. *scabies* produces melanin on peptone yeast extract iron medium; (B) S. *acidiscabies* produces a pH-sensitive red-yellow pigment on modified salts starch agar

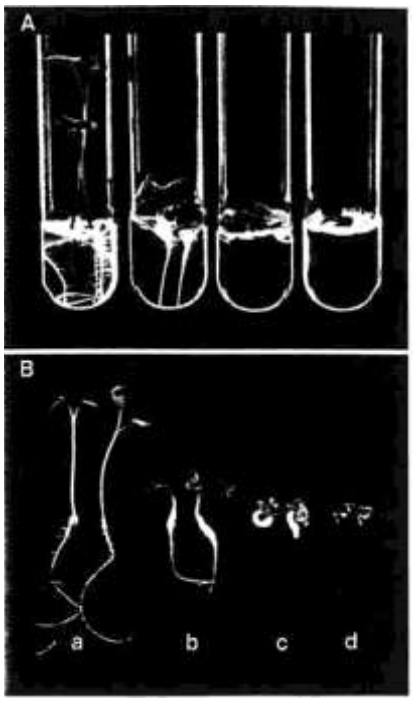


Fig. 3. Radish seedlings (A) in culture tubes and (B) removed from tubes, 7 days after treatment: seedlings were (a) untreated or treated with (b) 75 nM purified thaxtomin A, (e) filter-sterilized *Streptomyces scabies* culture supernatant (containing thaxtomin A but not the pathogen), causing stunting and radial swelling, and (d) whole S. *scabies* culture, causing necrosis and seedling collapse

Fig. 4. Endospores of *Bacillus* spp.

Fig. 6. Growth of *Clostridium puniceum* on potato slice

Fig. 5. Growth of *Clostridium puniceum* on potato infusion agar

Photo ~redits: Figs. 1-3, R. Loria. Fig. 4. J. Leary. Figs. 5 and 6, A. Kelman.

D. *Clostridium*

Barbara M. Lund and Arthur Kelman

1. INTRODUCTION

Anaerobic, spore-forming, rod-shaped bacteria in the genus *Clostridium* can be isolated frequently from decaying plant material. Although they usually stain Gram-positive (at least in the early stages of growth), some strains invariably appear to be Gram-negative (see p. 218, coryneform bacteria). Clostridia can be distinguished from aerobic or facultatively anaerobic *Bacillus* spp. because clostridia grow much better in anaerobic than in aerobic conditions. Anaerobic techniques are required for effective isolation and culture of clostridia (8, 12,35, 38). Strains differ in their sensitivity to oxygen, and if other conditions are optimal some strains are able to grow in the presence of2-4% oxygen (11, 17,24). Clostridia are not able to reduce sulfate to sulfide; anaerobic spore-forming bacteria that are capable of this reduction are classified as *Desulfotomaculum* spp. (11).

The clostridia implicated in the decay of plant tissue are strains capable of actively degrading pectic substances. Under the environmental conditions that usually lead to decay of plant material, aerobic and facultatively anaerobic bacteria, such as *Erwinia* spp., initially create an oxygen-depleted environment that favors growth of clostridia (15). In general, growth of species of *Clostridium*, like that of *Erwinia* spp., does not occur below a pH of approximately 3.8. Therefore, clostridia are not likely to cause decay of those fruits that have a low pH, unless their growth is facilitated by a rise in pH resulting from activities of other microorganisms.

Bacterial soft rot of potatoes is considered to be due mainly to strains of *Erwinia carotovora* and closely related species. In general, this decay occurs when tubers are depleted of oxygen, usually because the tuber surface remains wet. The potential importance of clostridia in decay of potatoes was demonstrated initially by research studies in England (14, 15, 16, 18, 21, 22,30). When potatoes from a particular source were induced to rot by incubation in an atmosphere depleted of oxygen without inoculating with *E. carotovora*, pectolytic clostridia were the main soft-rot bacteria isolated from the decayed tissue (21, 22). In the case of potatoes from a different source that were induced to rot by incubation in a mist chamber, both pectolytic clostridia and *E. carotovora* were isolated (20). Pectolytic clostridia were also isolated together with *E. carotovora* by Perombelon et al. (25) from potatoes induced to rot in the laboratory and by Campos et al. (3) from potatoes that decayed in a commercial storage facility. When pectolytic clostridia were inoculated into potatoes that were then incubated in conditions resulting in anaerobiosis, typical symptoms of soft rot were obtained and clostridia could be re-isolated from these decayed tubers (15, 25).

Several different types of pectolytic clostridia have been isolated from decaying potatoes

and from soil (2, 3, 16,26,27). Isolations have often been made at temperatures of25 - 30°C and many of the clostridia obtained from these and other situations grow optimally at temperatures between 30°C and 37°C. However, strains isolated by Brocklehurst and Lund (2) apparently were able to grow and cause rots in potato at 5 ° C. The optimum temperature for growth of some strains was lower than 22 ° C; some of the pectolytic clostridia isolated from soil and plant roots by Perry (27) grew well at 10°C.

The environmental conditions that favor rotting of potato tubers by *E. carotovora* also favor soft rot by pectolytic clostridia. It is likely that decay of other root crops, such as carrots, could involve infection by these bacteria (29). In view of (a) the widespread distribution in soil of species of *Clostridium* including pectolytic strains (27), (b) the frequent presence of pectolytic *Clostridium* spp. in the rhizosphere of plants (26), (c) the ability ofpectolytic strains to macerate plant tissue rapidly, and (d) the fact that isolations from decayed tissues are usually only made on media incubated in air, it is probable that the relative prevalence of clostridia in the decay of vegetable crops in field conditions, storage, and transit, has been overlooked generally, and their importance underestimated greatly.

Clostridia have been reported as possible causal agents in cavity spot of carrots (26, 27, 28), but there is evidence now that clostridia playa secondary role in lesions induced primarily by *Pythium violae* (9). Clostridia also have been associated with wetwood (32) and discoloration of heartwood of Tepa wood *(Lanreliopsis philippiana)* (23) and other symptoms such as discolored tissue in living trees (32, 33, 34, 36). A bacterium isolated from wetwood disease of *Populus* spp. and identified as *Clostridium butyricum* produced pectate lyase and pectinesterase in culture. These enzymes were also present in tissues affected by the wetwood disease (33). The pectin-rich tori in membranes of bordered pits were degraded, probably by means of the pectate lyase of the *Clostridium* strain involved. A disease affecting hornbeam has also been attributed to a strain of C. *butyricum (10).*

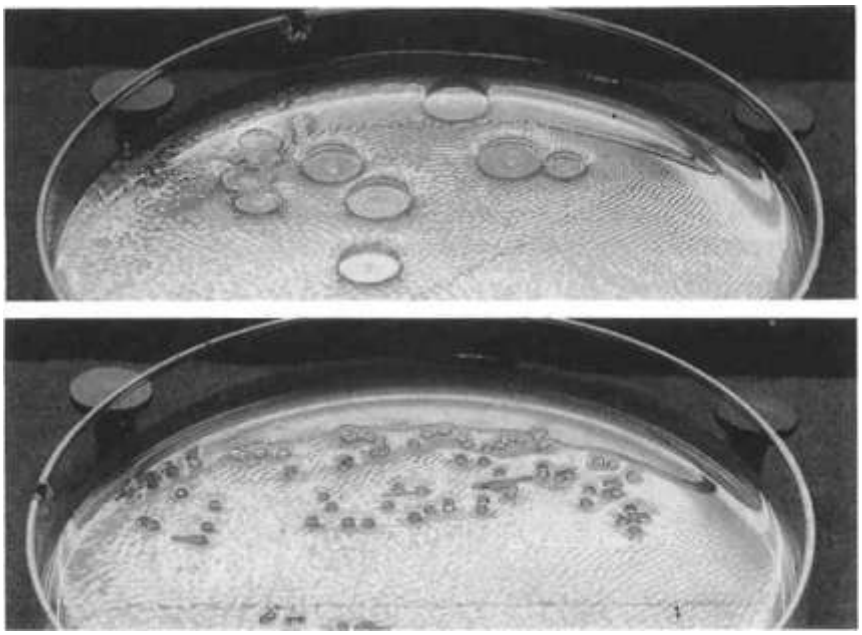
The majority of species of Clostridium occur in soil and are, therefore, potentially associated with plants; many of the strains isolated have not been identified. The main clostridia that may be associated with plants are summarized in Table 1.

2. ISOLA TION TECHNIQUES USING DIFFERENTIAL AND SEMISELECTIVE MEDIA

The isolation of pectolytic clostridia from decaying potatoes and carrots, and field and rhizosphere soils, is facilitated by plating onto PI medium, a double-layer pectate medium containing polymyxin (14) incubated in anaerobic jars under $H/C0_2$ (90: 10 by volume), or $N/H/C0_2$ (80: 10: 10 by volume). Pectolytic clostridia form colonies in pits (craters) on PI medium; some strains produce large pits (Fig. lA) within 2 days at 25 ° C and, in order to isolate the pectolytic clostridium, colonies must be transferred by streaking to a medium solidified with agar before liquefaction of the medium occurs. In contrast, other strains produce small pits (Fig. IB) without rapid liquefaction of the peetate layer. The polymyxin inhibits soft-rot erwinias,

which otherwise would produce small colonies in pits under these conditions. Eleven strains of pectolytic clostridia, previously isolated on an antibiotic-free medium, were shown not to be inhibited on the polymyxin-containing medium (14). The possibility that a minority of clostridia might be inhibited and that some pectin-degrading strains may not produce pits on this medium should be recognized, but there appear to be no reports of such strains in the literature.

a



b

Fig. 1. Colonies ofpectolytic *Clostridium* spp. on PI medium after incubation at 25 DC for 2 days under $Hz/C0_2$ (90: 10 by volume). Some strains typically form the large pits shown in (a), others form smaller pits, shown in (b).

Some pectolytic *Bacillus* spp., e.g. *B. polymyxa,* produce small colonies in pits when grown in anaerobic conditions on PI medium; it is important, therefore, to confirm that a suspected *Clostridium* grows in anaerobic, but not in aerobic conditions. When isolating from decayed tissue, sampling from the leading edge of the rot facilitates selection of bacteria, in particular clostridia, that are the primary cause of the rot. It is important in isolation of pectolytic clostridia from decayed tissue to determine whether other pectolytic bacteria (aerobes or facultative anaerobes) that may be present, have the ability to cause decay. The appropriate procedures for detection of these bacteria, in particular the soft rot erwinias, are outlined in other chapters of this manual and research papers (6, 20, 25).

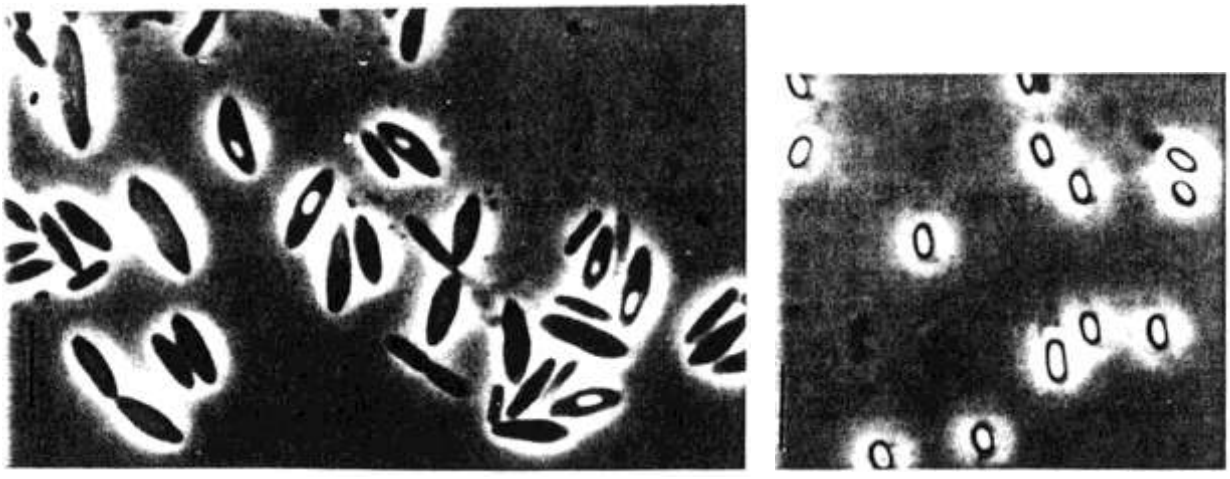
Pectolytic clostridia can be isolated from decayed potatoes by streaking a suspension of the decayed tissues onto NGA plates that are then exposed to chloroform for 10 minutes to eliminate vegetative bacteria (1). Many clostridia may be present in rots as vegetative bacteria rather than as spores. However, a wet mount can be examined under a phase-contrast microscope to detect the presence of bacterial spores. The NGA plates are incubated in anaerobic jars under an atmosphere containing either $Hz/C0_2$ (90: 10 by volume), or $NiH/C0_2$ (80: 10: 10 by volume),

for about 4 days at 28 ° C. Pectolytic clostridia have been isolated from potatoes following preparation of a water suspension of partially decayed tuber tissue and incubation of the suspension for one week at 28°C (3). Development of anaerobic conditions in the suspension probably allowed survival and spore formation by the clostridia.

If clostridia are isolated as colonies that form pits on a pectate medium (Fig. 1), strains should be serially plated several times on a standard optimal medium that does not liquefy, in order to ensure that a pure culture is obtained. A useful medium for this purpose is Potato Infusion Agar, which facilitates demonstration of pigment formation by certain strains.

Media suitable for maintenance of pectolytic clostridia are Potato Infusion medium, or Chopped 1J~eat Medium (12) with the addition of glucose, 2 gil. In order to maintain viable cultures, it may be necessary to subculture at monthly intervals.

Strains can be identified presumptively as peetolytic clostridia on the basis of (a) the ability to grow on media incubated in anaerobic conditions, but not on media exposed to the air, (b) formation of distinct pits or craters on Lund's pectate medium, and (c) formation of endospores (Fig.2b). Cells with endospores can be seen in wet mounts examined under the phase-contrast microscope. Endospores can be demonstrated more definitively using the malachite green stain of Schaeffer and Fulton (31).



a b

Fig. 2. Phase contrast photomicrographs of C. *puniceum* grown on Potato Infusion agar at 25 ° C for 2-4 days. (a) vegetative cells and sporulating cells. (b) free spores. Bar markers represent 5 *,urn.*

3. DIFFERENTIATION OF COMMONLY ISOLATED SPECIES

Potatoes affected by clostridial soft rot that has occurred in anaerobic conditions typically show a soft white or creamy rot. Blackening at the edge of the rot, due to oxidation of polyphenols, is likely to occur if the potato becomes aerobic, e.g. following drying after a rot has become established. Tissue attacked by clostridia is often transformed into a soft, viscous, slimy rot (Fig. J), but this is not always the case and some rots caused by clostridia have a soft, cheesy consistency. Many pectolytic clostridia are butyric acid-formers and would be expected, therefore, to cause malodorous rots, but much of the unpleasant odor associated with anaerobic rots may be due also to the presence of secondary bacteria.

Fig. 3. Potato tuber inoculated with a pectolytic strain of *Clostridium;* after incubation the potato was cut into halves that have been separated to show strands of slime characteristic of some clostridia during the decay of potato tubers.

The identification of a bacterium as a *Clostridium* sp. is based. in general. on (a) the requirement for anaerobic conditions. (b) formation of endospores (4, 3) and (c) inability to carry out a dissimilatory reduction of sulphate (11, 13). To identify a strain as a particular species is more difficult and this is due in part to the unsatisfactory state of characterization and taxonomy in this genus. Because of the need to maintain cultures under anaerobic conditions during characterization tests, procedures are necessary that require specialized techniques and equipment. A full description ofthese methods is beyond the scope ofthis manual and detailed information is provided in several publications (8, 11, 12,35, 38). The most generally accepted key is that presented by Cato et ai. (4); and the methods used are detailed in the Anaerobe Laboratory Manual of Holdeman et ai. (12). The non-medical clostridia are also described by Hippe et ai. (11). The results of identification tests naturally depend on the techniques used. In order to obtain valid results it is important to use the methods described by the originators of the test, or by the authors who have used these tests to devise an identification key, IDd to show that reference strains give typical results with the methods used.

A primary characteristic used to subdivide the clostridia into groups is the ability to degrade gelatin (4, 35). Other key tests that are used in identification include:

Determination of certain enzyme activities including tests for lecithinase, lipase. proteolysis, indole formation, effect on milk and nitrate reduction.
Determination of the ability to grow and form acid using specific carbohydrates as substrates.

J. Determination of the major fermentation products.

~. Position of the mature spore within the cell (terminal or subterminal).

Other properties that are useful in characterization of pectoiytic clostridia are the .emperature range for growth and the mol % G.or C content of the DNA.. In a study to determine the value of fatty acid formation in identification of strains of bacteria associated with postharvest diseases (37), the cellular fatty acid compositions of J strains of *Clostridium* isolated from a decay of tomato fruit were compared with fatty acids of 181 other strains of bacteria. These included strains from the following genera: *Bacillus, Cytophaga, Erwinia, Pseudomonas,* and *Xanthomonas.* One strain of *Clostridium histoiyticum* and five strains of C. *perfringens* were also included; these were air-tolerant strains that were not designated as capable of causing decay. A total of 78 different fatty acids were identified by gas liquid chromatography; mean percentages were analyzed statistically for each fatty acid and chemical class. The highest mean percentage
(63 9%) of saturated, straight-chain, even carbon fatty acids (Class A) characterized the strains of *Ciostrtdium.* This was significantly different from the percentage noted for all other genera with the exception of the *Erwinia* (40.9%). An expert system based on the different classes of fatty acids was developed which would make it possible to differentiate the ciostridia included in the data base from the strains in the other genera.

The establishment of a strain as a new species of *Clostridium* requires detailed study. The description of a group of pigmented, pectolytic strains as a new species, C. *puniceum* (19), was a

practical measure designed to highlight strains that were apparently quite distinct from other pigmented, pectolytic strains described in the literature, in particular from C. *felsineum* and C. *aurantibutyricum.* The relationship ofC. *puniceum* to the other species of *Clostridium,* based on 16S rRNA gene sequences, is shown in a study of the phylogeny of the genus *Clostridium* by Collins et al. (5).

The pectolytic clostridia isolated from rots of potatoes appear to be of three main types (16, 26):

Type 1. These bacteria form large pits on Lund's pectate medium incubated at 25°C (15) (Fig. la), form sub-terminal, oval spores and fail to produce pigment on Potato Infusion Agar. Some of these bacteria have characteristics similar to those of C. *butyricum.*

Type 2. Bacteria in this group are morphologically similar to type 1, but produce pink pigment on Potato Infusion Agar (plate 6, Fig. 5) or potato slices (plate 6, Fig. 6). These strains show other biochemical differences from C. *butyricum* and have been described as C. *puniceum (19).*

Type 3. This group of clostridia grow more slowly on pectate medium at 25 ° C and form smaller pits than types 1 & 2 (Fig. lb). Spores tend to be spherical and appear to be terminal or very near the end of the bacterium. Morphologically similar bacteria have been seen frequently in soft rots of potatoes (14,26), but they have not been characterized fully.

A pectolytic clostridium isolated from wetwood was identified as C. *butyricum* (33) as was the clostridium that was associated with a disease of hom beam (10). A clostridium associated with discolored tissues in living oaks has been described as C. *quercicolum (36).*

Perry (27) characterized the properties of 56 strains of pectolytic clostridia isolated from field and rhizosphere soils and from cavity spot lesions on carrots. He concluded that five strains had the same properties as C. *puniceum,* but that the majority of strains could not be assigned to known species on the basis of the usual biochemical tests, and could only be described as belonging to the butyric acid-forming group of bacteria.

Pectolytic clostridia are clearly capable of degrading plant tissue and there is conclusive evidence that they can initiate rots in the absence of *Erwinia* spp. in potato tubers that are depleted of oxygen. Whether they are able to act as primary pathogens on other hosts remains to be demonstrated. However, it should be emphasized that in many cases clostridia present in diseased plant tissue are not secondary invaders.

4. -DIAGNOSTIC MEDIA AND TESTS

a. Double layer pectate medium for isolation of pectolytic clostridia (Medium PI + polymyxin [14]).

1) Medium for basal layer

	perL
Tryptone Lab-	10.0 g
lemco powder	2.4 g
Yeast extract	5.0 g
$CaCl_z \cdot 6H_zO$	5.2 g
Cysteine hydrochloride	0.4 g
Bacto agar	19.0 g*
Polymyxin B sulphate solution (24,000 units/ml)	10,0 ml**

* Davis agar may be used at 15.0 g per L.
** The solution of polymyxin B sulphate is prepared by the aseptic addition of sterile water to polymyxin B sulphate to give 24,000 units polymyxin B sulphate perml.

The components, except for the polymyxin solution, are dissolved and the pH of the solution is adjusted to 6.8. The medium is sterilized by autoclaving at 121°C for 15 min; the polymyxin solution is added after cooling.

2) Medium for upper layer

	perL
Sodium polypectate'	20,0 g
Ethanol (absolute)	60.0 ml
EDT A, di-sodium salt	1.0 g

• A pectate with a high molecular weight is required.

To prepare medium for the upper layer, the sodium polypectate is mixed with the ethanol and a solution of EDT A is added slowly with constant stirring. The pH of the solution is adjusted to 7.4. The medium is sterilized by autoclaving at 121°C for 15 min.

To prepare plates, after sterilization medium for the basal layer is cooled to 5560°C and 10 ml ofa sterile aqueous solution of polymyxin B sulphate (24,000 units/ml) are added to 1 liter of medium and mixed. Twenty ml quantities of the basal medium are distributed into Petri dishes, 9 em diameter. When the basal layer has solidified, it is overlaid with 8,5 ml of the sterilized pectate solution (upper layer).

After allowing at least 4 h for the medium to solidify, the plates are placed open in a laminar flow hood for 45-60 min to *dry.* Adequate drying of the medium is critical for successful counts to be made of pectolytic clostridia. Immediately after drying, the plates are placed in an atmosphere of *Hi* CO_2 (90: 10 by volume) in anaerobic jars with a room temperature catalyst (Oxoid Ltd); the plates are stored at room temperature for at least 16 h before use, to ensure that the medium is reduced and to allow diffusion of the constituents from the basal layer into the upper layer.

b. Potato Infusion Medium and Potato Infusion Agar

These media have the same formula apart from the concentrations of agar. In preparing the liquid medium, however, the potato tissue is retained as a mash in the medium, whereas in preparing the solid medium the infusion prepared from the potatoes is strained and the insoluble potato tissue is discarded.

1) Potato Infusion Medium

	perL
Potatoes (white)	200.0 g
Glucose	5.0 g
(NH4)2 S04	1.0 g
$CaC0_3$	3.0 g
Cysteine HC I	0.5 g
Davis agar	0.5 g

D Potato Infusion agar

Prepare as for Potato Infusion Medium but use 15 g agar per L.

To prepare Potato Infusion Medium the potatoes are peeled, cut into pieces and immersed in a portion of the water, with the cysteine HCI. The potato pieces are passed through a mincer, returned to the liquid, and the slurry is then mixed briefly in a blender « 30 sec). Sufficient water should then be added to give a pourable slurry.

The glucose, (NH4)2S04, $CaC0_3$ and agar are added to the remainder of the water which is then heated to dissolve the agar. In order to dispense aliquots of the medium, appropriate volumes of the potato slurry and of the solution of the remaining components are added to a small flask (e.g. 500 ml), so that the components can be mixed well during distribution, and 20 ml volumes are poured into narrow-necked 20 ml bottles or tubes with metal screw caps to give a layer of potato solids 25 mm deep. The medium is sterilized by autoclaving at 121°C for 15 min. Before use, bottles of medium are immersed in a boiling water bath for 20 min, with the tops loose, to expel dissolved oxygen, the tops are then tightened and the medium cooled. This

medium should then be inoculated, using a relatively large inoculum, and if the tops are tightly closed the bottles can be incubated in air.

To prepare Potato Infusion Agar the potatoes are cut into pieces and cooked in half the water. The liquid is then strained through muslin to remove pieces of potato and the remaining components (except the agar) are added to the infusion. The agar is added to the remainder of the water and dissolved by heating at 100°C. The two solutions are combined and sterilized by autoclaving at 121°C for 15 min. The medium is then cooled to 50-55°C, mixed well to suspend the $CaC0_3$, and 18-20 ml volumes are distributed into Petri dishes (9 em diameter).

After the medium has set the plates are dried by exposure to a stream of sterile air for 45 min. on a laminar air flow clean bench as described above. Immediately after drying, the plates are placed in an atmosphere of$H/C0_2$ (90: 10 by volume), in anaerobic jars with a room temperature catalyst and stored at room temperature for at least 16 h before use.

5. INOCULATION TESTS FOR MACERATION OF TISSUE

The ability of clostridia to macerate potato tissue can be demonstrated by introducing a suspension of the test strain into bottles of sterile solution of cysteine-HCI (0.5 gil in phosphate buffer) containing an aseptically-prepared cylinder of raw potato tissue (18). After incubation at 25^0 C for up to 5 days, many strains cause maceration of the cylinder of tissue, whereas control, non-inoculated cylinders of tissue remain firm and intact. A control should also be set up using a culture of a clostridium known to degrade potato tissue.

In order to determine whether a strain can induce typical soft rot of potato tubers, a suspension of the *Clostridium* sp. can be injected into surface-sterilized tubers to a depth of 1-2 em with a needle and hypodermic syringe, after which the potatoes are wrapped in moist paper towels (6), wrapped tightly with a film of Saran plastic wrap to prevent drying and maintain anaerobiosis, and incubated at an appropriate temperature (6). Under these conditions pectolytic clostridia will produce rots at the points of inoculation. Samples of tissue from the leading edge of rots should be plated onto layered pectate plates, incubated anaerobically, and also onto suitable pectate media incubated aerobically, in order to determine whether other pectolytic bacteria, in addition to the clostridia, are involved in the decay.

6. LITERATURE CITED

1. Bauernfeind, A. and 1. R Burrows. 1978. Suggested procedure allowing use of plastic Petri dishes in bacteriocin typing. Appl. Env. Microbiol. 35:970.

2. Brocklehurst, T. F. and B. M. Lund. 1982. Isolation and properties ofpsychrotrophic and psychrophilic, pectolytic strains of *Clostridium.* 1. Appl. Bacteriol. 53:335-361.

3. Campos, E., E. A. Maher, and A. Kelman. 1982. Relationship of pee to lytic clostridia and

Erwinia carotovora strains to decay of potato tubers in storage. Plant Dis. 66:543-546.

4. Cato, E. P., W. L. George, and S. Finegold. 1986. Genus Clostridium, Prazmouski 1880. Pages 1141-1200 in: P. Sneath, H. A Mair, N. S., Sharpe, M. E. and 1. G. Holt, eds., Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams and Wilkins, Baltimore.

5. Collins, M. D., P. A. Lawson, A Willems, 1. 1. Cordoba, 1. Femandez-Garayzabal, P. Garcia, H. Hippe, and 1. A. E. Farrow. 1995. The phylogeny of the genus *Clostridium:* proposal of five new genera and eleven new species combinations. Int. 1. Syst. Bacteriol. 44:812-816.

6. De Boer, S. H. and A. Kelman. 1975. Evaluation of procedures for detection of pectolytic *Erwinia* spp. on potato tubers. Am. Potato 1. 52: 117-123.

7. Ercolani, G. L. 1997. Note: Occurrence and persistence of culturable clostridial spores on the leaves of horticultural plants. Lett. Appl. Microbiol. 82:137-140.

8. Gerhardt, P. (ed.). 1994. Manual of Methods for General and Molecular Bacteriology. Am. Soc. Microbiol., Washington, D.C.

9. Groom, M. R. and D. A. Perry. 1985. Induction of 'cavity-spot-like' lesions on roots of *Daucus carota* by *Pythium violae.* Trans. Br. Mycol. Soc. 84:755-757.

10. Gvozdjak, R. I., S. F. Khodos, V. V. and Lipshu. 1976. Biological properties of *Clostridium butyricum* varieties nova, an agent of hom beam disease. Mikrobiol. Z. (Kiev) 38:288-292 (in Russian).

11. Hippe, H., 1. R. Andreesen, and G. Gottschalk. 1992. The genus *Clostridium* - Nonmedical. Pages 1801-1866 in: A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer, eds., The Prokaryotes, Second Ed. Vol. II. Springer-Verlag, New York.

12. Holdeman, L. v., E. P. Cato, and W. E. C. Moore. 1977. Anaerobe Laboratory Manual. 4th ed. VPI Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

13. Holt, 1. G., N. R. Krieg, P. H. A Sneath, 1. T. Staley, and S. T. Williams. 1994. Genus *Clostridium.* Pages 560-562 in: Bergey's Manual of Determinative Bacteriology. 9th ed. Williams and Wilkins, Baltimore.

14. Lund, B. M. 1979. Isolation ofpectolytic clostridia from potatoes. 1. Appl. Bacteriol. 33:609-614.

15. Lund, B. M. 1979. Bacterial soft-rot of potatoes. Pages 14-49 in: Plant Pathogens. D. W. Lovelock and R. Davies, eds., Soc. Appl. Bacteriol. Technical Series 12. Academic Press, London.

16. Lund, B. M. 1982. Clostridia and plant disease: new pathogens? Pages 263-283 in: M. S. Mount, and G. H. Lacy, eds., Phytopathogenic prokaryotes. Vol. 1. pp. 263-283. Academic Press, New York.

17. Lund, B. M. 1986. Anaerobes in relation to food of plant origin. Pages 351-372 in: E. M. Barnes and G. C. Mead, eds., Anaerobic Bacteria in Habitats Other Than Man. Soc. Appl. Bacteriol. Symp. Ser. No. 13. Blackwell Sci. Pub!. Oxford, UK.

18. Lund, B. M. and T. F. Brocklehurst. 1978. Pectic enzymes of pigmented strains of *Clostridium.* J. Gen. Microbiol. 104:59-66.

19. Lund, B. M., T. F. Brockelhurst, and G. M. Wyatt. 1981. Characterization of strains of *Clostridium puniceum* sp. nov., a pink-pigmented pectolytic bacterium. J. Gen. Microbiol. 122: 17-26.

20. Lund, B. M. and A. Kelman. 1977. Determination of the potential for development of bacterial soft rot of potatoes. Am. Potato J. 54:211-225.

21. Lund, B. M. and J. C. Nicholls. 1970. Factors influencing the soft-rotting of potato tubers by bacteria. Potato Res. 13:210-214.

22. Lund, B. M. and G. M. Wyatt. 1972. The effect of oxygen and carbon dioxide concentrations on bacterial soft rot of potatoes. I. King Edward potatoes inoculated with *Erwinia carotovora* var. *atroseptica.* Potato Res. 15: 174-179.

23. McLemore, J. B., W. C. Bridges, Jr., A. W. C. Lee, and F. H. Taintor. 1999. Selected mechanical and physical properties of Chilean tepa wood affected by butterfly stain. Forests Products J. 49:59-64.

24. Morris, J. G. and R. W. O'Brien. 1971. Oxygen and clostridia: a review. Pages 1-37 in: A. N. Barker, G. W. Gould, and J. Wolf, eds., Spore Research, 1971. Academic Press, London.

25. Perombelon, M. C. M., J. Gullings-Handley, and A. Kelman. 1979. Population dynamics of *Erwinia carotovora* and pectolytic *Clostridium* sp. in relation to decay of potatoes. Phytopathology 69: 167-173.

26. Perry, D. A. 1982. Pectolytic *Clostridium* spp. in soils and rhizospheres of carrot and other arable crops in east Scotland. J. Appl. Bacteriol. 52:403-408.

27. Perry, D. A. 1985. Characteristics ofpectolytic clostridia isolated from soil and plants in Scotland. 1. Appl. Bacteriol. 58:293-302.

28. Perry, D. A. and 1. G. Harrison. 1977. Pectolytic anaerobic bacteria cause symptoms of cavity spot in carrots. Nature 269:509-510.

29. Perry, D. A. and 1. G. Harrison. 1979. Cavity spot of carrots. II. The effect of soil conditions and the role of pectolytic anaerobic bacteria. Ann. Appl. BioI. 93: 109-115.

30. Rudd-Jones, D. and W. 1. Dowson. 1950. On the bacteria responsible for soft-rot in stored potatoes and the reaction of the tuber to invasion by *Bacterium carotovora* (Jones) Lehmann and Neumann. Ann. AppI. BioI. 37:563-569.

31. Schaeffer, A. B. and M. Fulton. 1933. A simplified method of staining endospores. Science 77: 194.

32. Schink, B., 1. C. Ward, and 1. G. Zeikus. 1981. Microbiology of wet wood: role of anaerobic bacterial populations in living trees. J. Gen. Microbiol. 123:313-322.

33. Schink, B., 1. C. Ward, and 1. G. Zeikus. 1981. Microbiology of wet wood: importance of pectin degradation and *Clostridium* species in living trees. AppI. Env. Microbiol. 42: 526-532.

34. Shigo, A. L., J. Stankewich, and B. 1. Cosenza. 1971. *Clostridium* sp. associated with discolored tissue in living oaks. Phytopathology 61: 122-123.

35. Smith, L. D. S. and G. Hobbs. 1974. *Clostridium.* Pages 551-572 in: R. E. Buchanan, and N. E. Gibbons, eds., Bergeys Manual of Determinative Bacteriology. 8th ed. Williams and Wilkins, Baltimore.

36. Stankewich, 1., B. 1. Cosenza, and A. L. Shigo. 1971. *Clostridium quercicolum* sp.n., isolated from discolored tissues in living oak trees. Antonie Van Leeuwenhoek, 1. Microbiol. SeroI. 37:299-302.

37. Wells, 1. M., 1. E. Butterfield, and L. G. Revear. 1993. Idenzification of bacteria associated with postharvest diseases of fruits and vegetables by cellular fatty acid composition: an expert system for personal computers. Phytopathology 83:445-83

38. Willis, A. T. 1969. Techniques for the study of anaerobic spore-forming bacteria. Pages 79-115 in: 1. R. Norris and D. W. Ribbons, eds., Methods in Microbiology. Vol. 3B. London: Academic Press.

7. CHEMICAL LIST

Chemicals	Source
Unless stated otherwise, all chemicals in this list can be obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.	
Beef extract	
$CaCl_2$ (anhydrous)	
$CaCI_2 \cdot 2H_20$	
Cysteine hydrochloride	
Davis agar	Davis Gelatin New Zealand Ltd.
Esculin	
Ethanol	
Ethylenediaminotetraacetate (EDT A)	
Ferric ammonium citrate	
Gelatin	
K_2HP0_4	
KH_2P0_4	
Lab-lemco powder	Oxoid Ltd
$MgS0_4 \cdot 7H_20$	
NaCI	
$NaHC0_3$	
Peptone	
Polymyxin B sulfate	
Sodium hydroxide	
Sodium polypectate (high molecular weight)	M. Burger Associates, 25 Eton Ridge Rd. Madison, WI 53705
Tryptone	Oxoid, Ltd. or Difco
Yeast extract	Oxoid, Ltd. or Difco

IV. Fastidious Phloem-Limited Bacteria

Michael J. Davis

A. INTRODUCTION

Bacteria in this group are associated with plant diseases (Table 1) but have not been grown in axenic culture precluding the completion of Koch's postulates (10). They have often been referred to as rickettsia-like organisms (RLO), rickettsia-like bacteria (RLB), or bacteria-like organisms (40), but have not been taxonomically classified and their affiliation with other bacteria is uncertain with the exception of the bacteria associated with citrus greening disease (huanglongbing) (17, 18), yellow vine disease of cucurbits (1), and papaya bunchy top disease (12). Phylogenetic analyses based on gene sequences have indicated that citrus greening and papaya bunchy top bacteria are members of the K subdivision of the *Proteobacteria.* The citrus greening bacteria *(Candidatus Liberobacter asiaticum* and *L. africanum)* are related most closely to members of the *a-2* subgroup ofthe *Proteobacteria,* but they are distinct from this subgroup (18). The papaya bunchy top bacterium is a member of the K-I subgroup, and its closest relatives are early diverging members ofthe genus *Rickettsia* (12). The yellow vine disease of cucurbits bacterium is a member of the y - 3 subdivision ofthe *Proteobacteria* (1).

Table I. Plant diseases associated with phloem-limited bacteria.

Disease Association	Plant Host	Geographit Location	Citation
Pollen sterility of garlic	*Allium sativum*	Germany	22
Sugar beet latent rosette	*Beta vulgaris*	Europe	31
Papaya bunchy top	*Carica papaya*	Caribbean Islands, Cent. Am.	11
Yellow vine disease of cucurbits	*Citrullus lanatus, Cucumis melo, Cucurbita spp.*	USA	5
Citrus greening	*Citrus* spp., *Poncirus trifoliata,* and other rutaceous plants	Asia and Africa	1 8
Coconut palm decline	*Cocos nucifera*	Tanzania	35
Carrot yellows	*Daucus carota* subsp. *sativus*	USSR	13
Strawberries yellows Strawberry marginal chlorosis Brown blast of	*Fragaria* X *ananassa Fragaria* X *ananassa*	Australia France and Spain	17 32
rubber trees Hop crinlde	*Hevea brasiliensis*	China	44
Larch witches' broom	*Humulus lupulus*	Eastern Europe	38
Tomato stolbur-like	*Larix decidua*	Germany	2
Proliferation and stunting	*Lycopersicon esculentum*	Eastern Europe	9
Potato leaflet stunt	*Melaleuca armilaris*	Israel	13
Little leaf	*Solanum tuberosum*	Israel	1
Spinach witches' broom	*Sida cordifolia*	Puerto Rico	9
Rugose leaf curl of clover	*Spinacia oleracea*	Italy	21
Yellows of clover	*Trifolium* spp.	Australia	2
Clover club leaf	*Trifolium repens*	Canada	6
Wheat yellow leaf curl	*Trifolium repens*	USA and England	6
Yellows disease of grapevine	*Triticum* spp.	China	2
Infectious necrosis of grapevine	*Vitis vinifera*	Germany, Greece	3
Shoot proliferation	*Vitis vinifera* *Wissadula periplocifolia*	Czechoslovakia Jamaica	24,27 43 30 37 8

In general, these bacteria are very small (0.1-0.5 by 1.0-5.0 um), with a Gram-negative type cell wall. They are obligately endophytic and intracellular. They often appear to be tissue specific, being observed most frequently by electron microscopy in the phloem of diseased plants. However, some appear not to be limited to a specific tissue, sometimes being found in both the xylem and phloem. Some inhabit specific cell types, such as sieve tubes and laticifers. Some xylem-limited bacteria once considered as part of this group of organisms have been isolated in axenic culture, taxonomically described, and their role as plant pathogens confirmed (see *Clavibacter xyli,* 3, p. 223).

B. ISOLATION TECHNIQUES USING SELECTIVE AGAR MEDIA

The bacterium associated with grapevine yellows has been cultivated in chick embryos (30), but attempts to cultivate other bacteria of this group in a similar manner or in tissue cultured cells, have not been reported. Failure of plant disease-associated bacteria to grow on common bacteriological media or other media for fastidious bacteria, such as the PW medium developed for *Xylella fastidiosa* or SC medium developed for *Clavibacter xyli,* suggests that the bacteria might be obligate endophytes.

c. DIFFERENTIA nON OF COMMONLY ISOLATED SPECIES

Identification is based on symptoms, host range, vector specificity, electron microscopy, and PCR using prokarytic specific primers. Diseases associated with these bacteria have sometimes been described as yellows or yellows-like because of similarities to diseases, such as aster yellows, caused by phytoplasmas (4). Both phytoplasmas and fastidious bacteria have been observed within different plants of the same species with similar symptoms; for example, a phytoplasma (6), and a phloem-limited bacteria (31) have been observed in sugar beet with rosette disease and latent rosette disease, respectively, and similarly in strawberries with yellows diseases (17). Furthermore, both types of organisms have been found together in the same carrot plants with yellows symptoms (13), tomato plants with stolbur-like symptoms (13), and proliferated *Wissadula periplocifolia* (9). Symptoms of the diseases associated with these bacteria vary considerably even when incited by the same agent in closely related host species or cultivars. Entire plants often exhibit stunting, die-back, degeneration, or death. Roots may be brown to black and stunted (32). The primary phloem of plants may be dead or degenerate (34,36,42). Leaves may be deformed or reduced in size (2,3, 10, 19,23,24,33). Leaves may be chlorotic, exhibiting general chlorosis, marginal chlorosis, or interveinal chlorosis. Flowers may be dwarfed, proliferated or exhibit viresensce (3).

The host range of these bacteria is not well established. Both insect vector and host specificity exist. Symptomatic plants were observed in species of five plant families following transmission of the clover club leaf pathogen by its leafhopper vector, *Agaliopsis novella,* but closely related leafhoppers were unable to transmit the pathogen (4). The phloem-limited bacterium associated with rugose leaf curl of clover (2), but not that associated with a yellows-

type disease of strawberries (16), is transmitted by the leafhopper, *Austroagallia torrida*. The citrus greening bacteria can be experimentally transmitted to numerous *Citrus* spp. and other rutaceous plants by graft or insect transmission, but attempts to transmit it to several other plant species via dodder and its insect vectors have been unsuccessful, except for periwinkle (8).

In general, the bacteria are rod-shaped in longitudinal section and round in cross section when observed in plant tissues by transmission electron microscopy. Occasionally, small spherical bodies are found associated with rod-shaped cells (19, 40). The bacteria appear to divide by binary fission. Granular bodies resembling ribosomes and fibrilar strands resembling DNA are frequently observed in the cytoplasm ofthe bacteria. The bacteria are bound by an outer membrane and cytoplasmic membrane both of which are trilaminar in structure and measure 5-8 nm in width. The membranes are separated by a periplasmic space varying from 2-28 nm in
width. The outer membrane in longitudinal section usually has an undulating outline without regularity, however some bacteria have a rippled outline (19,37). When in the phloem, the bacteria are frequently within sieve tubes but are occasionally also in parenchyma and companion cells. In rubber trees with brown blast disease, the bacteria were observed in both sieve tubes and laticifers (44), and in papaya with bunchy top, they appeared to be limited to laticifers (11). When in the xylem, the bacteria are frequently within tracheids.

D. DIAGNOSTIC MEDIA AND TESTS

The association of these bacteria with plant diseases has been established by their detection within tissues of diseased, but not healthy, plants by either transmission electron microscopy and/or peR. Other than presumptive diagnoses based on symptoms, electron microscopy still remains as the only practical means of detection with few exceptions. Phasecontrast microscopy has been used to detect the bacteria associated with clover club leaf disease(21) and papaya bunchy top disease (10) in extracts from plants. Epifluorescence microscopy using DNA-binding fluorochromes has been used to detect the fastidious bacteria in extracts from plants (11, 25) and within tissues of diseased papaya (11) and strawberries (32).

Plants infected by these bacteria often exhibit a remission of disease symptoms when treated with penicillin or tetracycline (2, 17,27, 37, 38, 40, 41, 42). Permanent cures are not obtained, and cessation of treatment results in the re-occurrence of the disease. Since penicillin acts by inhibiting peptidoglycan synthesis in bacterial cell walls, especially those of Gram-negative bacteria, symptom remission due to penicillin treatment suggests that a disease might be caused by a bacterium with a cell wall and not a wall-free prokaryote, such as a phytoplasma or spiroplasma.

E. PATHOGENICITY TESTS

The means of transmission of these bacteria in nature, ifknown, is usually from plant to plant by insects. Nematode transmission is suspected for the bacterium associated with yellows disease of grapevine (33), and soil-borne transmission was reported for the bacterium associated with stunted and witches' broom diseased larch trees (29). Graft and dodder transmission have

been reported for some of these bacteria. Mechanical or seed-borne transmission has not been reported. Insect vectors of these bacteria are often leafhoppers; however psyllids (8, 16,28) and a piesmid (31) have been found to be the vectors of individual bacteria.

F. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

No commercial diagnostic materials or methods are available for detection of these bacteria. Monoclonal antibodies for *Liberobacter* spp. (citrus greening) (15) and polyclonal antibodies for the grapevine yellows disease bacterium (30) have been developed for serological detection. DNA hybridization (39) was developed for detection of *Liberobacter* spp. PCR (Table 1) was developed for detection of *Liberobacter asiaticum* and *L. africanum* (18), papaya bunchy top bacterium (11) and yellow vine disease of cucurbits bacterium (1).

Table 2. PCR primers for detection of phloem-limited bacteria.

Specificity	Primer designation	Sequence	Size (bp)	Reference
Papaya bunchy top	PBTFI	(5' AAAGGTICTGATIGGTI AGGTG3')		12
bacterium	PBTRI	(5'ATCTITATGCTCTCCAACTCCTC 3')	705	
Yellow vine disease	YVI	(5 'GGGAGCTTGCTCCCCGG3')		
bacterium	YV2	(5 'CGCTACACCTGGAATICTAC3')	643	
	YV3	(5'GGTI ACCTITGTI ACGACTTCA3')	1433	
Citrus greening	all	(5'GCGCGTATGCAATACGAGCGGCA3')		
bacteria	0I2c	(5 , GCCTCGCGACTTCGCAACCCAT3 ')	1160	18

G. CULTURE PRESERVA nON

Isolates are maintained in host plants by plant to plant transmission or propagation of cuttings from disease plants. Periwinkle *(Catharanthus roseus)* has been found to be a convenient experimental host for the clover club leaf (23,40) and citrus greening (14) agents by graft transmission after initial transmission to this host by leafhoppers and dodder, respectively. However, numerous attempts to establish the bacterium associated with marginal chlorosis of strawberries in periwinkle by dodder transmission were unsuccessful (32).

H. LITERATURE CITED

1. Avila, F. 1., B. D. Bruton, 1. Fletcher, 1. L. Sherwood, S. D. Pair, and U. Melcher. 1998. Polymerase chain reaction detection and phylogenetic characterization of an agent associated with yellow vine disease of cucurbits. Phytopathology 88:428-436.

2. Behncken, G. M., and D. H. Gowanlock. 1976. Association ofa bacterium-like organism with rugose leaf curl disease of clovers. Aust. 1. Biolog. Sci. 29: 137-46.

3. Benhamou, N., and R. C. Sinha. 1981. Association of a rickettsia-like organism with a disease of white clover. Can. 1. Plant Pathol. 3: 191-196.

4. Black, L. M. 1993. Clover club leaf revisited. Plant Dis. 77: 102-103.

5. Bruton, B. D., 1. Fletcher, S. D. Pair, M. Shaw, and H. Sittertz-Bhatkar. 1998. Association of a phloem-limited bacterium with yellow vine disease in cucurbits. Plant Dis. 82:512-520.

6. Canova, A., M. G. Bellardi, A. Bertaccini, and V. Vicchi. 1990. Rosette-disease and witches' broom in sugar beet and spinach in Italy. Phytopathol. Mediter. 24:39-43.

7. Capoor, S. P., D. G. Rao, and S. M. Viswanath. 1967. *Diaphorina citri* Kuway., a vector of the greening disease of citrus in India. 1. Agric. Sci. 37:572-576.

8. da Graca, 1. V. 1991. Citrus greening disease. Annu. Rev. Phytopathol. 29: 109-136.

9. Dabek, A. 1., H. Waters, and D. Lavin. 1983. *Wissadula* proliferation in Jamaica. I. Symptoms and associated prokaryotic structures. Phytopath. Z. 107:336-344.

10. Davis, M. 1. 1991. Fastidious bacteria of plant vascular tissue and their invertebrate vectors. Pages 4030-4049 in: A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer, eds. The Prokaryotes, Second Ed., Vol. IV. Springer-Verlag, New York.

11. Davis, M. 1., 1. B. Kramer, F. H. Ferwerda, and B. R. Brunner. 1996. Association of a bacterium and not a phytoplasma with papaya bunchy top disease. Phytopathology 86: 102-109.

12. Davis, M. 1., Z. Ying, B. R. Brunner, A. Pantojas, and F. H. Ferwerda. 1998. Rickettsia relative associated with papaya bunchy top disease. CUff. Microbiol. 36:80-84.

13. Fedotina, V. 1976. Mycoplasma-like and rickettsia-like organisms in the tissues of yellows-infected plants. Sel'Skokhozyaistvennaya Biologiya 11 :259-266.

14. Garnier, M., and *I.* M. Bove. 1983. Tranmission of the organism associated 'with citrus greening disease from sweet orange to periwinkle by dodder. Phytopathology 73: 1358-1363.

15. Garnier, M., and J. M. Bove. 1987. Monoclonal antibodies against the bacteria-like organism associated with citrus greening disease. Ann. Inst. PasteurlMicrobiol. (Paris) 138:639-650.

16. Giannotti, J., C. Louis, F. Lelant, G. Marchoux, and C. Vago. 1974. Infection due to mycoplasmas and to micro-organisms with rickettsia pattern in a plant affected by proliferation and in the psyllid vector of the disease. Comptes Rendus Hebdomadaires Des Seances De L'Academie Des Sciences. D 278, no. 4:469-471.

17. Greber, R. S., and D. H. Gowanlock. 1979. Rickettsia-like and mycoplasma-like organisms associated with two yellows-type diseases of strawberries in Queensland. Aust. J. Agric. Res. 30: 1101-1109.

18. Jagoueix, S., J. M. Bove, and M. Garnier. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the Proteobacteria. Int. *I.* Syst. Bacteriol. 44:379-386.

19. Klein, M., S. Dabush, and M. Bar-Joseph. 1979. A preliminary report on the occurrence of bacteria-like organisms in both phloem and xylem tissues of stunted *Melaleuca armilaris* plants. Phytoparasitica 7: 169-175.

20. Klein, M., S. Dabush, and S. Zimmerman-Gries. 1979. Association of bacteria-like organisms with a disease of sugar beets. PI. Prot. Bull. *FAD* 27: 12-18.

21. Klein, M., S. Zimmerman-Gries, and B. Sneh. 1976. Association of bacteria-like organisms with a new potato disease. Phytopathology 66:564-569.

22. Konvicka, 0., F. Nienhaus, and G. Fischbeck. 1978. Investigations on the causes of pollen sterility in *Allium sativum* L. Zeit. pflanzenzuchtung 80:265-276.

23. Lee.T. M., and R. E. Davis. 1983. Phloem-limited prokaryotes in sieve elements isolated by enzyme treatment of diseased plant tissues. Phytopathology 731: 1540-1543.

24. Liu, H. Y., and L. M. Black. 1974a. Study of the rickettsia-like organism of clover club leafby phase contrast microscopy. Proc. Am. Phytopathol. Soc. 1:97. (Abstr.).

25. Liu, H. Y., and L. M. Black. 1974b. The clover club leaf organism . Int. Congr. Mycoplasmas of Man, Animals, Plants and Insects. Les Colloques De I'Institut National De La Sante Et De La Rescherche Medicale. INSERM, Paris, Les Mycoplasmes/Mycoplasmas 33:97-98. (Abstr.).

26. Maramorosch, K., H. Hirumi, M. Kimura, 1. Bird, and L. A. Jr. 1975. Mollicutes and Rickettsia-like plant disease agents (Zoophytomicrobes) in insects. Ann. New York Acad. Sci. 266:276-292.

27. Markham, P. G., R. Townsend, and K. A. Plaskitt. 1975. A rickettsia-like organism associated with diseased white clover. Ann. AppI. BioI. 81:91-93.

28. McClean, A. P. P. and P. C. 1. Oberholzer. 1965. Citrus psylla, a vector of the greening disease of sweet orange. So. Afr. 1. Agric. Sci. 8:297-298.

29. Nienhaus, F., H. Brussel, and U. Schinzer. 1976. Soil-borne transmission of Rickettsialike organisms found in stunted and witches' broom diseased larch trees *(Larix decidua).* Z. Pflanzenkrankh. Pflanzenschutz 83:309-316.

30. Nienhaus, F. and I. Rumbos. 1978. First results in the cultivation of rickettsia-like organisms of yellows diseased grapevines in chick embryos. Z. Pflanzenkrankh. Pflanzenschutz 85: 113-117.

31. Nienhaus, F. and H. Schmutterer. 1976. Rickettsia like organisms in latent rosette (witches' broom) diseased sugar beet *(Beta vulgaris)* and spinach *(Spinacia oleracea)* plants and in the vector *Piesma quadratum* Fieb. Z. Pflanzenkrankh. Pflanzenschutz 83:641-646.

32. Nourrisseau,1. G., M. Lansac, and M. Garnier. 1993. Marginal chlorosis, a new disease of strawberries associated with a bacterium-like organism. Plant Disease 77: 1055-1059.

33. Rumbos, I., R A. Sikora, and F. Nienhaus. 1977. Rickettsia-like organisms in *Xiphinema index* Thome & Allen found associated with yellows disease of grapevine. Z. Pflanzenkrankh. Pflanzenschutz 84: 240-243.

34. Sadovskii, Yu. P. and V. I. Meshcheryakov. 1984. Rickettsia-like organisms in peach plants with symptoms ofleafvein browning. MikrobioI. Z. 46:24-29.

35. Steiner, K. G., F. Nienhaus, and K. 1. Marschall. 1977. Rickettsia -like organisms associated with a decline of coconut palms in Tanzania. Z. Pflanzenkrankh. Pflanzenschutz 84:345-351.

36. Subandiyah, S. 1994. Transmission of citrus vein phloem degeneration (CVPD) to periwinkle through dodder. Agrivita 17:21-22.

37. Ulrychova, M., G. Vanek, M. Jokes, Z. Klobaska, and O. Kralik. 1975. Association of rickettsia-like organisms with infectious necrosis of grapevines and remission of symptoms after penicillin treatment. Phytopath. Z. 82:254-265.

38. Vanek, G., M. Ulrychova, G. Blattny, M. Jokes, and M. Novak. 1976. Rickettsialike structures in hop plants with crinkle disease and therapeutic effect of antibiotics. Phytopath. Z. 87:224-230.

39. Villechanoux, S., M. Gamier, 1. Renaudin, and M. Bove. 1992. Detection of several strains of the bacterium-like organism of citrus greening disease by DNA probes. CUlT. Microbial. 24:89-95.

40. Windsor, I. M. and L. M. Black. 1973a. Evidence that clover club leaf is caused by a rickettsia-like organism. Phytopathology 63: 1139-1148.

41. Windsor, I. M. and L. M. Black. 1973b. Remission of symptoms of clover club leaf following treatment with penicillin. Phytopathology 63:44-46.

42. Wu, S. P. and 1. S. Li. 1986. Preliminary studies on the pathogens of blotchy yellows and yellow shoot disease of citrus. Sci. Agric. Sin. 1: 73 -76.

43. Wu, X. 1., W. C. Zhang, and C. H. et al. Lou. 1981. Rickettsiae-like organisms - a new pathogen severely attacking wheat in China. Nature 1. 4:238.

44. Zheng, G. B., M. R Chen, S. W. Yung, Z. Y. Chen, and 1. Y. Shen. 1988. A further report on the study of causative agents and control of brown blast disease of rubber trees. 1. So. China Agric. Univ. 9:22-23.

v. CELL-WALL FREE BACTERIA

Spiroplasmas and Phytoplasmas

Ing-Ming Lee, Robert E. Davis, and Jacqueline Fletcher

A. INTRODUCTION

Spiroplasmas and phytoplasmas (formerly termed mycoplasma-like organisms or MLOs) are prokaryotes (class *Mollicutes)* lacking a true cell wall (14,39,50). Each cell is bounded by a trilaminar unit membrane about 8-10 nm thick. In plants these organisms are associated with yellows type diseases and various little leaf, virescence, phyllody, stunting, bunchy top, witches'broom, and proliferation disorders. These pathogens are limited to phloem tissue in infected plants. They are transmitted from plant to plant in nature by phloem-feeding leafhoppers and may be experimentally transmitted by grafting or by the parasitic plant, dodder *(Cuscuta* spp.) (4, 37, 57,61). Spiroplasmas and phytoplasmas are sensitive to heat treatment in diseased plants and insect vectors; and treatment of infected plants with tetracycline antibiotics results in remission of disease whereas penicillin has no such effect (14, 50).

The study of cell wall-free prokaryotes that affect plants began in 1967 (26). Earliest data on the nature of these plant pathogens came from light and electron microscopy and from antibiotic treatment of infected plants. Few ofthe plant-inhabiting cell wall-free prokaryotes have been cultivated *in vitro* and characterized (14). Those that have been cultivated and shown with certainty to be pathogenic in plants have all proven to be spiroplasmas (8, 14,27, 84). Methodology used for identification of *Mycoplasma* spp. and spirochetes has been adapted to the identification of spiroplasmas (10, 85). The following phytoplasmas and spiroplasmas are included (Table 1).

Table 1. Representative phytoplasma 16S rRNA groups and spiroplasma species.

Strains	Diseases	Location	References
Pbytopluma Groups:			
Group I (aster yellows)			
Subgroup I-A Subgroup	tomato big bud (BB) Maryland	Arkansas	11,51
I -B 1 Subgroup I-B2	aster yellows (AY1) maize	US	51
Subgroup I-C Subgroup	bushy stunt (MBS) clover	Mexico, US	34
I-D Subgroup I-E1	phyllody (CPh)	Canada	51
Subgroup I-E2	paulownia witches' -broom (PaWB)	Taiwan	56
Group n (peanut witches'-broom)	blueberry stunt (BBS1)	Michigan	53,56
Subgroup II-A	blueberry stunt (BBS3)	Arkansas	53,56
Group m (X-disease)			
Subgroup III-A	peanut witches' -broom (Pn WB)		56
Subgroup III-B			
Subgroup III-C	peach X-disease (CX)	Canada	54
Subgroup III-D	clover yellow edge (CYE)	Canada	54
Subgroup III-E	pecan bunch (PB)	US	6,33
	golden rod yellows (GRY,GR1)	US	30
	spirea stunt (SP1)	US	30

Subgroup III-F	milkweed yellows (MWY, MWI)	US	30
Subgroup nr-o	walnut witches' -broom (WWB)	US	6,33
Subgroup III-H	poinsettia branch-inducing (PoiBl)	US	57
Group IV (coconut lethal yellows)			
Subgroup IV-A	coconut lethal yellows (L Y3)	US	35
Group V (elm yellows)			
Subgroup V-A	elm yellows (BYl)	US	52
Subgroup V-B	jujube witches' -broom (JWE)	China	88
Subgroup V-Cl	rubus stunt (RS)	Italy	45
Subgroup V-C2	grapevine yellows (GVY)	Italy	2
Group VI (clover proliferation)			
Subgroup VI-A	clover proliferation (CP)	Canada	25
Group VI (ash yellows)			
Subgroup VII-A	ash yellows (AshY)	US	22
Group VIII (loofah witcbes'-broom)			
Subgroup VIll-A	loofah witches'-broom (LfWB)	Taiwan	56
Group IX (pigeon pea witcbes'-broom)			
Subgroup IX-A	pigeon pea witches' -broom (PPWB)	US	36
Group X (apple proliferation)			
Subgroup X-A	apple proliferation (AP-A)	Germany	42,62
Subgroup X-B	apricot chlorotic leaf roll (ACLR)	Italy	44
Subgroup X-C	pear decline (PD)	Italy	44
Group XI (rice yellows dwarf)			
Subgroup XI-A	rice yellow dwarf (RYD)	India	58
Subgroup XI-B	sugarcane white leaf (SCWL)	Thailand	58
Group XU (stolbur)			
Subgroup XII-A	celery yellows (CeY)	Italy	53
Group xm (Mexican periwinkle virescence)			
Subgroup XIII-A	Mexican periwinkle virescence (MPV)	Mexico	33
Group XIV (Bermuda grass white leaf)			
Subgroup XIV-A	Bermuda grass white leaf (BGWL)	Thailand	58
Spiroplasma Species:			
Spiroplasma citri	Citrus stubborn		75
Spiroplasma kunkelii	Com stunt		83
Spiroplasma phoenicium	Periwinkle host		76

Phytoplasmas have never been successfully cultured in cell-free media. Because the pleomorphic morphology and ultrastructure resemble those of animal mycoplasmas (class *Mollicutesy,* until recently they were referred to as mycoplasma-like organisms or MLOs (7,39, 50). Recently, comprehensive phylogenetic studies revealed that phytoplasmas form a monophyletic group and are new members of the class *Mollicutes* (32, 59, 60, 69, 78, 80). The term "phytoplasma" was officially adopted to replace the older term "mycoplasma-like organism" in 1994 at 10th International Congress of the International Organization for Mycoplasmology (lOM). Due to the inability to obtain pure cultures, the identification ofphytoplasmas is primarily based on molecular data (1,2,6, 7, 19,20-22,24,25,28-36,38-58,62,63,64,66-68, 73, 77-80, 87-89).

B. ISOLATION TECHNIQUES

1. Spiro plasmas

The plant pathogenic spiroplasmas can be isolated in artificial culture from diseased plants and from infected insect vectors. The isolation in culture of some spiroplasmas from plants can depend heavily on the tissue chosen as spiroplasma source. For example, S. *citri* can be isolated and cultivated from surface-sterilized young leaves and shoots, seeds, or fruit columella (E. C. Calavan, personal communication) of stubborn diseased citrus. However, the spiroplasma is unevenly distributed in aerial parts of infected plants (3). S. *citri* can also be isolated from leaf or root tissue of other hosts, including horseradish, turnip, and periwinkle (27, 65, 75). The original discovery ofa natural insect vector of S. *citri* resulted from studies involving isolation of the pathogen in culture from infected insects collected in the field (46). S. *phoenicium* has been isolated in culture from tissues of plants of periwinkle (76).

S. *kunkelii,* com stunt spiroplasma, can be isolated from leaves, stems, roots, or tassels of diseased plants of com *(Zea mays* L.). In the case of com stunt spiroplasma, helical spiroplasma cells usually can be observed, by dark field optical microscopy (1,000 to 12,560X), in the undiluted, filtered homogenate of plant tissues. Helical S. *kunkelii* cells can even be seen, by dark field microscopy, in crude juice freshly expressed from com stunt-infected plant tissues and in hemolymph withdrawn from infected insect vectors (15, 18). Primary isolation and *in vitro* culture of the com stunt spiro plasma can also be achieved from infected insect vectors (83).

a) Procedure for primary isolation of spiroplasmas in culture:

(1) Carry out the isolation procedure in a **biological safety cabinet.**

(2) If plant material will be the source of spiroplasma, select plant tissues such as fruit columella or symptomatic young leaves and shoots for isolation of S. *citri.* Symptomatic leaf tissues are generally used for ioslation of S. *kunke/ii* or S. *phoenicium.*

(3) Using a sterile scalpel, cut 0.1 to 0.2 gm tissue from fruit columella or young leaves and shoots of citrus, or from young leaves or shoots of periwinkle, or from midvein of symptomatic com leaf

(4) Surface sterilize small piece of tissue from young leaf or vein and place in a dry, sterile Petri dish, and keep the dish covered with its lid until ready for spiroplasma extraction.

(5) Transfer the plant tissue to a plate containing 4-5 mlliquid culture

medium and chop the tissue into 1 mnr' fragments.

(6) Using a sterile 5 ml syringe without needle, aseptically remove the liquid suspension of plant tissue fragments and spiroplasma cells and pass it through a sterile 0.45 ,*uM* filter syringe assembly. Aseptically collect the filtrate in a sterile 15 ml culture tube.

(7) Transfer 0.5 ml of the filtrate to 4.5 ml offresh, sterile culture medium (see Table 1), then pipette 0.1 ml of the original filtrate and diluted filtrate onto the surface of the agar medium. After spreading the liquid on the surface with a sterile L shaped glass rod, incubate the seeded plate at 30-32°C.

(8) Helical spiroplasma cells in liquid medium primary cultures, and minute colonies of spiroplasma on agar medium, may be seen after one to two weeks of incubation, depending upon the spiroplasma strain present in the source tissues. The cells in liquid medium may be viewed live by dark field microscopy. Colonies on agar may be seen by the unaided eye when fully developed, and when very small may be observed using a binocular dissecting microscope.

(9) In parallel with primary isolations, always incubate unseeded, sterile liquid medium and agar medium controls to check for possible contaminants in the medium.

Notations:
Spiroplasma growth is usually accompanied by production of acid from sugars and, in weakly buffered or unbuffered medium containing phenol red, a shift in color of the medium from red to orange-yellow occurs.

b) Media

Several media (Table 2) have been successfully employed for *in vitro* cultivation of plant pathogenic spirolasmas (7, 8, 12,27,48,49). Medium C3G is a simple medium commonly used (8). Medium LD8A3 yields superior growth oftbe plant pathogens (48, 49); serum free medium LD59 is especially recommended for primary culture attempts with com stunt spiroplasma since LD59 supports good growth of strains that grow poorly if at all in media containing serum (49). Use offreshly prepared media is strongly recommended. Ease of culture in some media may vary with the strain of spiroplasma. Incubation of cultures in candle jar or under 5% CO_2 or N_2 in BBL anaerobic jars improves growth of many spiroplasma strains in some media.

Table 2. Recipes for medium C3G, medium LD8A3, and serum-free medium LD59[1],

Component	C3G	**LD8A3**	**LD59**
PPLObroth	15.0	15.0	15.0
Fructose		1.0	1.0
Sucrose	120.0	120.0	120.0
L-arginine		0.6	0.6
L-asparagine		0.6	0.6
L-methionine		0.4	0.4
alpha-Ketoglutarate		0.4	0.4
Pyruvate		0.4	0.4
Lactalbumin hydrolysate		2.0	2.0
HEPES buffer		7.0	7.0
Horse serum	200.0		
Fetal bovine serum		100.0	
Serum substitute (BSA-lipids solution?			100.0
Deionized water	665.0	720.0	720.0

ı Amounts of serum, serum substitute, and water are given in ml. Amounts of all other ingredients are given in grams.

Medium preparation should be carried out in a biological safety cabinet, for all steps where this is feasible, to avoid microbial contamination of mediwn.

2 Method for preparation of serum substitute is given below.

Preparation of serum substitute for spiroplasma culture medium LD59:

1) Preparation of lipid stock 1:

Dissolve 0.6 ml Tween 80, 20.0 mg phosphatidic acid, 0.075 ml linoleic acid, 60 mg palmitic acid, and 40 mg cholesterol in 2 ml absolute ethanol.

2) Preparation oflipid stock II:

Dissolve 12 mg lysophosphatidyl choline (from egg yolk), 12 mg lysophosphatidyl choline (from soybean), and 20 mg phosphatidyl choline (from egg yolk) in 10 ml absolute ethanol.

3) Preparation of serum substitute (100 ml BSA-lipids solution):

Dissolve 0.66 ml of Lipid Stock I and 3.5 ml of lipid stock II in warm (37°C) bovine serum albumin (BSA) solution (9 g BSA in 100 ml of deionized water). Vortex BSA solution and add drop-by-drop the lipid stock into the solution. The solution should remain clear. This serum substitute is then added to the basal medium as prepared above.

c) Preparation of spiroplasma culture media:

1) Liquid medium

(a) Dissolve all components (except serum or serum substitute) in deionized water and adjust the pH of each medium to 7.5 using 2N NaOH.
(b) To the autoclaved and cooled basal medium in serum bottles, aseptically add the appropriate volume of serum or serum substitute. Swirl to mix thoroughly.
(c) Aseptically dispense 4.5 ml aliquots of the liquid medium into sterile 15 m' culture tubes with screw caps and store at 4 ° C up to 2 weeks, or at -40 ₀C up to 2 months.

2) Agar-solidified medium

(a) Add 1.0 g of Noble agar to the basal medium (medium components minus serum or serum substitute) before autoclaving.
(b) After cooling to 57 ° C in a water bath, add serum or serum substitute, swirl to mix thoroughly and dispense 5 ml into each 6 ern diameter Petri plate.
(c) Store agar plates at 4°C before use. Allow plates to warm to room temperature just before use.

2. Phytoplasmas

Because phytoplasmas are non-cultivable, procedures are designed to enrich or partially purify phytoplasmas and to obtain good quality of DNA or nucleic acid preparations from their plant or insect hosts. The phytoplasma-enriched fractions are used for DNA extraction and genomic characterization. Successful detection and identification of phytoplasmas associated with plant and insect hosts largely depends on the quality of DNA or nucleic acid preparations (e.g. high ratio ofphytoplasma DNNhostDNA).

a) Preparation of phytoplasma-enriched fractions by differential centrifugation (40).

1) Cut up 1.5 g of leaf midribs, roots, or phloem scrapings in small pieces and place in pre-cooled mortar containing 8 ml of phytoplasma grinding buffer. Incubate on ice for 10-20 minutes.
2) Grind thoroughly with cold pestle, add another 5 ml of grinding buffer to the mortar, and grind again until the tissue is pulverized.
3) Transfer the homogenate to a cold 15-ml glass Corex tube and centrifuge at 2,000 *g* for 5 minutes at 4°C.
4) Transfer the supernatant to a cold 15-ml Corex tube and centrifuge at 20,000 g for 25 to 30 minutes.
5) Discard the supernatant and drain the tube for 1-2 minutes to get rid of the rest of supernatant. Resuspend the pellet (phytoplasma-enriched fraction) in a desired buffer.

Phytoplasma grinding buffer:

	perL
$K_zHP0_4 \cdot 3H_zO$	21.7 g
KH_ZP0_4	4.1 g
Sucrose	100.0 g
BSA (fraction V)	1.5 g
PVP-l 0 (Polyvinylpyrrolidone)	20.0g

Filter sterilize. Store in 100 ml aliquots at 4 ° C for up to 2 months. Just before use, bring to room temperature and add 0.53 g L-ascorbic acid per 100 ml buffer, and adjust pH to 7.6 with 2N NaOH.

b) Isolation of phytoplasma by enzyme treatment (47). This method is particularly suitable for preparing phytoplasma-enriched fractions from phytoplasma-infected periwinkle plants.

1) Collect 50-100 g of young periwinkle leaves showing early stage symptoms. Surface-sterilize the leaves in a large beaker or clean plastic bag with 20% bleach (approximately 1 % sodium hypochlorite) for 5-10 minutes. Drain the bleach and rinse leaves twice with sterile distilled water.

2) Place the leaf right-side up on a cover of sterile plastic petri plate. Strip midribs of leaves longitudinally with EM grade sharp forceps by pressing (at about 45 ° angle) the forceps parallel on the petiole and score down along the midvein. Make sure that cortex tissues on both sides of the midribs are cut open and REMOVED.

3) Transfer the stripped vascular tissues (midribs) into Petri plates containing the maceration enzyme solution. Incubate the Petri plates overnight in the dark at 4°e.

Maceration enzyme solution for 100 ml:

Cellulase R-lO	0.8%
Macerozyme R-l 0 (or Macerase)	0.4%
$CaCl_2$	1.0 mM
PVP-40	0.5%
Mannitol	0.6M

Filter through a O.4S-l1m (pore size) membrane filter.

4) Transfer each of the partially digested vascular bundles (translucent in appearance) to a Petri plate containing suspending medium. Separate the phloem tissue (green parts of the bundles) with forceps and transfer to a separate Petri plate containing fresh suspending medium (25 ml for phloem tissue prepared from 50 g ofleaves) and incubate at room temperature for 2-3 hours.

Suspending medium for 1 liter:

Mannitol	0.5 M
HEPES	30.0 mM
PVP-40	0.1%

Adjust the solution to pH 7.0 and autoclave it.

5) With forceps or a plastic pipette, transfer digested sieve elements and other plant organelles into the suspending medium.

6) Release phytoplasmas from sieve elements by gently rupturing the sieve elements with glass tissue homogenizers first thoroughly with a conical tissue homogenizer and subsequently briefly with a Tenbroeck tissue grinder (four to six strokes).

7) Obtain phytoplasmas by differential centrifugation.

c) **Preparation of total DNA and RNA from phytoplasma-infected hosts without steps for enriching phytoplama** ceUs.

There are several procedures (40, 52) that work well for extracting total

nucleic acids from both herbaceous and woody plants as well as insects.

Procedure

a) Place 1-1.5 g fresh or frozen plant tissue (or 0.3 g dried tissue) in a pre-cooled mortar. Add liquid nitrogen and pulverize tissue with porcelain pestle.

b) Allow tissue to warm slightly at room temperature. Add 10-14 m1 phytoplasma grinding buffer and grind thoroughly.

c) Centrifuge at 13,000 rpm (SS-34 rotor) (20,000 *g)* for 20 minutes at 4°C.

d) Discard the supernatant. Suspend the pellet with 8 m1 of Extraction buffer and 160 III proteinase K.

Extraction buffer:
100 mM Tris-HC1, pH 8.0
100 mMEDTA, pH 8.0
250mMNaCl

e) Add 88011110% Sarkosyl (final concentration is 1%) to the mix and incubate for 1-2 hours at 55°C.

f) Centrifuge lysate for 15 minutes at 8,000 rpm (7,500 g) at 4°C. Save the supernatant.

g) Add 0.6 volume isopropanol to the supernatant and mix thoroughly. Incubate at -20°C for 30 minutes. A nucleic acid precipitate should be visible.

h) Centrifuge 15 minutes at 8,000-10,000 rpm at 4°C. Discard the supernatant.

i) Resuspend the pellet in 3 m1 TE buffer (10 mM Tris, 1.0 mM EDTA, pH 8.0). Add 75 III of20% SDS and 60 III of proteinase K stock solution (5mglml). Mix thoroughly and incubate 30-60 minutes at 37 ° C.

j) Add 525 1l15M NaCl and mix thoroughly. Add 420 III CTABlNaCI and mix very thoroughly. Incubate 10 minutes at 65° C and then on ice for 10 minutes.

2% CTAB buffer (for 100 ml):

2% cetyltrimethyamrnonium bromide (CT AB):
1.4 MNaCI
20 mM EDTA, pH 8.0
100 mM Tris, pH 8.0
Sterile H_20

CTABlNaCI solution: 10% CTAB in 0.7 MNaCI:

Dissolve 4.1 g NaCI in 80 ml water. Slowly add 10 g CTAB while heating and stirring. Adust final volume to 100 ml. Store at room temperature.

k) Add equal volume of chloroform/isoamyl alcohol (CIA) to the mix. Mix thoroughly and centrifuge at 8,000 for 5 minutes. Repeat this step as needed.

1) Transfer the aqueous supernatant to a clean 30-ml Corex tube. Add equal volume of phenol/CIA (1: 1). Mix thoroughly and centrifuge at 8,000 rpm for 5 minutes.

m) Transfer the aqueous supernatant to a clean 15-ml Corex tube. Add 0.6 volume of isopropanol to precipitate nucleic acids. Gently mix and incubate at -20°C for 30 minutes or at 4°C overnight. Centrifuge 15 minutes at 10,000 rpm at 4 °C to pellet nucleic acid.

n) Discard the supernatant. Wash the pellet with 70% ethanol to remove residual CTAB. Centrifuge 10 minutes at 8,000 rpm at 4°C.

0) Air-dry and resuspend the pellet in sterile TE buffer (100-200 Ill).

g) Add 0.66 volume of 5M ammonium acetate to the sample and then add two volumes ofice-cold, 95% ethanol and incubate at -20°C for 1-2 hours.

h) Centrifuge, wash with 80% ethanol, dry the pellet, and resuspend the nucleic acids in TE.

C. DIFFERENTIATION OF SPECIES OR GROUPS

1. Spiro plasmas

Spiroplasma strains have been classified into distinct groups and subgroups based on serological and other criteria (16, 17, 72, 81, 83, 85, 86). The plant pathogenic spiroplasmas, S. ***citri,*** S. *kunkelii,* and S. *phoeniceum,* comprise three separate subgroups in a single major group, serogroup I, that also contains honey bee spiroplasma *(8. melliferum)* (10) and several other *Spiroplasma* species. In agreement with original proposals (17), it has been well accepted that each major serogroup represents at least one distinct species, and that each distinct subgroup, such as those that contain S. *citri,* com stunt spiroplasma, and honeybee spiroplasma, represents a separate Spiroplasma species (17). Accordingly, in addition to S. *citri,* S. *melliferum,* S. *kunkelii,* and S. *phoeniceum* have been recognized as separate species and named (10, 16,83).

Differentiation of S. *citri,* S. *kunkelii,* S. *phoenicium,* and other spiroplasmas, may be achieved through the use of serology or polyacrylamide gel electrophoresis (PAGE) analysis of whole cell or membrane proteins (10, 13-18, 75, 76, 81-86).

Broth cultures should first be examined by phase contrast or dark field optical microscopy at 1,000X to 1,250X to observe helical spiro plasma cells. Since motility of spiroplasma cells is often lost in older cultures (12), cultures should be examined during log phase. Absence of cell wall in suspected spiroplasmas is determined by ultrathin section electron microscopy of a cloned strain.

Because mixed cultures may be obtained from initial isolations for spiroplasmas from diseased plants or infected insects, **a culture should be triply cloned** prior to identification and characterization procedures.

a) Triple filter-cloning of a spiroplasma strain:

The objective of this procedure is to obtain a culture that is derived from a single viable spiroplasma cell. Filtering of broth cultures, before seeding agar-solidified medium, minimizes the number of colonies initiated by clumps of spiroplasma cells.

1) All operations are carried out aseptically in a biological safety cabinet.

2) Filter a log phase (24 to 48 h) broth culture through a 0.22 *f..lm* pore diameter filter and collect filtrate in a sterile culture tube.

3) Prepare a 10-fold dilution series (usually to 10-6) of the filtrate,

using sterile complete medium, sterile basal medium, or sterile 10% sucrose solution as diluent. This step can conveniently be accomplished by transferring 0.5 ml filtrate or diluted filtrate serially into 4.5 ml sterile liquid in culture tubes.

4) Pipette 0.1 ml of selected dilutions (usually 10^{-5} and 1 O~ dilutions) of filtrate onto the surface of agar-solidified (1 % agar) medium in Petri plates. Seed three plates with each dilution.

5) Select a single colony developing on agar medium seeded with high dilution of filtrate. Using a sterile loop or needle, remove a portion of the colony from the agar.

6) Place the selected colony/portion of colony into 4.5 to 5 ml of fresh, sterile liquid medium, and incubate at 30-3rC.

7) Repeat these steps twice more. The third liquid culture (third performance of step 5) will be a triply filter-cloned spiroplasma culture.

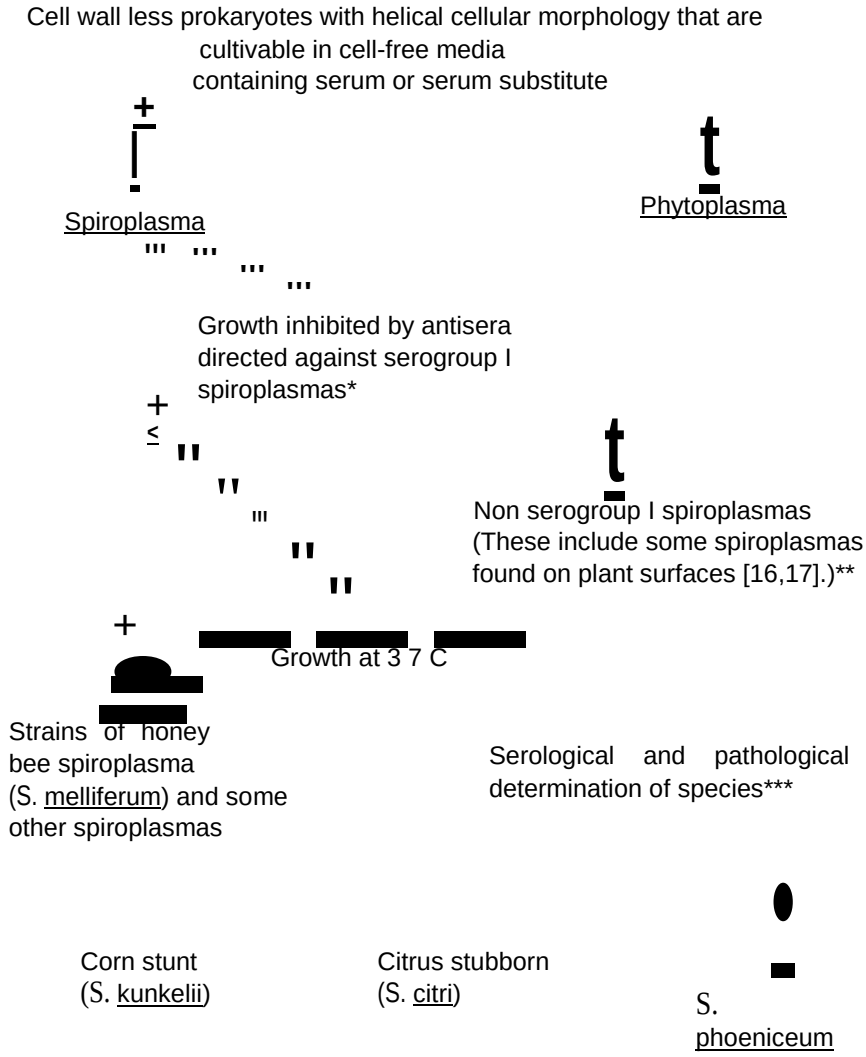
b) Initial differentiation of wall-free prokaryotes in plants:

A flowchart (Fig. 1) can be used as a guide for initial differentiation of cell wall free prokaryotees (spiroplasmas and phytoplasmas) from plants.

c) ELISA test for *Spiroplasma* identification:
(Please see enzyme-linked immunosorbent assay below.)

Figure 1

GUIDE FOR INITIAL DIFFERENTIATION
OF CELL WALL FREE PROKARYOTES
(SPIROPLASMAS AND PHYTOPLASMAS) FROM PLANTS



*AII spiro plasmas known to inhabit plant interiors belong to Group I, but Group I also contains spiroplasma types found only on plant surfaces in their plant associations.

**For example, some Spiroplasma species are harbored on the surface of flowers. There is no evidence linking them with disease in plants.

***See serology, p. 338. Tests of insect transmissibility and of pathogenicity in plants may not identify species. At least one strain of corn stunt spiroplasma, passaged in Drosophila prior to cultivation in vitro, was nontransmissible to corn. See also, pathogenicity tests, p. 299.

Phytoplasmas

Because it has been not possible to isolate phytoplasmas in artificial culture media, for many years type of symptoms induced in infected plants, characteristics of'transmission by specific insect vectors, and similarities and differences in plant host ranges were the only criteria available for differentiation of various phytoplasmas (7, 39, 50). These procedures were time-consuming, laborious, and sometimes unreliable. The less laborious molecular-based methods introduced in the past decade have proved to be more accurate and reliable (1, 6, 20-22, 24, 25, 28-36, 38-45, 50-58, 62, 67-69, 73, 77-80, 87-89). The use of molecular probes, such as phytoplasma-specific cloned DNA and monoclonal antibodies, made it possible to classify phytoplasmas on the basis of DNA-DNA homology and serological data (6, 7,25,36,38,41,43,50-52,54,66,67). The use oflabeled DNA probes in dot hybridizations and restriction fragment length polymorphism (RFLP) analyses of chromosomal DNA revealed genomic cluster relationships among various phytoplasmas. These relationships led to a genotype-based system for classification of phytoplasmas (50). Each genomic cluster contains closely related phytoplasmas.

Current systems of phytoplasma classification are based on phylogenetic relationships derived from analysis of highly conserved 16S rRNA gene (32, 59, 60, 69, 79). Comprehensive analyses of 16S rRNA gene sequences from representative phytoplasmas yielded a phylogenetic tree (phytoplasma clade) and distinct phylogenetic lineages (subclades) that coincided with genomic clusters that were recognized through the use of DNA hybridization probes (32,53, 69, 79). Each distinct subclade was proposed to represent at least a species. Because it has not been possible to isolate phytoplasmas in pure culture, the convention of *"Candidatus* Phytoplasma" species has been adopted to refer to distinct lineages ofphytoplasmas (19, 89). In practice, phytoplasma 16S rDNA detected by the use of peR can be analyzed by RFLP analysis using selected restriction enzymes. The grouping of phytoplasmas based on RFLP analysis of 16S rRNA genes is consistent with phylogenetic classification based on nucleotide sequence analyses (32, 53). (see molecular techniques p. 303)

D. DIAGNOSTIC TESTS

1. Detection in plant tissue by light microscopy

a) Dienes' Stain

Dienes' stain (23) may be used to detect mollicutes in plant tissue. The stain, originally developed for detection of mycoplasma colonies on agar plates, is taken up by the mollicutes. Tissues of plants with several other pathogens, including fungi, bacteria, and viruses, do not stain (23).

Materials: Single-edge razor blades, glass slides, cover slips, paper towels,

Pasteur pipettes, light microscope, and Dienes' stain (23) (in 100 ml water dissolve 2.5 g methylene blue, 1.25 g azure II, 10.0 g maltose, and 0.25 g sodium carbonate. Filter mixture through Whatman #1 paper. For use, dilute this concentrate to 0.2% in water).

I) Cut fresh thin sections of plant stems, petioles, or midribs with a new single-edge razor blade into a drop of water, or use a freezing microtome to cut sections of 100 *J.lm.* Add a cover slip.

2) Using absorbent paper, draw water from under the cover slip on one side while adding 0.2% Dienes' stain with a dropper at the other side.

3) The time required for staining varies with section integrity and thickness, from immediate to 10-15 minutes. Observations may be begun immediately and when staining is complete, replace the stain with water.

4) Xylem vessels will stain deep blue in healthy or diseased material and this helps the observer to locate the phloem area. A positive reaction is seen as patches of blue-stained sieve cells within the phloem area.

5) It is important to include negative (and, where possible, positive) controls. It is also recommended that a diagnosis based on Dienes' stain be confirmed by another method of mollicute detection.

b) DAPI (4', 6-diamidino-2-phenylindole)

DAPI, a fluorochrome dye that binds nonspecifically to DNA, is now considered a standard for mollicute detection. The following protocol was adapted from that of Seemuller and Kirkpatrick (78).

1) Use freshly collected plant parts for DAPI staining. Roots, stems, petioles, or midribs may contain relatively higher titers of mollicutes in their sieve tubes. Excise sections up to 8 rom long and 2 rom thick, and split them to allow greater stain infiltration.

2) Fix tissues in glutaraldehyde (5% in 0.1 M phosphate buffer, pH 7.0) and refrigerate.

3) Wash samples in 0.1 M phosphate buffer and cut sections (15-25 rom thick) with a freezing microtome (preferably) or by hand.

4) Place sections on a glass slide and cover with DAPI stain (0.1 mg stain in 100 ml phosphate buffer, dissolved by agitating 30 minutes at room temperature passed through a 0.22 ml filter, and stored refrigerated in the dark).

5) Stain 2-5 min, then blot and seal cover slip edges with fingernail polish.

6) Examine sections immediately (best) or within 48 h. A microscope equipped for epifluorescence is required.

Seemuller and Kirkpatrick recommend: a high pressure mercury lamp (HB050 or HBOIOO), exciter filter transmitting at 300-400 nm (eg., UG 1), barrier filter transmitting above 400 nm (eg., K400 or K430) and two objectives of 10 and 65-80X.

7) DAPI does not distinguish between DNA from mollicutes, other pathogens, or plants. Mature plant sieve tubes lack nuclei, but young sieve tubes may still contain them. In addition, DAPI will stain the DNA of other plant pathogens. It is recommended that healthy controls be used for comparison, and that any diagnosis based on DAPI staining be confirmed by another method of mollicute detection.

2. In situ visualization of spiro plasma and phytoplasma by enzyme treatment (47)

a) Enzyme solution, pH 5.5

	per 100 ml
Cellulase	0.8 g
Macerozyme	0.4 g
$CaCl_2a2H_20$	1.0 mM
PVP-40	0.5 g
Mannitol	12.5 g

First, dissolve cellulase and macerozyme in water until the solution is clear. Add mix ofPVP-40 and mannitol while vortexing. It takes time to dissolve PVP-40. Adjust pH to 5.5 with O.IN NaOH. Filter the solution through a 0.45 zzrn pore size Nalgene filter.

b) Buffer solution, pH 7.2

	per 100 ml
HEPES	0.05M
$CaCl_z \cdot 2H_20$	1.0 roM
Mannitol	12.5 g

Adjust pH to 7.2 with 2N NaOH

c) Procedure

1) Surface sterilize young leaves (periwinkle plant) showing early symptoms with 20% Clorox for 5 min, and then rinse twice in sterile distilled water.
2) Strip the veins or veinlets with a sharp forceps (EM grade). Place the leaf right side up on a cover of plastic Petri plate. Strip the midribs longitudinally by pressing forceps (opened about 1 mm wide, and secured by pressing the forceps with your index finger) at a 30° angle on the center portion of the petiole. Move along both edges of the midrib so that only the central portion is removed.
   The vascular tissues on both sides of the stripped midvein must be VIsmLE in order to be fully digested by enzymes.
3) Transfer the stripped veins, veinlets or midribs into Petri plates containing the macerating enzymes in solution.
4) Incubate the petri plates at 24 - 27°C in dark for 3-5 h or at 4°C overnight. At this point, the tissues should have a water-soaked appearance; the vascular bundles (central greenish portions) can be easily parted from surrounding parenchymatous cell mass.
5) Peel off the vascular bundles with two sharp forceps and transfer them into Petri plates containing new batches of enzyme solution. Allow to be further digested for 30-60 min at room temperature.
6) Gently transfer the digested vascular bundles to the buffer solution (pH 7.2) and incubate at room temperature at least 30 min.
7 Carefully strip the phloem tissues, consisting of layers of sieve elements, from the xylem tissues. Observe the phytoplasma (bluish white short filamentous or in clumps) or spiroplasma (helical shape) in the translucent sieve elements by dark-field microscopy (1,000 or 1,250X).

E. PATHOGENICITY TESTS

a. Transmission by insect vectors.

Since phytopathogenic mollicutes cannot be mechanically transmitted to plants,

pathogenicity tests with cultured spiroplasmas require infected insect vectors. Insects can be injected with finely drawn glass needles (65). Healthy vector insects may also acquire the spiroplasma through feeding on liquid suspensions or through parafilm membranes (74). Following an appropriate latent period in the insect vector, the spiroplasma can be transmitted by the vector to test plants during an inoculation access period (lAP). Usually, plants are held at 30° to 32°C (night temperatures of 26°C can be permitted for S. *citri;* E. C. Calavan, personal communication) and observed for subsequent development of characteristic disease symptoms (65, 70, 71). Pathogenicity of a given spiroplasma strain should be tested using a log phase broth culture of that strain.

Cultures are produced by inoculating 5 ml sterile broth medium with 0.1 ml of a log phase broth culture filtrate (0.45 *,urn* pore diam.). Filtration removes clumped spiroplasma cells from the inoculum. Estimates of titer during early log phase are often more accurate when broth is seeded with filtered spiroplasma. Incubate the seeded broth medium at 30-32°C for 24 to 72 h (depending on the growth rate of the strain being tested) to produce a high titer log phase culture. Estimate the viable colony forming units per ml (CFU/ml) by duplicate plating of serial ten-fold dilutions (using sterile broth medium as diluent) on sterile agar plates.

Concentrated suspensions of spiroplasma cells for membrane feeding tests may be prepared by centrifuging a broth culture at 10,000 g for 20 min. Resuspend pelleted cells in sterile 5% sucrose - 0.02 M phosphate buffer, pH 7.2 (74) to give approximately 10^9 CFU/ml. Suspensions that are more dilute may be necessary if the spiroplasma studied is pathogenic to the insect vector. Actual CFU/ml in this concentrate can be determined by plating dilutions on agar, but many individual colonies may have arisen from clumps of cells resulting from the centrifugation. The spiroplasma suspension is placed in a reservoir made with a stretched parafilm bottom (74). Healthy leafhopper vectors (in the case of S. *citri*, starved 8 to 12 h) adults of *Circulifer tenellus,* or adults or nymphs of *Scaphytopius nitridus* (74) are confined beneath the parafilm and allowed to feed in an inverted position, through the parafilm membrane for an acquisition access period (AAP) of 6 to 8 h at 28-30°C under continuous light (74). The insects are then caged with test plants of a susceptible species under appropriate conditions. In the case of C. *tenellus* (89), plants may include a healthy seedling of *Citrus sinensis* (Madame Vinous sweet orange) and a healthy sugar beet plant. Plants are maintained at 26-30°C for at least 5 months to observe symptoms. S. *nitridus,* after acquiring S. *citri* by membrane feeding, have been held on orange seedlings for 28 days to inoculate the plant (74). Since inoculation efficiency is low, use at least 20 plants, each exposed to 20 to 30 insects during at least the fourth, fifth, and sixth weeks (74) after the acquisition feeding. The duration of the latent period in the insect, between acquisition access and ability to transmit the spiroplasma to plants,
is expected to vary with the spiroplasma dose ingested or injected. Inoculation of the plants by insect vectors should be confirmed by re-isolation, i.e. culture of the spiroplasma from the plants.

Micro-injection ofleafhoppers with spiroplasma suspensions may be accomplished using finely drawn glass capillary needles having an orifice of about 10 to 20 *,urn* in diameter (65). The insects can be immobilized by restraining devices, by cooling (freezing for 1-2 minutes in a closed container until they become torpid; the exact times vary for each insect species), or by anesthesia (such as a stream of'Cu, gas flowing over the insects) while they are positioned for injection. For injection, insects may be placed on a moist filter paper pad (65) or on a vacuum stage. A simple vacuum stage may be constructed from a plastic Petri dish by cutting a circular orifice in one side for insertion of a vacuum hose. Excise a small rectangular region of plastic (large enough to contain 5-10 leafhoppers) in the top, over which a fine-mesh cloth is glued (A. Wayadande and J. Fletcher, unpublished). With the latter device, torpid insects can be positioned, ventral side up, onto the screen while a very gentle vacuum secures the insects in place. A volume of approximately 0.1 ~l of spiroplasma suspension is injected into a shallow ventral puncture usually made between the third and fourth abdominal sternites. Pressure for the injection may be provided through a rubber or polyethylene tube, from a rubber syringe bulb, air line, peristaltic or other pump, or the experimenter's mouth. An excellent account of the microinjection procedure has been published (65). Following an incubation (latent) period (two to four weeks, depending on the dose injected) on suitable host plants, the insects are caged on test plants for an inoculation feeding period of from several days to two weeks. Inoculation of young, vigorously growing test plants is recommended.

It should be noted that a spiroplasma strain may lose pathogenicity during prolonged subcultivation *in vitro.* Pathogenicity should be tested using a culture having as brief a subculture history as possible, provided that sufficient dilution has taken place during subculture to eliminate any non-multiplying infectious agent that may have been present in the primary culture inoculum.

2. Transmission by grafting.

Phytopathogenic spiroplasmas and phytoplasmas may be transmitted from one plant to another by grafting. This technique works well with some plant species, including the common mollicute maintenance host, periwinkle *(Catharanthus roseus),* but not with others.

a) Most commonly, a young terminal symptomatic shoot (1-2 in, in length) of an infected plant is cut from the parent plant to serve as a scion.

b) Flowers and all but a few terminal leaves are gently stripped away, and the stem end is shaped with a sharp razor blade into a V-shape.

c) The recipient plant is ideally a seedling whose main stem is approximately the same diameter as the scion. The terminal tip (1-2 in) ofthis main stem

is removed and the remaining stem serves as the stock.

d) A slit, bisecting the stock into two equal-diameter sections connected at the lower end, is made with a sharp blade; its depth should match the length of the "V" in the scion.

e) Finally, the V-shaped scion is fitted into the prepared stock so that the phloem tissues of both plants are in contact, and the two stems are stabilized by wrapping the junction several times with stretched parafilm.

f) It is helpful to place newly grafted plants out of direct sunlight and into a humid environment (such as a plastic bag secured to the pot rim) for one to two days after the grafting.

3. Transmission by dodder *(Cuscuta* spp.)

This method is useful in transmitting mollicutes between plants that cannot be grafted. There are at least 17 species of dodder, chlorophyll-less parasites for which different host ranges are known. Different species may also vary in effectiveness. The two species, C. *compestris* and C. *subinclusa,* are most widely used for transmitting mollicutes from infected to healthy plants (4,37, 57,61). The following steps are recommended:

a) To exclude any of several known dodder-borne viruses, dodder should be started from seed. If necessary, scarify seeds by rubbing with a file, then germinate on moist filter paper in a sterile petri dish.

b) When golden threads are 2-4 em long, place them in contact with stem or petiole of the host plant. Place plant with dodder into a humid chamber or enclose in a plastic bag to retain humidity.

c) For transmission, remove or train a thread of dodder of 10-20 ern and place on an infected plant keeping the growing tip in contact with the plant. Keep in a humid environment until pegs form.

d) After 10-14 days, train some strands of the dodder onto a test plant (place it next to infected plant). Alternatively, remove a 10-20 em recipient strand from the infected plant and transfer to the test plant. Keep test plants in a humid environment until pegs form.

e) Observe test plants for symptom development.

F. MOLECULAR, SEROLOGICAL, AND COMMERCIAL AUTOMATED TECHNIQUES

1) Molecular techniques

a). Nucleic acid-based assays

Because of its high sensitivity, PCR assays using phytoplasma-specific "universal primers", designed from 16S rRNA gene or other conserved sequences, are useful for diagnosis and confirmation ofphytoplasmal infections (Table 3), which are preliminarily based on symptomatology or on the results ofDAPI or Diene's stain (1,22, 53, 56, 68, 77, 80). Identification of putative phytoplasmas can be achieved by RFLP analysis ofPCR products. Nested PCR may be performed by using two universal primer pairs or using one universal followed by a phytoplasma specific primer pair (31, 55). It provides both broad and specific detection ofphytoplasmas associated with plants and insect vectors. For detection and identification of a particular phytoplasma group or strain of interest, group-or strain-specific primers may be used in the PCR assay. For a detailed procedure, see the previous sections.

Southern hybridization assays (25,30,36,42,43,51,52,54,62) with cloned DNA probes are useful for differentiation of strains within a given phytoplasma group.

b) RFLP analysis ofPCR-amplified ribosomal DNA

The procedure is based on PCR amplification ofphytoplasma 16S rDNA using universal primers (Table 3) that recognize 16S rDNA sequences from all phytoplasmas but not from other prokaryotes or plant sources. Phytoplasma groups or subgroups are differentiated based on RFLP analysis of peR-amplified phytoplasma 16S rDNA sequences using a number of frequently cutting restriction endonucleases (53,56, 77). Each phytoplasma group or subgroup represents a distinct collective RFLP pattern type (Fig. 2). The accuracy of identification relies on the purity ofPCR products. Often nested PCR is necessary to obtain pure phytoplasma 16S rDNA from infected woody plants (31,53,55).

See Appendix A for additional details.

8)

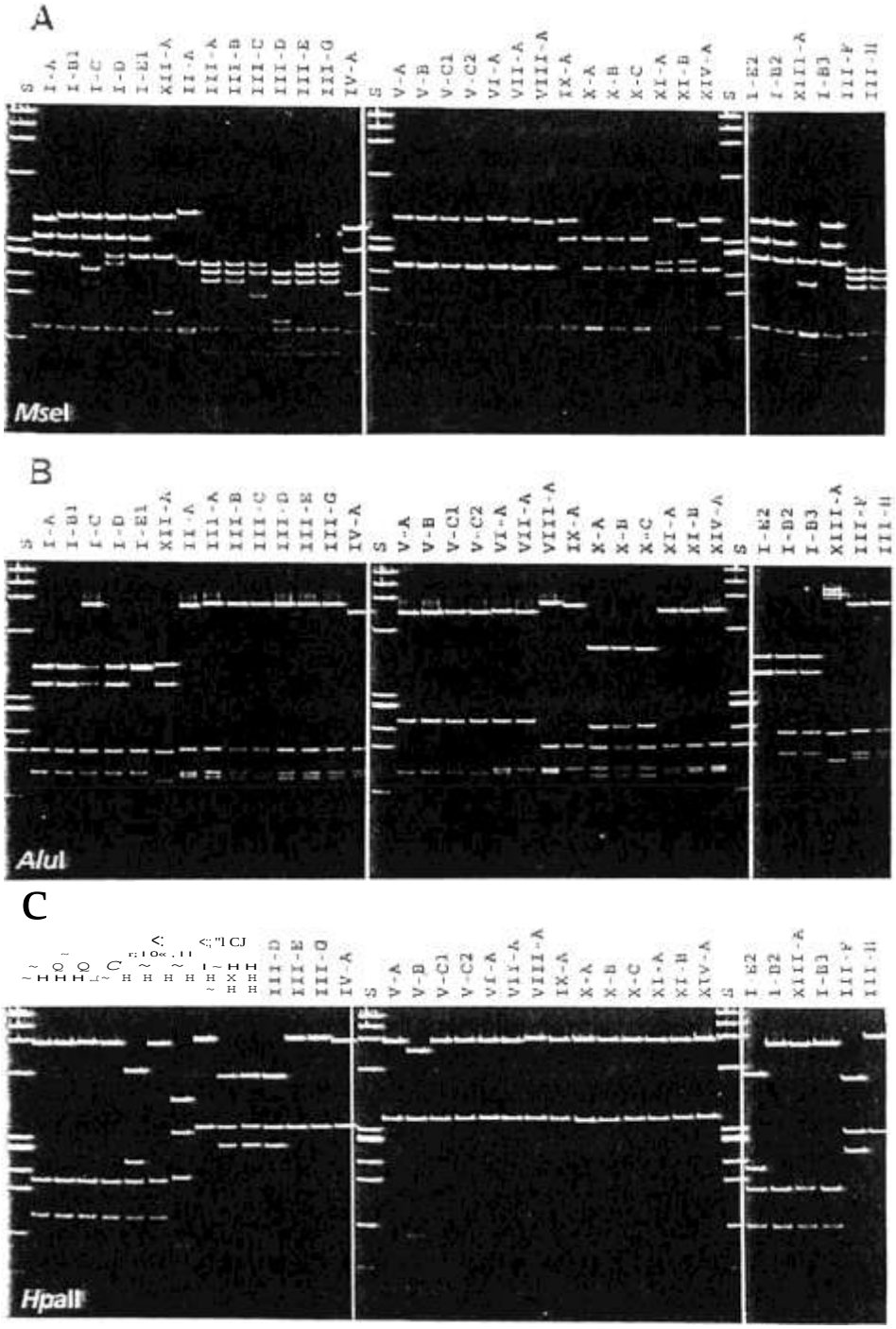
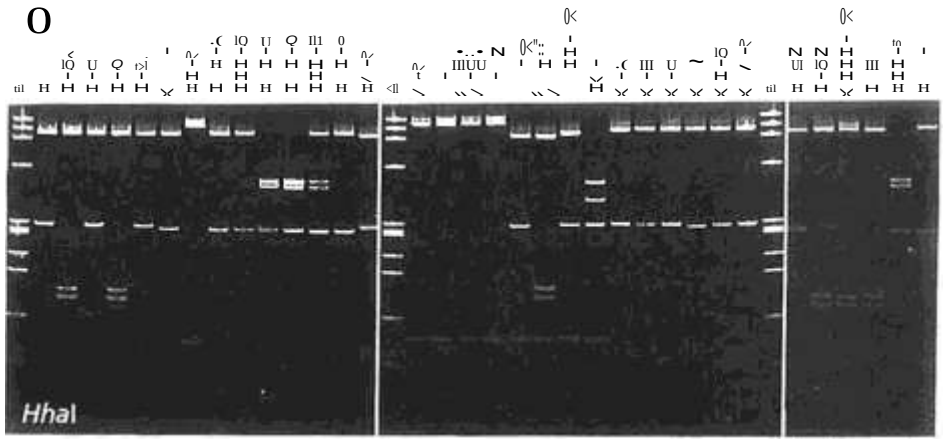
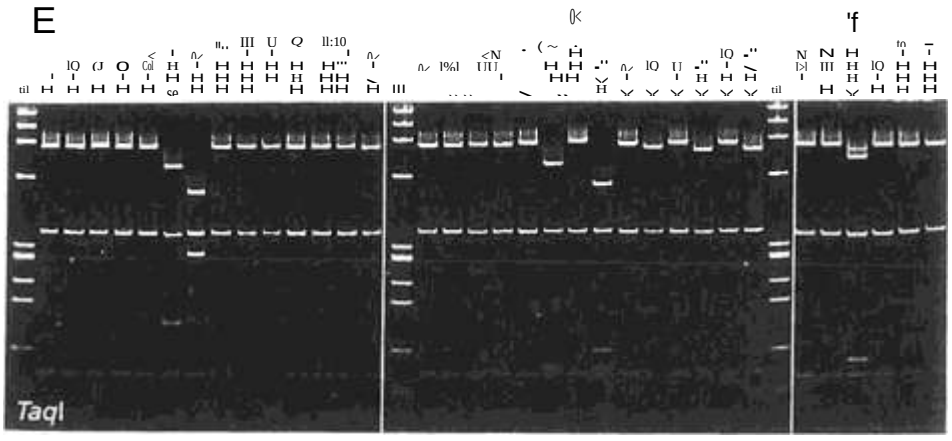


Fig. :2a, h. RFLP patterns of representative phytoplasma 16SrRNA groups and subgroups derived from analyses of 16S rDNAs *(RI6F2nJR16R2* nested peR products). DNA products were digested with *AlseI* (A), *AluI* (B), *HpaII* (C), *HhaI* (D), *TaqI* (E), or *RsaI* (F) and separated by electrophoresis through a .5% polyacrylamide gel. Lane S, <!>X174 RFI DNA *HaeIII* digest, ç.J (1.:.iT/+ b tt, „ '3"~ 1078 °72 603 110 "'181 ·-'71 "'1'14 '9.:1 118 -') lragmem SIzes \DP) ,.om cop to 0 om. 1 ~.J, , II " F or codes of each subgroup strain (ie., IA ill 1) see Table 1, p. 283. Roman numerals indicate 16S rRNA group, letters indicate subgroups, and numerals indicate different strains in a subgroup.


O
HhaI


E
TaqI

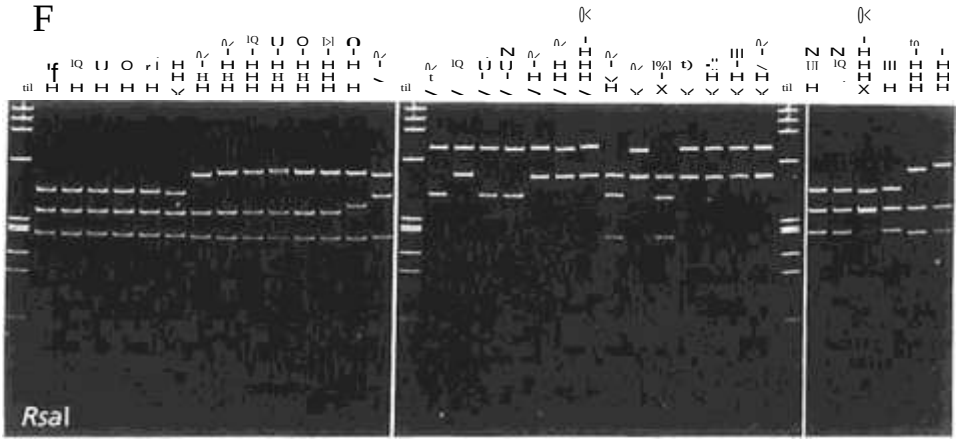

F
RsaI

Table 3 Oligonucleotide primers designed for phytoplasma detection

Primer	Target[a]	Primer sequence (5'-3')	Expected size of PCR product (bp)	Specificity	Reference
	16SrDNA SR	aagagtttgatcctggctcaggatt (11-30)/ cgtccttcatcggctctt (23S rDNA))		All phytoplasmas	24, 80
RI6F1/RI6R0	6SrDNA	aagacgaggataacagttgg (129-149)/ ggataccttgttacgacttaacccc (1483-1458)		All phytoplasmas	
RI6mF2/ R16mR1		catgcaagtcgaacgga (53-69)/ cttaaccccaatcatcgac (1487-1469)		All phytoplasmas	
RI6F2/ R16R2	16SrDNA	acgactgctgctaagactgg (152-168)/ tgacgggcggtgtgtacaaaccccg (1397-1373)		All phytoplasmas	
RI6F2n/ R16R2	16SrDNA	gaaacgactgctaagactgg (149-168)/ tgacgggcggtgtgtacaaaccccg (1397-1373)		All phytoplasmas	
fU5/rU3	16SrDNA	cggcaatggaggaaact (369-386)/ ttcagctactctttgtaaca (1251-1231)		All phytoplasmas	
Pl/Tint	16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ tcaggcgtgtgctctaaccagc (SR-t RNA)		All phytoplasmas	
	16SrDNA	gtttgatcctggctcaggatt (1-21)/ cctcagcgtcagtaa (746-732)		All phytoplasmas	
16R758F/ 16R1232R	16SrDNA	gtctttactgacgctgaggc (738-758)/ cttcagctacccttttgtaac (1251-1232)		All phytoplasmas	

16SrDNA	taaaagacctagcaatagg (278-297)/ caatccgaactgagactgt (1297-1279)	
16SrDNA	tttaagcaattaaacttta (63-81)/ aaccccgagaacgtattcacc (1368-1348)	
16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ tacaatttgcaagcaagttac (SR)	
16SrDNA	aagagtggaaaaactccc (457-474)/ tccgaactgagattga (1355-1340)	
16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ gacagtgcttataacttttta (SR)	
16SrDNA	ttaaaagaccttcttcgg (204-221)/ ttcaatccgtactgagactacc (1359-1338)	
16SrDNA, SR	gaccttcaaaggtcttag (73-91)/ cgtcttttatataagagaaaca (SR)	
16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ gatgattttagtatatatagtcc (SR)	
16SrDNA, SR	gaccttcaaaggtcttag (73-91)/ gcaggaccgtttatattaatc (SR)	
16SrDNA	gacccgcaagtatgctgagagatg (210-233)/ caatccgaactgagactgt (1358-1339)	

9)

Table 3. (continued)

Primer pair	Target[a]	Primer sequence (5'-3')[b]	Expe... PCR product (bp)	Specificity
fAT/rAS	16SrDNA SR	ccatcatttagttgggcactt (1113-1131)/ ggcccccggaccattatttatt (SR)		
P1/PYLRint	16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ cccggccattattaatttttatc (SR)		
fPD/rPDS	16SrDNA, SR	gacccgtaaggtatgctga (205-324)/ cccggcaattattaatttta (SR)		
	16SrDNA	gtttgatcctggctcaggatt (1-21)/ atgcaccacctgtatcc (1031-1015)		
rpF1/rpR1	rp genes	ggacataagttaggtgaattt (rps19)/ acgatatttagttctttttgg (rpl16)		
rpF2/rpR2	rp gene	tctcgtacttttcgtgg (rps19)/ accttagctcttggaa (rpl22)		
rp3/rp4	rp gene	aacttctagcacaaaacttgc(rpl22)/ gtctgttaggagtgttagaa(rps3)		
	rp gene	agaaaggcattaaacatatgaat/ cgctgttcataatttagcct		
VrpF1/rpR1	rp gene	tcgcggtcatgcaaaaggcg/ acgatatttagttctttttgg		
	Cloned non-rDNA	gggttaaggctagaaatggatcttg/ tatcagatatcattggcgaaggact		

BB88F1/ BB88R1	Cloned non-rDNA	caccatcacacgcgtgatgaccgcctttcc/ gccttacaactacaccatcagtttgaggag
CK6/CK8	Plasmid DNA	cctctttaggcccaatagcag/ ctcatgttttcagcttgggttc
	Cloned non-rDNA	aacccaaactatcaaaag/ tgtttctacttcttcttg
AY19p/AY19m	Cloned non-rDNA	taacatcagaataaatgg/ gacttacgttggtgaagg
G35p/G35m		taacactgtggaagctca/ cgtcaatggctaatcgat
MBS-F1/MBS-R1	Cloned non-rDNA	aatgtcgaactaacaggcgg/ ttggcgatttggttttgg
EY11-F1/ Ey11-R1	Cloned non-rDNA	gactcteggatgagagtcttgggtg/ cttgggattatggccctgttataccag
	Cloned non-rDNA	tcttttacctaaatttgaggtaattc/ ttgtgttatcgcaaggggctttagg
LY-F1/LY-R1		catatttttatttccttgcaatctg/ tcgttttgataatcttcatttgac
	Cloned non-rDNA	gccatcattaagttgggga/ agatgtgacctatttttggtgg

2) Serological techniques

a) Growth inhibition serological test for *Spiroplasma* identification:

(1) Prepare filter paper discs containing sera:

(a) Cut filter discs (7 mm diam.) from filter paper, and sterilize the discs by autoclaving.

(b) Aseptically place several discs in a sterile Petri plate; keep the discs separated from one another.

(c) Aseptically pipette 0.025 ml of undiluted rabbit antiserum (prepared against a serogroup I spiroplasma such as S. *citri)* onto each sterile filter paper disc, allow antiserum to dry on discs, and store discs dry at 20-25°C. (See flow diagram illustrating steps in *Spiroplasma* identification, p. 295.)

(d) Prepare a separate set of discs using preimmune rabbit serum (normal serum) to use as controls.

(2) Set up the test:

(a) Use 6-cm diameter Petri plates each containing 5 ml of agar-solidified culture medium. Assure that there is no liquid on the surface of the agar; the agar surface may be dried by partially removing the Petri plate covers for a few min in a biological safety cabinet.

(b) Using broth culture medium or sterile 10% sucrose, aseptically prepare a 10- fold dilution series of the unknown *Spiroplasma* sp. Begin with a log phase culture of the spiroplasma.

(c) Onto the surface of each agar-solidified culture medium in Petri plates, aseptically pipette 0. 1 ml of spiroplasma culture diluted to yield 1000 to 5000 colonies per plate. In practice, this may be most conveniently be accomplished by preparing three plates with each of two or three dilutions of spiroplasma culture, giving a total of six to nine seeded plates. In parallel with the test, serial dilutions of the original broth culture are plated out on agar-solidified culture medium to estimate the colony forming units (CFU) on the agar plates seeded for the growth inhibition test.

(d) Hold plates, seeded with diluted spiroplasma culture, at 30-32°C, for 2 h to permit the agar to absorb excess liquid.

(e) Place a filter paper disc saturated with normal serum or antiserum on the surface of the agar medium, in the center of the Petri plate.

(t) Incubate the plates at 30-32°C.

(3) Reading test results:

(a) Using a dissecting microscope, observe the Petri plates for development of spiroplasma colonies. Some spiroplasma strains develop visible colonies within 5 to 7 days; other strains require incubation for ten days to two weeks.

(b) Measure zones of growth inhibition (from edge of filter paper disc to growth of colonies) with the aid of a dissecting microscope. Preimmune (normal) sera should result in zones of inhibition of less than 1 mm. Zones of growth inhibition that are induced by antisera that was prepared against cells of homologous *Spiroplasma* sp. are often 12 to 15 mm or greater in width and may be bordered by a ring of precipitation. Cells of strains of S. *citri* cross react (partial identity) with antisera against cells of S. *kunkelii* strains (17, 83).

b) Enzyme-Linked Immunosorbent assay ELISA for detection of spiroplasma and phytoplasma.

The enzyme-linked immunosorbent assay can be used for detection of specific organisms from infected tissue (5, 9). See Appendix A for a description of the technique.

See Appendix B for details.

3) Commercial automated techniques.

Elisa kits are available commercially.

See Appendix C for details.

G. LITERATURE CITED

1. Ahrens, u., and E. Seemuller. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82:828-832.

Bertaccini, A., M. Vibio and E. Stefani. 1995. Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). Phytopathol. Mediter. 34: 137-141.

3. Calavan, E. C., E. O. Olson, and D. W. Christianson. 1972. Transmission of the stubborn pathogen in citrus by leaf-piece grafts. Pages 11-14 in: W. C. Price, ed., Proc. 5th Conference International Organization of Citrus Virologist, University of Florida. University of Florida Press, Gainesville.

4. Carraro, L., N. Loi and M. A. Favali. 1991. Transmission characteristics of the clover phyllody agent by dodder. 1. Phytopathol. 133: 15-22.

5. Cassell, G. H. and M. B. Brown. 1983. Enzyme-linked irtununosorbent assay (ELISA) for detection of anti-mycoplasma antibody. Pages 457-469 in: S. Razin and 1. G. Tully, eds., Methods in Mycoplasmology Vol. 1. Academic Press.

6. Chen, 1. c., C. 1. Chang, R. Jarret, and N. Gawel. 1992. Isolation and cloning of DNA fragments from mycoplasma-like organism associated with walnut witches' -broom disease. Phytopathology 82:306-309.

7. Chen, T. A., 1. D. Lei, and C. P. Lin. 1989. Detection and identification of plant and insect mollicutes. Pages 393-424 in: R. F. Whitcomb and 1. G. Tully ed., The Mycoplasmas Vol. 5. Academic Press, New York.

8. Chen, T. A. and C. H. Liao. 1975. Com stunt spiroplasma: Isolation, cultivation, and proof of pathogenicity. Science 188: 1015-1016.

9. Clark, M. F. and A. N. Adams. 1977. Characteristics of the microplate method of enzyme linked immnosorbent assay for detection of plant viruses. 1. Gen. Virol. 34:475483.

10. Clark, T. B., R. E. Whitcomb, 1. G. Tully, C. Mouches, C. Saillard, 1. M. Bove, H. Wroblewski, P. Carle, D. L. Rose, R. B. Henegar, and D. L. Williamson. 1985. *Spiroplasma melliferum,* a new species from the honeybee *(Aphis mellifera).* Int. J. Syst. Bacteriol. 35:296-308.

11. Dale, 1. L. and L. D. Smith. 1975. Mycoplasma-like bodies observed in tomato plants with big bud in Arkansas. Plant Dis. Rept. 59:455-458.

12. Daniels, M. 1., P. G. Markham, M. Meddins, A. K. Plaskitt, R. Townsend, and M. Bar-Joseph. 1973. Axenic culture of a plant pathogenic spiroplasma. Nature (London). 244:523-524.

13. Davis, R. E. 1978. Spiroplasma associated with flowers of tulip tree *(Liriodendron*

tulipifera L.). Can. 1. Microbiol. 24:954-959.

14. Davis, R. E. 1979. Spiroplasmas: Newly recognized arthropod-borne pathogens. Pages 441-448 in: K. Maramorosch and K. F. Harris, eds., Leafhopper Vectors and Plant Disease Agents. Academic Press, New York.

15. Davis, R. E. 1974. Spiroplasma in com stunt-infected individuals of the vector leafhopper *Dalbulus maidis.* Plant Dis. Rept. 59: 1109-1112.

16. Davis, R. E. and I.-M. Lee. 1982. Comparative properties of *Spiroplasma* and emerging taxonomic concepts: A proposal. Rev. Infect. Dis. 4:(Supplement) 122-128.

17. Davis, R. E., I.-M. Lee, L. K. Basciano. 1979. Spiroplasmas: Serological grouping of strains associated with plants and insects. Can. 1. Microbiol. 25:861-866.

18. Davis, R. E., 1. F. Worley, R. F. Whitcomb, T. Ishijima, and R. L. Steere. 1972. Helical filaments produced by a mycoplasma-like organism associated with com stunt disease. Science 176:521-523.

19. Davis, R. E., E. L. Dally, D. E. Gundersen, I.-M. Lee, and N. Habili. 1997. "Candidatus Phytoplasma australiense", a new phytoplasma taxon associated with Australian grapevine yellows. Int. 1. Syst. Bacteriol. 47:262-269.

20. Davis, R. E., and I.-M. Lee. 1993. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasma-like organisms. Phytopathology 83: 1008-1011.

21. Davis, R. E., 1. P. Prince, R. W. Hammond, E. L. Dally, E. L., and I.-M. Lee. 1992. Polymerase chain reaction detection ofItalian periwinkle virescence mycoplasma-like organism (MLO) and evidence for relatedness with aster yellows MLOs. Petria 2: 184-193.

22. Davis, R. E., W. A. Sinclair, I.-M. Lee, and E. L. Dally. 1991. Cloned DNA probes specific for detection of a mycoplasma-like organism associated with ash yellows. Mol. Plant-Microbe Interact. 5: 163-169.

23. Deely, 1., W. A. Stevens, and R. T. V. Fox. 1979. Use of Dienes' stain to detect plant diseases induced by mycoplasma-like organisms. Phytopathology 69: 1169-1171.

24. Deng, S., and C. Hiruki. 1991. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. 1. Microbiol. Meth. 14:53-61.

25. Deng, S. and C. Hiruki. 1991. Genetic relatedness between two non-culturable

mycoplasma-like organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81: 1475-1479.

26. Doi, Y. M., M. Teranaka, K. Yora, and H. Asuyama. 1967. Mycoplasma or PLT-grouplike microorganisms found in the phloem elements of plants infected with mulberry dwarf potato witches' -broom, aster yellows, or paulownia witches' -broom. Ann. Phytopathol. Soc. Jpn. 33:259-266.

27. Fudl-Allah, A. E. S. A. ,E. C. Calavan, and E. C. K. Igwegbe. 1972. Culture ofa mycoplasma-like organism associated with stubborn disease of citrus. Phytopathology 62:729-731.

28. Gibb, K. S., A. C. Padovan, and B. D. Mogen. 1995. Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plant species in northern Australia. Phytopathology 85: 169-174.

29. Goodwin, P. H., B. G. Xue, C. R. Kuske, and M. K. Sears. 1994. Amplification of plasmid DNA to detect plant pathogenic mycoplasma-like organisms. Ann. Appl, BioI. 124:27-36.

30. Griffiths, H. M., D. E. Gundersen, W. A. Sinclair, I. -M. Lee, R. E. Davis. 1994. Mycoplasma-like organisms from milkweed, goldenrod, and spiraea represent two new 16S rRNA sub-groups and three new strain subclusters related to peach X-disease. Can. 1. Plant Pathol. 16: 255-266.

31. Gundersen, D. E. and I. -M. Lee. 1996. Ultrasensitive detection ofphytoplasmas by nested-PCR assays using two universal primer pairs. Phytopath. Medit. 35: 114-151.

32. Gundersen, D. E., 1. -M. Lee, S. A. Rehner, R. E. Davis, and D. T. Kingsbury. 1994. Phylogeny of mycoplasma-like organisms (phytoplasmas): a basis for their classification. 1. Bacteriol. 176:5244-5254.

33. Gundersen, D. E., 1. -M. Lee, D. A. Schaff, N. A. Harrison, C. J. Chang, R. E. Davis, and D. T. Kingsbury. 1996. Genomic diversity and differentiation among phytoplasma strains in16S rRNA group I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). Int. 1. Syst. Bacterioi. 46:64-75.

34. Harrison, N. A. 1996. PCR assay for detection of the phytoplasma associated with maize bushy stunt disease. Plant Dis. 80:263-269.

35. Harrison, N. A., P. A. Richardson, 1. B. Kramer, and 1. H. Tsai. 1994. Detection of the mycoplasma-like organism associated with lethal yellowing disease of palms in Florida by polymerase chain reaction. Plant Pathoi. 43:998-1008.

36. Harrison, N. A., 1. H. Tsai, C. M. Bourne, and P. A. Richardson. 1991. Molecular cloning and detection of chromosomal and extrachromosomal DNA of mycoplasma-like organisms associated with witches'-broom disease of pigeon pea in Florida. Mol. Plant-Microbe Interact. 4:300-307.

37. Hibben, C. R. and B. Wolanski. 1971. Dodder transmission of a mycoplasma from ash witches' -broom. Phytopathology 61: 151-156.

38. Jarausch, W., C. Cellaret, F. Dosba, and 1. M. Bobe, 1994. Differentiation of mycoplasma-like organisms (MLOs) in European fruit trees by PCR using specific primers derived from the sequence of a chromosomal fragment of the apple proliferation MLO. Appl. Environ. Microbiol. 60:2916-2923.

39. Kirkpatrick, B. C. 1991. Mycoplasma-like organisms-plant and invertebrate pathogens. Pages 4050-4067 in: A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer, eds., The Prokaryotes. Vol. 3. Springer, New York.

40. Kirkpatick, B. C., N. A. Harrison, I-M. Lee, H. Neimark, and B. B. Sears. 1995. Isolation of mycoplasma-like organism DNA from plant and insect hosts. Pages 105-117 in: S. Razin and 1. G. Tully, eds., Molecular and Diagnostic Procedures in Mycoplasmology. Vol. 1. Academic Press, New York.

41. Kirkpatrick, B. C., D. C. Stenger, T. 1. Morris, and A. H. Purcell. 1987. Cloning and detection of DNA from nonculturable plant pathogenic mycoplasma-like organism. Science 238:197-200.

42. Kison, H., B. Schneider, and E. Seemuller. 1994. Restriction fragment length polymorphisms within the apple proliferation mycoplasma-like organism. 1. Phytopath. 141:395-401.

43. Kuske, C. R., B. C. Kirkpatrick, and E. Seemuller. 1991. Differentiation ofvirescence MLOs using western aster yellows mycoplasma-like organism chromosomal DNA probes and restriction fragment length polymorphism analysis. 1. Gen. Microbiol. 137:153-159.

44. Lee, I.-M., A. Bertaccini, M. Vibio, and D. E. Gundersen. 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 85:728-735.

45. Lee, I.-M., A. Bertaccini, M. Vibio, D. E. Gundersen, R. E. Davis, L. Mittempergher, M. Conti, and F. Gennari. 1995. Detection and characterization ofphytoplasmas associated with disease in *Ulmus* and *Rubus* in northern and central Italy. Phytopathol. Mediterr. 34: 174-183.

46. Lee, I.-M., G. Cartia, E. C. Calavan, and G. H. Kallostian. 1973. Citrus stubborn disease organism cultured from beet leafhopper. Calif Agric. 27: 14-15.

47. Lee, I.-M. and R. E. Davis. 1983. Phloem-limited prokaryotes in sieve elements isolated by enzyme treatment of disease plant tissues. Phytopathology 73: 1540-1363.

48. Lee, I.-M. and R. E. Davis. 1984. New media for rapid growth of *Spiroplasma citri* and com stunt spiroplasma. Phytopathology 74:84-89.

49. Lee, I.-M. and R. E. Davis. 1989. Serum-free media for cultivation of spiro plasmas. Can. J. Microbiol. 35: 1091-1099.

50. Lee, I.-M., and R. E. Davis. 1992. Mycoplasmas which infect plants and insects. Pages 379-390 in: J. Maniloff, R. N. McElhansey, L. R. Finch, and J. B. Baseman, eds., Mycoplasmas: Molecular Biology and Pathogenesis. American Society for Microbiology, Washington, D.C.

51. Lee, I.-M., R. E. Davis, 1. A. Chen, L. N. Chiykowski, J. Fletcher, C. Hiruki, and D. A. Schaff 1992. A genotype-based system for identification and classification of mycoplasma-like organisms (MLOs) in the aster yellows MLO strain cluster. Phytopathology 82: 977 -986.

52. Lee, I.-M., R. E. Davis, W. A Sinclair, N. D. DeWitt, and M. Conti. 1993. Genetic relatedness of mycoplasma-like organisms detected in *Ulmus* spp. in the United States and Italy by means of DNA probes and polymerase chain reactions. Phytopathology 83:829833.

53. Lee, I.-M., D. E. Gundersen-Rindal, R. E. Davis, and I. M. Bartoszyk. 1998. Revised classification scheme ofphytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. 48: 1153 -1169.

54. Lee, I.-M., Gundersen, D. E., Davis, R. E .. and L. N. Chiykowski. 1992. Identification and analysis of a genomic strain cluster of mycoplasma-like organisms associated with Canadian peach (eastern) X-disease, western X-disease, and clover yellow edge. J. Bacteriol. 174:6694-6698.

55. Lee, I.-M., D. E. Gundersen, R. W. Hammond, and R. E. Davis. 1994. Use of mycoplasma-like organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology 84:559566.

56. Lee, I.-M., R. W. Hammond, R. E. Davis, and D. E. Gundersen. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of

mycoplasma-like organisms. Phytopathology 83: 834-842.

57. Lee, I.-M., M. Klopmeyer, I. M. Bartoszyk, D. E. Gundersen-Rindal, T. -So Chou, K. L. Thomson, and R. Eisenreich. 1997. Phytoplasma induced free-branching in commercial poinsettia cultivars. Nature Biotechnol. 15: 178-182.

58. Lee, I.-M., M. Pastore, M. Vibio, A. Danielli, S. Attothom, R. E. Davis, and A. Bertaccini. 1997. Detection and characterization of phytoplasma associated with annual blue grass *(Poa annua)* white leafin southern Italy. Eur. 1. Plant Pathol. 103:251-254.

59. Lim, P. O. and B. B. Sears. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasma-like organisms are evolutionarily distinct from animal mycoplasmas. 1. Bacteriol. 171:5901-5906.

60. Lim, P. -0. and B. B. Sears. 1992. Evolutionary relationships ofa plant-pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. 1. Bacteriol. 174:2606-2611.

61. Loi, N., L. Carraro, R. Musetti, I. Pertot, and R. Osler. 1995. Dodder transmission of two different MLOs from plum trees affected by" leptonecrisis". Acta Horticulturae 386:465-470.

62. Lorenz, K. -H., B. Schneider, U. Ahrens, and E. Seemiiller. 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology 85:771-776.

63. Maixner, M., U. Ahrens, and E. Seemuller, 1995. Detection of the German grapevine yellows (Vergibungskrankheit) MLO in grapevine, alternative hosts, and a vector by a specific PCR procedure. Eur. 1. Plant Pathol. 101:241-250.

64. Marcone, C., A. Ragozziono, and E. Seemiiller. 1997. Identification and characterization of the phytoplasma associated with elm yellows in southern Italy and its relatedness to other phytoplasmas of the elm yellows group. Eur. 1. Forest Pathol. 27:45-54.

65. Markham, P. G., and R. Townsend. 1979. Experimental vectors of spiro plasmas. Pages 413-445 in: Maramorosch, K. and K. F. Harris, eds., Leafhopper Vectors and Plant Disease Agents. Academic Press, New York.

66. Maurer, R. E. Seemuller, and W. A. Sinclair. 1993. Genetic relatedness of mycoplasmalike organisms affecting elm, alder, and ash in Europe and North America. Phytopathology 83:971-976.

67. Nakashima, K., S. Kato, S. Iwanami, and N. Murata. 1993. DNA probes reveal

relatedness of rice yellow dwarf mycoplasma-like organisms (MLOs) and distinguish them from other MLOs. Appl. Environ. Microbiol. 59: 1206-1212.

68. Namba, S., S. Kato, S. Iwanami, H. Oyaizu, H. Shiozawa, and T. Tsuchizaki. 1993. Detection and differentiation of plant-pathogenic mycoplasma-like organisms using polymerase chain reaction. Phytopathology 83: 786-791.

69. Namba, S., H. Oyaizu, S. Kato, S. Iwanami, and T. Tsuchizaki. 1993. Phylogenetic diversity of phytopathogenic mycoplasma-like organisms. Int. 1. Syst. Bacteriol. 43:461467.

70. Nault, L. R., and O. E. Bradfute. 1979. Com stunt: Involvement ofa complex of leafhopper-bone pathogens. Pages 561-586 in: Maramorosch, K. and K. F. Harris, eds., Leafhopper Vectors and Plant Disease Agents. Academic Press, New York.

71. Oldfield, G. N., G. H. Kaloostian, H. D. Pierce, E. C. Calavan, A. L. Granett, R. L. Blue, G. L. Rana, and D. 1. Gumpf 1977. Transmission of *Spiroplasma citri* from citrus to citrus by *Scaphytopius nitridus.* Phytopathology 67:763-765.

72. Padhi, S. B., A. H. Mclntosh, and K. Maramorosch. 1977. Characterization and identification of spiro plasmas by polyacrylamide gel electrophoresis. Phytopath. Z. 90:268-272.

73. Prince, 1. P., R. E. Davis, T. K. Wolf, 1. -M. Lee, B. D. Mogen, E. L. Dally, A. Bertaccini, R. Credi, and M. Barba. 1993. Molecular detection of diverse mycoplasma-like organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. Phytopathology 83: 1130-1137.

74. Rana, G. L., G. H. Kaloostian, G. N. Oldfield, 1. -M. Lee, and D. 1. Gumpf 1975. Acquisition of *Spiroplasma citri* through membranes by homopterous insects. Phytopathology 65: 1143-1145.

75. Saglio, P., M. L'Hospital, D. Lafleche, G. Dupont, 1. M. Bove, 1. G. Tully, and E. A. Freundt. 1973. *Spiroplasma citri* gen. and sp. nov: A mycoplama-like organism associated with «stubborn" disease of citrus. Int. 1. Syst. Bacteriol. 23:191-204.

76. Saillard, C., 1. C. Vignault, 1. M. Bove, A. Raie, 1. G. Tully, D. Williamson, A. Fos, M. Garnier, A. Gadeau, P. Carle, and R. F. Whitcomb. 1987. *Spiroplasma phoeniceum* sp. nov., a plant-pathogenic species from Syria. Int. 1. Syst. Bacteriol. 37:106-115.

77. Schneider, B., U. Ahrens, B. C. Kirkpatrick, and E. Seernuller, 1993. Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis ofPCR-amplified 16S rDNA. 1. Gen. Microbiol. 139:519-527.

78. Seemuller, E. and B. C. Kirkpatrick. 1996. Detection ofphytoplasma infections in plants. Pages 299-311 in: Molecular and Diagnostic Procedures in Mycoplasmology, Vol. II. Academic Press, Inc., New York.

79. Seemuller, E., B. Schneider, R. Maurer, U. Ahrens, X. Daire, H. Kison, K -H. Lorenz, G. Firrao, L. Avinent, B. B. Sears, and E. Stackebrandt. 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. Int. 1. Syst. Bacteriol. 44:440-446.

80. Smart, C. D., B. Schneider, C. L. Blomquist, L. 1. Guerra, N. A. Harrison, U. Ahrens, K-H. Lorenz, E. Seemuller, and B. C. Kirkpatrick. 1996. Phytoplasma-specific peR primers based on sequences of 16S-23 S rRNA spacer region. Appl. Environ. Microbiol. 62:2988-2933.

81. Tully, 1. G., RF. Whitcomb, 1. M. Bove, and P. Saglio. 1973. Plant mycoplasmas: Serological relation between agents associated with citrus stubborn and corn stunt disease. Science 182:827-829.

82. Tully, 1. G., R. F. Whitcomb, H. F. Clark, D. L. Williamson, and D. L. Rose. 1977. Pathogenic mycoplasmas: Cultivation and vertebrate pathogenicity of a new spiroplasma. Science 195:892-894.

83. Whitcomb, R F., T. A. Chen, D. L. Williamson, C. Liao, 1. G. Tully, 1. M. Bove, C. Monchas, D. L. Rose, M. E. Coan, and T. B. Clark. 1986. *Spiroplasma kunkelii* sp. nov.: Characterization of the etiologic agent of com stunt disease. Int. 1. Syst. Bacteriol. 36:170-178.

84. Williamson, D. L. and R F. Whitcomb. 1975. A cultivable spiroplasma causes corn stunt disease. Science 188: 1018-1020.

85. Williamson, D. L., R F. Whitcomb, and 1. G. Tully. 1978. The spiroplasma deformation test: A new serological method. Curro Microbiol. 1:203-207.

86. Williamson, D. L., R F. Whitcomb, 1. G. Tully, G. E. Gasparich, D. L. Rose, P. Carle, 1. M. Bove, K J. Hackett, 1. R Adams, R. B. Henegar, M. Konai, C. Chastel, and F. E. French. 1998. Revised group classification of the genus *Spiroplasma.* Int. 1. Syst. Bacteriol. 48:1-12.

87. Yoshikada, N., H. Nakamura, N. Shashi, T. Kubono, K Katsube, T. Shoji, and T. Takahashi. 1994. Amplification and nucleotide sequences of ribosomal protein and 16S rRNA genes of mycoplasma-like organism associated with paulownia witches' -broom. Ann. Phytopath. Soc. Japan. 60:569-575.

88. Zhu, S., A. Hadidi, I. -M. Lee, D. E.Gundersen-Rindal, and C. Zhang. 1998. Characterization of the phytoplasmas associated with cherry lethal yellows and jujube witches' -broom diseases in China. Acta Horticulturae 472: 70 1-714.

89. Zreik, L., Carle, P., Bove. J, M., and Garnier, M. 1995. Characterization of the mycoplasma-like organism associated with witches' -broom disease oflime and proposition of a *Candidatus* taxon for the organism, *"Candidatus* Phytoplasma *aurantifolia"* Int. J. Syst. Bacterial. 45:449-453.

H. CHEMICALS LIST

CHEMICAL	**SOURCE**
Unless stated otherwise, all chemicals in this list can be obtained from Sigma Chemical Co., St. Louis, MO 63178.	
K-ketoglutarate	
Arginine	
Asparagine	
Bovine serum albumin	Life Technologies, Gaithersburg, MD
Cysteine	
Fetal bovine serum	
Glutamine	
HEPES buffer	
Horse serum	
Isopropanol	
Lipids	Life Technologies, Gaithersburg, MD
Methionine	
PPLO broth	
Phenol red	
Proteinase K	Difco
Pyruvate	
10% Sarkosyl (wt/vol)	
Yeast extract fresh 25%	Difco
Yeastolate	Difco

APPENDIX

A. MOLECULAR TECHNIQUES

F. J. Louws and D. A. Cuppels

1. INTRODUCTION

DNA-based molecular methods are an indispensable tool to identify plant pathogenic bacteria and will increase in importance and utility as techniques advance and associated expenses and personnel expertise decrease. The key advantage of DNA-based methods is that reliability of the identification assay is not dependent on the environmental conditions, age, or physiological state of the target pathogen. Some techniques are, however, dependent upon the quality of DNA extracted.

Important criteria that impact the adoption of DNA-based identification assays, common with other identification protocols, include specificity, sensitivity, and speed. Specificity can be understood as the ability to correctly identify the target pathogen with no false negatives when it is present and no false positives when it is not present. Specificity is largely determined by DNA probe or primer selection and hybridization or amplification conditions, respectively. For example, primers have been designed that are specific for all prokaryotes (12, 13), that are genus specific (e.g. for pseudomonads or xanthomonads), or group specific, as with phytoplasmas (see chapter V). Plant pathologists and practitioners often seek identification of pathogens that are associated with a specific disease complex and therefore primers and protocols must be specific at the species (e.g. *X fragariae;* p.192), pathovar (e.g. *P. syringae* pv. *phaseolicola;* p. 110) or even the strain level (e .. g. *X campestris* pv. *citri* group "A" strains; p.191). Specific identification ofa pathogen can be based on the amplification of a single DNA product or based on the pattern of amplified products (e.g. DNA fingerprint patterns) after electrophoretic separation and/or restriction analysis. Normally a probe should be used to confirm the identity of the amplified product.

F or detection techniques, sensitivity can be a complex issue and criteria could include the lowest number of cells (CFU's) detectable in the actual sample or relate to a threshold level known to be associated with predicted economic losses (2,35,38). Sensitivity depends on the nature of the DNA-based protocol, including sampling protocols and sample preparation. Reliable sampling protocols can be expensive because of the "one in ten-thousand problem" (18), where one infested seed in ten thousand may be below the detection level but sufficient to result in economic losses (8,37).

Speed can be a crucial issue for the practitioner. Often a decision to use a seed lot, vegetatively propagated material, sell a crop, or implement practices to limit disease risk, is time-sensitive. Recent protocols such as semi-automated systems (such as TaqMan ™) to detect amplified PCR fragments enhance speed without compromising specificity or sensitivity.

2. DNA EXTRACTION

The most rapid method (direct PCR) for obtaining DNA from bacterial cells for PCR involves transferring the cells directly to the PCR tube where they are lysed during an extended denaturation step prior to cycling. Liquid cultures can be added direct using a pipette tip or washed first. For washed cells, spin down 2 aliquots of 1.5 ml (3 ml) of liquid culture (OD_{600} 0.65-0.95) for 5 min in a micro centrifuge tube. Discard the supernatant and wash the pellet 1-2 times with 1 M NaCI and centrifuge again. Plant pathogens that produce excess polysaccharides (e.g. xanthomonads) benefit from 1-2 additional washes with 1 M NaCl. Resuspend the pellet in 100 III H_20 and use 1-2 III per 25 III PCR reaction mixture.

To purify larger quantities of DNA, the CrAB protocol (1) and modifications thereof (e.g. 15, 16) is a recommended protocol for both Gram negative and positive bacteria. A DNA extraction method developed by Pitcher et al. (24) and modified by Rademaker and de Bruijn (27) yields high quality DNA useful for most applications in bacteriology (e.g. DNA homology studies, RFLP analysis and PCR).

F or whole-cell PCR from solid media, whole colonies may be selected using a I,ul-inoculating loop (Lab Product Sales, Rochester, NY) or toothpick if the colony size is 1-2 mm. Otherwise the loop or toothpick should gently touch the edge oflarger colonies. Adhering cells are released by gentle rotation of the loop or toothpick in the sample tube containing the PCR reaction mixture. Young colonies are ideal but 2 wk or older cultures have been used successfully. The type of medium will impact amplification success. Some media favor polysaccharide production or contain media components that will inhibit PCR amplification. It is common to collect excess cells that inhibit amplification resulting in a smeared background. Care should be taken to collect a small quantity of cells for analysis.

Depending upon the target bacteria, whole-cell PCR may not always be that effective. PCR-ready DNA also can be procured by a freeze-boil method in which the bacteria (suspended in 1% Triton X-IOO, 0.5% Tween 20, 1 mM EDTA, 10 mM Tris, pH 8.0) are frozen in liquid nitrogen or an ultra-low freezer (-70°C or lower) for 3 min and then boiled for 3min (30). Cell debris is removed by a brief spin in a microfuge and the supernatant is transferred to a new tube and stored at 4°C. Whole-cell rep-PCR analysis from difficult-to-lyse pathogens (e.g. *Streptomyces)* is also enhanced using alkaline lysis. Suspend cells (portion of a colony or 10 ,ul of a liquid culture as described above) in 100 ,ul of 0.05 M NaOH and incubate the mixture in the thermal cycler for 15 min at 95 ° C. Centrifuge for 2 minutes to pellet cell debris and use I ,ul (or other optimum amount) of the supernatant for PCR analysis. DNA purification kits also are available from scientific supply companies (see below).

When working with plant samples, it is critical that the target DNA be purified away from potential PCR inhibitors. Some plants are high in polyphenolic compounds and polysaccharides which can interfere with DNA extraction and utilization. Currently, several kits are available for extracting DNA from plant material. The following are a few examples: Plant DNA Isolation Kit (Roche Diagnostics), MasterPure™ Plant Leaf DNA Purification Kit (Epicentre), DNeasy Plant

Mini Kits (Qiagen Inc.), Easy-DNA kit (Invitrogen). Protocols may also include use of compounds such as polyvinyl-pyrrolidone (PVP), polyvinyl-polypyrrolidone powder (PVPP), sodium ascorbate, and/or ficoll, BLOTTO, or citric acid in the template extraction methods during the template extraction or PCR protocols (reviewed in (18)).

DNA template preparation can be successfully achieved direct from plant tissue (36). Such techniques may provide specific pathogen identification but are not likely sensitive to low cell numbers. Collect one lesion (e. g. from tomato fruit or leaf) with no symptoms of secondary organisms, surface sterilize, and grind by hand with a polypropylene grinder in a 1.5 ml micro centrifuge tube at room temperature. Add 400 ~l extraction buffer (200mM Tris HCL, pH 7.5,250 mM NaCl; 25 rnM EDTA; and 0.5 % SDS) and vortex for 15 seconds. Allow the mixture to stand 5 min, or until all samples are prepared, and centrifuge them 13,000 rpm for 3 min. Transfer 300 ~l of the supernatant to a clean microcentrifuge tube and add 300 ul isopropanol. Let the mixture stand at room temperature for 10 min. Centrifuge at 13,000 rpm for 5 min, pour off the supernatant, air dry the pellet, and dissolve it in 100 ~l H_20 or TE. Determine the concentration of DNA, adjust to 150 ng/ul and use 1 ul for PCR analysis. Plant tissue known to be bacteria-free should be prepared in a similar manner as a control. Plant lesions can be occupied with a near -pure culture of the pathogen and bacterial cells can be collected directly from the lesion or infected tissue. Surface sterilize the lesion and surrounding tissue using 70 % ethanol for 1 min followed by 10%-20% bleach for 1 min and three rinses in SDW. Finely cut infected tissue with a sharp scalpel in the presence of200 ~l 0.1 M phosphate buffer (pH 7.0), 0.01 M $MgS0_4$ buffer (pH 7.0; preferred for xanthomonads) or H_20. Allow the preparation to stand for 10-20 min for the bacteria to stream out of the tissue. Use 1 ,ul of the suspension in the PCR reaction mixture. Avoid excess plant tissue in the l,ul sample to avoid inhibitory compounds as highlighted above. Surface sterilization is not always necessary and success has also been achieved using bacterial suspensions that stream directly from xylem tissue (e.g. bacterial wilt and cabbage black rot pathogens).

For additional details on isolation of DNA, see Appendix A-I.

3. peR-BASED METHODS

Diagnostic PCR has been described extensively (10, 11, 18,23,26). PCR primers must anneal specifically to the target DNA of the bacterial strains or species of interest. Target DNA can be sequences involved in known pathogenicity genes *(hrp* or phytotoxin genes), repetitive sequences, rRNA sequences, 16S-23S rRNA spacer regions or even cryptic sequences (of unknown function) known to be specific for the bacterium of interest. Critical parameters for PCR include the $MgCl_2$ concentration, reagent purity, primer selection, thermal cycling parameters and template DNA preparation. The ingredients required for PCR amplification *(Taq* DNA polymerase, dNTP stock solution, reaction buffer, and $MgCl_2$ stock solution) can be purchased separately or through a kit (see below). Detailed protocols can generally be acquired from the original publications. Commercial kits are available for several organisms (see pp. 341342). This list does not constitute an endorsement or recommendation.

a. **BIO-PCR.** The sensitivity ofPCR assays can be enhanced up to 100-fold by employing the BID-PCR (34) assay to identify bacteria in seed washes (31, 34), soil samples (11), and plant tissues (21,33). In this assay, source samples are spread on agar plates and then incubated for 24 to 72 h, depending upon the organism. The plates are then washed with water and the washwater assayed by PCR.

b. **Immunocapture Plus PCR.** The efficiency of target (template) DNA preparation for PCR can be improved by using superparamagnetic particles coated with antibodies generated against the target bacterial cells to separate the cells from plant material and potential PCR inhibitors. To date, this promising method has not been used extensively with phytopathogenic bacteria (9,25,42) but enhances sensitivity (fewer false negatives) and specificity of the assay.

c. **Real- Time PCR/Quantitative PCR.** It is now possible, using the Roche LightCycler™ System or the PE Biosystems ABI Prism® 7700 Sequence Detection System (TaqMan™), to simultaneously quantify and analyze the product during PCR amplification ('real time' analysis). These two systems employ fluorogenic probes or the fluorescent dye SYBR® Green I to monitor amplification. Although the required instrumentation is expensive, both systems are rapid and efficient. Schaad et al. (33) reported that TaqMan in combination with BID-PCR was an extremely effective method of screening potato tubers for *Clavibacter michiganensis* subsp. *sepedonicus.* However, the detection sensitivity of TaqMan PCR alone was similar to that of classical PCR. An important advantage of the hybridization-based TaqMan system is the elimination of a Southern blot to confirm the identity of the amplified product, as described below.

d. Detection of PCR Products

PCR products usually are visualized by agarose gel electrophoresis and/or DNA dot blots. Both methodologies are described by Sambrook et al. (32). A typical PCR reaction mixture is 25 or 50 ul; 10 ul of the reaction mixture plus 2 ul of loading buffer/well is usually sufficient to detect the amplified fragment. The sample is subjected to electrophoresis at 7.5 V/cm for 2 h (or less, depending upon the size of the PCR product) and then stained with ethidium bromide. DNA blots are performed to confirm the identity of the PCR product. Ten microliters of the PCR product are mixed with 40 ul of water, denatured, loaded into a dot blot apparatus, and then cross-linked to a nylon membrane. Probe (the target sequence for the PCR primers) is tagged with a nonradioactive label such as Roche's digoxigenin-ll-dUTP (DIG) and then hybridized to the blot (3). The DIG-labeled DNA can be visualized colorimetric ally or by chemiluminescence.

4. REP-PCR GENOMIC FINGERPRINT ANALYSIS TO CHARACTERIZE AND IDENTIFY PHYTOBACTERIA

The rep-PCR employs primers designed to anneal to repetitive sequences distributed around the genome of diverse bacteria (6,40). The possibility of using rep-PCR to characterize, classify, and identify bacteria, including clinical, industrial, environmental and agricultural related bacteria has been recently reviewed (18, 28). Several publications provide detailed methods to perform rep-PCR (41) and it can be effectively used for identification ofunknowns(19, 28).

Rep-PCR is universally applicable to generate complex genomic fingerprints from phytobacteria using template DNA from actinomycetes such as *Streptomyces* (5), Gram-positive bacteria such as *Clavibacter* (15), and Gram negative bacteria such as *Burkholderia* (8), *Erwinia (22), Pseudomonas* (14,41), and *Xanthomonas* (16). Rep-PCR provides a powerful capability to identify phytobacteria because, in general, each host-adapted pathogen, classified at the species or pathovar level, is comprised of strains that have one or more distinctive and therefore diagnostic rep-PCR genomic fingerprints (15,16, 41). In some cases, a host adapted population includes more than one distinct genotype (few or no comigrating rep-PCR bands) able to incite similar symptoms, as observed for strains that cause bacterial spot on tomato (4,17). The rep-PCR method has also proven useful to detect diversity within a species or pathovar population based on unique polymorphisms observed at the strain level (4, 14, 15, 17,39). The amount of genetic diversity varies considerably within different populations. Some pathovars, or other host-adapted populations, constitute strains that all have near-identical rep-PCR genomic fingerprint profiles. Other pathovars include strains that have genomic fingerprints that share numerous rep-PCR amplified bands but also contain numerous strain-specific polymorphisms (referred to as haplotypes or lineages 17).

The genomic fingerprint patterns are analogous to "bar codes" (20) in a grocery store and therefore can be exploited to identify bacterial pathogens. Two approaches have been used. Laboratories that fingerprint one to several pathogens simply use template DNA from a known strain in each PCR experiment and visually compare the genomic fingerprint of the unknown(s) to the positive control. Alternatively, ifmany different pathogens are evaluated, the rep-PCR genomic fingerprint patterns are analyzed using computer -assisted pattern analysis and then catalogued in a library database (28, 29). GelCompar software (Applied Maths, Kortrijk, Belgium 16) has been used for such analyses and detailed protocols are available (29). Once a library has been constructed, the identity of unknown strains can be accomplished rapidly ifthe rep-PCR genomic fingerprint pattern matches known strains in the database. If a match does not occur, other taxonomical or identification approaches need to be used. However, rep-PCR is an efficient first-step method to characterize populations of culturable bacteria, even if little is known about the identity or taxonomy of the strains (18). The genetic diversity of the population can be mapped quickly based on cluster analysis of the genomic fingerprint patterns. Once the diversity of the population has been determined and if considerable diversity is present, representative strains of each cluster can be further characterized using other methods described in this manual.

Rep-PCR genomic fingerprinting is rapid and can be used routinely. Amplification in the thermal cycler requires 5-6 h and optimum results for resolution in standard agarose gels is achieved by use oflong (-25 em) gels. Each gel of30 lanes requires 20 min to load and electrophoresis requires 6 to 18 h.

a. Preparation of Stock and Working Solutions

Stock solutions outlined below are prepared from molecular biology-grade ingredients and/or are autoclaved. Care must be taken to avoid contamination of all PCR ingredients. Stock solutions can be dispensed into working solutions and frozen or stored appropriately.

5x Gitschier Buffer: Prepare and autoclave stock solutions of each ingredient and then combine to obtain a final solution of 83 roM (NH4)2S04; 335 roM Tris HCI pH 8.8; 33.5 mM MgC12; 33.5 ~M EDTA pH 8.8; and 150 roM Il-mercaptoethanol. To prepare 200 rnl of 5x buffer combine the following stock solutions: 16.6 rnl ofa 1 M (NH4)2S04; 67 ml ofa 1 M Tris-HC~ pH 8.8; 6.7 rnl ofa 1 M $MgCI_2$; 1.3 ml ofa 1:100 dilution ofa 0.5 MEDTA, pH 8.8; 2.08 rnl of a 14.4 M p-mercapto-ethanol commercial. Adjust to 200 ml final volume using approximately 106 ml of water, mix well, aliquot into 1.5 ml amounts and store at -20°e.

BSA: Aliquot the BSA in 20 III amounts and freeze at -20°C. (e.g. use Boehringer #711 454 molecular biology grade BSA, 20 mglml).

DMSO: Dimethyl sulfoxide is added to the PCR mixture at a final concentration of 10% v/v to allow for reduced annealing temperatures. (e. g. use FLUKA #41640).

dNTPs: Use e.g. Pharmacia #27-2035-01 ultrapure set of 100 roM in H_20, pH 7.5. Mix each dNTP 1: 1: 1 : 1 to obtain a working solution of 25 roM of each dNTP and store 50 j..Ll aliquots at -20°C.

Taq Polymerase: 5 units/Ill (e.g. Perkin and Elmer #N808-0070). The enzyme is aliquotted in 20 III amounts and stored (up to several months) at -20°e.

Tween 20: Tween 20 is added to a final concentration of 1 % v/v in the 25 III reaction mixture and in some cases enhances whole-cell lysis and the quality of the rep-PCR fingerprints generated.

Mineral oil: Thermal cyclers that do not have a heated lid may require mineral oil over the reaction mixtures to limit evaporation (e.g. Sigma M3516 light white mineral oil).

Water: HPLC purified water (e.g. Fisher, Pittsburgh, PA) can be used to ensure the absence of contaminating DNA. However, double distilled water, dispensed as 2.5 ml aliquots in 5 ml screw cap vials and autoclaved; can be used.

Primers: BOX-PCR employs a single primer whereas REP- and ERIC-PCR employ two primers per reaction mixture. Primers are quantified and diluted to 0.3 J.lg/ J.ll.

10)

	Sequence (5'----------3')	
REP-PCR	IIIICgICgICATCIggC	REP1R
	ICgICTTATCIggCCTAC	REP2I
ERIC-PCR	ATgTAAgCTCCTggggATTCAC	ERIC1R
	AAgTAAgTgACTggggTgAgCg	ERIC2
BOX-PCR	CTACggCAAggCgACgCTgACg	BOXA1R

b. Rep-PeR Amplification of Genomic DNA

Prepare a master mix with the following amounts per sample: 5 ~l of 5x Gitschier buffer; 0.2 ul BSA; 2.5 ~l DMSO; 12.65 J.ll of water for REP- and ERIC-PCR or 13.65 J.ll for BOX-PCR; 1.25 111 of 1: 1: 1: 1 dNTP solution; 1 111 of each primer solution (e.g. 1 111 each ofREPIR & REP2I OR ERICIR & ERIC2 OR 1 111 BOXAIR); 2 units of *Taq* DNA polymerase; and an optional 0.25 111 of Tween 20. Mix by inverting several times. The master mix can be prepared on ice in a sterile 1.5 ml micro centrifuge tube or a 5 ml glass vial and then 24 J.ll aliquots dispensed into each rep-PCR reaction sample tube. Add 1 111 of genomic DNA or whole-cell preparation to each sample. If necessary, overlay the reaction mixture with an equal volume of mineral oil.

Load the samples and use the following cycling regimes for compressor-based thermal cyclers (e.g. Perkin Elmer 480):

- 1 initial cycle at 95°C for 7 min to lyse cells or denature DNA;
- followed by 30 to 35 cycles using denaturation conditions of 94°C for 1 min; annealing for 1 min at 48°C for REP-PCR, 52°C for ERIC-PCR and 53°C for BOX-PCR; extension step of 6 5°C for 8 min;
- a single final extension cycle at 65 ° C for 15 min and a final waiting temperature of 4°C.

For Peltier element-based thermal cyclers initiate cell lysis and DNA denaturation with an incubation of 9 5°C for 2 min, followed by 30 to 35 cycles of:

- 94 ° C for 3 sec
- 92°C for 30 sec

40°e .or : min for REP-PCR and 50°C for ERIC- and BOX-PCR

- 65 ° C tor S min,

Terminate the PCR reaction using a final extension cycle at 65 ° C for S min and a final waiting temperature of 4 ° C.

Thirty cycles is sufficient when using purified DNA as template. For crude DNA preps or whole-ceil rep-PCR use 35 cycles. Amplified PCR products can be stored for long periods of time at 4°C or -20°e with no apparent DNA degradation, or load products on gels for immediate analysis.

Gel Electrophoresis of rep-peR Products

Rep-PCR amplified fragments can be successfully resolved using agarose-based matrices although acrylamide based systems can also be employed for enhanced precision. For best resolution in agarose gels, 20 em x 24 em gels (e.g. BRL H4 horizontal gel apparatus Gibco BRL #1025RD) should be used. Thirty- or 20-tooth combs can be used and the wells should be thin (:::: 1 rnm) to ensure that the resolved rep-peR. bands are sharp.

Prepare a 1.5% agarose gel using IX TAE. (Prepare i X lAE from a 50X *'TAE* buffer stock solution that includes per litre 242 g Tris Base, 57.1 ml glacial acetic acid, and 18.61 g *N~EDTA.),* Load 6 to 10 ,ul ofrep-PCR amplified product per sample combined with 1.2 to :2 ,,:,:1 of 6x loading dye (bromoohenol blue 0.25% *wiv* in dH_20; xylene cyanol 0.25% *wlv* and Ficoll 15% *w/v* Type 400). Include a 1 kb ladder (Gibco BRL) or other suitable marker in the middle and end lanes, at a minimum, *if* computerassisted analysis is to be conducted. Gels can be run in IX TAE for 5-6 h at a constant voltage of 105 V (approximately :55 rnA) at room temperature or for 16-18 h at a constant voltage of 70 V (approximately 23 rnA) and 4 ▫C fur optimal results.

After electrophoresis, transfer the gel to an ethidium bromide bath (60 ~1 of 10 *mgirnl* stock solution per L of T AE buffer) for 30 rrun and destain in 1 X T AE buffer or ater for 30 min. hotograph the gel on an ultraviolet transilluminator, using an orange UV filter and positive rilm 'e.g. olaroid type 57) or 111m that provides a negative and positive e.g. olaroid type 55). Alternatively, gel images can be captured electronically with a video recorder and saved as a TIFF file, oy using the appropriate software. Digital images are more suited to computer-assisted pattern analysis.

3. LITERA TURE CITED

l . Ausubei, F. M., R. Brent, R. E. Kingston, D. D. Moore, 1. G. Seidman, *1.A.* Smith, and K Struhl. i 992. Current protocols in molecular biology. Vol. 1. Greene Publishing .vssociates and Wiley Interscience, New York.

fl>.A';(~() &'t;)~ [1,\, ~I·n'-{JfL .] n Af;.')

I'? ')/'0'. ~ "//1)./// :23

2. Bain, R. A. and M. C. M. Perombelon. 1990. Blackleg development and tuber yield in relation to numbers of *Erwinia carotovora* subsp. *atroseptica* on seed potatoes. Plant Pathol. 39:125-133.

3. Boehringer Mannheim GmbH, Biochemica. 1995. The DIG System User's Guide for Filter Hybridization. (Boehringer is now called Roche Molecular Biochemicals).

4. Bouzar, H., J. B. Jones, R. E. Stall, F. J. Louws, M. Schneider, J. L. W. Rademaker, F. J. De Bruijn, and L. E. Jackson. 1999. Multiphasic analysis ofxanthomonads causing bacterial spot disease on tomato and pepper in the Caribbean and Central America: Evidence for common lineages within and between countries. Phytopathology 89:328-335.

5. Clark, C. A., C. Chen, N. Ward-Ramey, and G. S. Pettis. 1998. Diversity within *Streptomyces ipomoeae* based an inhibitory interactions, rep-PCR, and plasmid profiles. Phytopathology 88: 1179-1186.

6. de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58:2180-2187.

7. Frey, P., J. J. Smith, L. Albar, P. Prior, G. S. Saddler, D. Trigaletdemery, and A. Trigalet. 1996. Bacteriocin typing of *Burkholderia (pseudomonas) solanacearum* race 1 of the French West Indies and correlation with genomic variation of the pathogen. Appl. Environ. Microbiol. 62:473-479.

8. Gitaitis, R. D., R. W. Beaver, and A. E. Voloudakis. 1991. Detection *ofClavibacter michiganensis* subsp. *michiganensis* in symptomless tomato transplants. Plant Dis. 75:834838.

9. Helias, v., A. C. Le Roux, Y. Bertheau, D. Andrivon, J. P. Gauthier, and B. Jouan. 1998. Characterisation of *Erwinia carotovora* subspecies and detection of *Erwinia carotovora* subsp. *atroseptica* in potato plants, soil and water extracts with PCR-based methods. Eur. J. Plant Pathol. 104:685-699.

10. Honeycutt, R. and M. McClelland. 1996. Application of the polymerase chain reaction for the detection of plant pathogens. Pages 187-201 in: B. W. S. Sobral (ed.), The Impact of Plant Moleular Genetics. Birkhauser, Boston.

11. Ito, S., Y. Ushijima, T. Fujii, S. Tanaka, M. Kameya-Iwaki, S. Yoshiwara, and F. Kishi. 1998. Detection of viable cells of *Ralstonia solanacearum* in soil using a semis elective medium and a PCR technique. J. Phytopathol. 146:379-384.

12. Jagoueiz, S., J. M. Bove, and M. Garnier. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the proteobacteria. Int. J. Syst. Bacteriol. 44:379-386. *Sfe-l-/~'c fn'p1t(/l tf dN !'ft,J:.fi/~(ttLI*

13. Jagoneiz, S., J. Bove, and M. Garnier. 1996. PCR detection of two *Candidatus liberobacter* species associated with greening disease ?-f citrus. Molec .. Cell. Probes 10:43-50. *~ f' e* lif-vl'C *f1);iiltf; r':L tic I* ıı *Lt !I'e* Atll'Jt'"fd .

14. Little, E. L., R. M., Bostock, and B. C. Kirkpatrick. 1998. Genetic characterization of *Pseudomonas syringae* pv. *syringae* strains from stone fruits in California. Appl. Environ. Microbiol. 64:3818-3823.

15. Louws, F. J., 1. Bell, C. M. Medina-Mora, C. D. Smart, D. Opgenorth, C. A. Ishimaru, M. K. Hausbeck, F. 1. de Bruijn, and D. W. Fulbright. 1998. rep-PCR-mediated genomic fingerprinting: A rapid and effecfve{method to identify *Clavibacter michiganensis.* Phytopathology 88:862-868. *fA'r/* 401 *C?ua.l rl1r'e4 "I /J vn -A-- IJ P·* ell *U.-V~1 (~ ,II.*

16. Louws, F. 1., D. W. Fulbright, C. T. Stephens, and F. 1. de Bruijn. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generatedJwit repetitive sequences and PCR. Appl. Environ. :Microbiol. *60:2286-2295.* P'Iril'") *N)}' ~1f;J·'"'rt'1I vi • .;. t1N/J U~/'1JJ(J) + ep- L Id 1/'*

17. Louws, F. J., D. W. Fulbright, C. T. Stephens, and F. 1. de Bruijn. 1995. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria.* Phytopathology 85:528-536.

18. Louws, F. 1.,1. L. W. Rademaker, and F. J. De Bruijn. 1999. The three Ds ofPCR-based genomic analysis of phytobacteria: Diversity, detection, and disease diagnosis. Ann. Rev. Phytopathol. 37:81-125. *Iii* ",,'J) . ı It *IL*

19. Louws, F. 1., M. Schneider, and F. 1. de Bruijn. 1997. Assessing genetic diversity of microbes using repetitive sequence-based PCR (rep-PCR). Pages 63-94 in: E. G. Toronzos. ed., Nucleic acid amplification methods for the analysis of environmental microbes. Technomic Publishing Co., Lancaster, P A. J *rrf,{ ~zÅt 4"" liet-Ie,{ to ,'c-kri,l{(j* t,'l«IIfl ...• " *!a--ttuoe.flA·*

20. Lupski, J. R. 1993. Molecular epidemiology and its clinical application. Jama 3 J. of Amer. Med. Assoc. *270:1363-1364.*

21. Manulis, S., F. Kleitman, O. Dror, 1. David, and D. Zutra. 1998. Characterization of the *Erwinja amylovora* population in Israel. Phytoparasitica 26:39-46. *11'1: ~4.e* 1.(*fl II'!, f* c IL *~d{ 1/01* *t,'J-;1/1*

22. McManus, P. S. and A. L. Jones. 1995. Genetic fingerprinting of *Erwinia amy/ovora* strains isolated from tree-fruit crops and *Rubus* spp. Phytopathology 85: 1547-1553.

23. Persing, D. H. 1993. In vitro nucleic acid amplification techniques. Pages 88-104 in: D. H. Persing, T. F. Smith, F. Tenover and T. 1. White, eds., Diagnostic Molecular Microbiology: Principles and Applications. American Society for Microbiology, Washington, D. C.

24. Pitcher, D. G., N.A. Saunders, and R. 1. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8: 151-156.

25. Pooler, M. R., I. S. Myung, 1. Bentz, 1. Sherald, and 1. S. Hartung. 1997. Detection of *Xylella fastidiosa* in potential insect vectors by immunomagnetic separation and nested polymerase chain reaction. Lett. Appl. Microbiol. 25: 123-126.

26. Prosen, D., E. Hatziloukas, N. W. Schaad, and N. 1. Panopoulos. 1993. Specific detection of *Pseudomonas syringae* pv. *phaseolicola* DNA bean seed by polymerase chain reactionbased amplification of a phaseolotoxin gene region. Phytopathology 83: 965-970.

27. Rademaker, 1. L. W. and F. 1. de Bruijn. 1997. Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis. Pages 151-171 in: G. Caetano-Anolles and P. M. Gresshoff, eds., DNA markers: Protocols, Applications and Overviews. 1. Wiley & Sons, Inc.

28. Rademaker, 1. L. W., F. 1. Louws, and F. 1. de Bruijn. 1998. Characterization of the diversity of ecological important microbes by rep-PCR genomic fingerprinting. Pages 1-26 in: A. D. L. Akkermans, 1. D. van Elsas, and F. 1. de Bruijn, eds., Molecular Microbial Ecology Manual. Chapter 3.4.3 Kluwer Academic Publishers, Dordrecht, The Netherlands.

29. Rademaker, 1. L. W., F. 1. Louws, and F. 1. de Bruijn. 1999. Computer assisted pattern analysis of electrophoretic fingerprints and database construction. Pages 1-32 in: A. D. L. Akkermans, 1. D. van Elsas, and F. 1. de Bruijn, eds., Molecular Microbial Ecology Manual. Chapter 7.1.3. Kluwer Academic Publishers, Dordrecht, The Netherlands.

30. Reischl, u., M. Pulz, W. Ehret, and H. Wolf 1994. PCR-based detection of mycobacteria in sputum samples using a simple and reliable DNA extraction protocol. Biotechniques 17:844-845.

31. Samac, D. A., R. 1. Nix, and A. E. Oleson. 1998. Transmission frequency *ofClavibacter michiganensis* subsp. *insidiosus* to alfalfa seed and identification of the bacterium by PCR. Plant Dis. 82: 1362-1367.

32. Sambrook, 1., E. F. Fritsch, and T. Maniatis. 1989. Agarose Gel Electrophoresis. Pages 6.3-6.19 in: Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

33. Schaad, N. W., Y. Berthier-Schaad, A. Sechler, and D. Knorr. 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIo-PCR and an autom.ated real-time fluorescence detection system. Plant Dis. 83: *109₀-1100.*

34. Schaad, N. W., S. S. Cheong, S. Tamaki, E. Hatziloukas, and N. 1. Panopoulos. 1995. A combined biological and enzymatic amplification (BIo-peR) technique to detect *Pseudomonas syringae* pv *phaseoiicoia* in bean seed extra ts. Phytopathology 85:243-248.

35. Schaad, N. W., W. R. Sitterly, and H. Humaydan. 1980. Relationship of incidence of ~ edbome *Xi ,. homonas caJ11festr!s* to ,black rot of crucifers in the field. Plant Dis. 64: 91-92.

36. Schneider, M. and F. 1. Debruijn. 1996. Rep-PCR mediated genomic fingerprinting of *Rhizobia* and computer-assisted phylogenetic pattern alysis. W rld 1. Microbiol. Biotechnol. 12:163-174.

37. Sutton, M. D. and V. R. Wallen. 1970. Epidemiological and ecological relations of *Xanthomonas phaseoli* and *X phaseoii* var. *fuscans* on bean in southwestern Ontario 19611968. Can. 1. Bot. 48: 1329-1334.

38. Umesh, K. c., R. M. Davis, and R. L. Gilbertson. 1998. Seed contamination thresholds for development of carrot bacteri blight caused by *Xanth mo as campestris* v. *carotae.* Plant Dis. 82:1271-1275.

39. Vera Cruz, C. M., E. Y. Ardales, D. Z. Skinner, 1. Talag, R. 1. Nelson, F. 1. Louws, H. Leung, T. W. Mew, and 1. E. Leach. 1996. Measurement of haplotypic variation in *Xanthomonas oryzae* within a single field by rep-PCR and RFLP analyses. Phytopathology 86: 1352-1359.

40. Versalovic, 1., T. Koeuth, and 1. R. Lupski. 1991. Distribution of repetitive DNA-sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19:6823-6831.

41. Versalovic, 1., M. Schneider, F. 1. de Bruijn, and J. R. Lupski. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell BioI. 5:25-40.

42. Walcott, R. R. and R. D. Gitaitis. 2000. Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using illlIlJunomagnetic separation and the polymerase chain reaction. Plant Dis. 84:470-474.

43. Weingart, H. and B. Volksch. 1997. Genetic fingerprinting of *Pseudomonas syringae* pathovars using ERIC-, REP-, and IS50-PCR. 1. Phytopathol. 145: 339-345.

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APPENDIX

A-I DNA ISOLATION PROCEDURE

1. INTRODUCTION

This procedure yields high quality DNA useful for most applications in bacteriology (e.g. DNA homology studies, RFLP analysis and PCR). Simpler protocols are available but may yield DNA suited for PCR applications only. This procedure is based on Pitcher et aI. 1989. "Rapid extraction of bacterial genomic DNA with guanidium thiocyanate". Letters in Applied Microbiology 8: 151-156, slightly modified by Luc Vauterin (Belgium) and used in all work of Rademaker, Louws and de Bruijn.

a. MATERIALS AND SOLUTIONS

Note: All materials need to be sterilized by autocIaving except solutions # 5, 8, 9, and 10.

1. 10 *,ul* disposable inoculation loop — Sirnport L200-2

2. 0.5 M EDTA pH 8.0

Prepare 1000 ml using: 186.1 gN~EDTA 20
gNaOH
800 ml deionized water
Adjust pH to 8.0 with 5 NaOH
Adjust final volume to 1000 ml with water

3. 100x 1.0 M Tris, 0.1 M EDTA

Prepare 1000 ml using: 121 g Tris
600 ml water
Adjust to pH 8.0 by adding 42 ml concentrated HCl 200 ml 0.5 M EDTA pH 8.0
Adjust to 1000 ml with water

4) Ix TE Buffer, 10 mM Tris, 1 mM EDTA, pH 8.0

Prepare 1000 ml using:
10 ml100x TE Tris, pH 8.0
990 ml water

5) GES Solution - Guanidine thiocyanate-EDTA-Sarkosyl

Note: Guanidine thiocyanate is harmful; use suitable protective wear.

Prepare 100 ml GES solution:
60 g guanidine thiocyanate — Sigma #G-9277
20 ml 0.5 M EDTA pH 8.0
Add 20 ml water
Dissolve all ingredients at 65°C, cool down

Add 1 g N-Iauryl sarkosine Sigma #L-5125
Adjust volume to 100 ml with water
Filter sterilize using a 0.45 *j..lm* filter, store at room temperature

6) Resuspension buffer 0.15 M NaCI, O. aIM EDT A, pH 8. a
Prepare 1000 ml using: 8.77
gNaCI
20 ml 0.5 M EDTA, pH 8.0
Adjust to 1000 ml with water

7) Ammonium Acetate 7.5 M
Prepare 1000 ml using:
578.1 g NH_4Ac in 1000 ml water

8) Chloroforrnliso-amyl-alcohoI2411 (v/v)
Note: Chloroform is highly toxic; wear suitable protective clothing and work under a fumehood. Depending on how many extractions are to be done, we often prepare 200 ml amounts.
Prepare 1000 ml using:
40 ml iso-amyl-alcohol
960 ml chloroform

9) RNAse Solution (2.5 mg/ml)
50 mg RNAse per 20 ml sterile water
Incubate 10 minutes at 100°C, aliquot in working solutions and store at -20°C

10) RNAse Solution (250 j..lg/ml)
Prepare 1 ml using:
100,u1 RNAse solution (2.5 mg/ml)
900 ,ul sterile water

b. STEPS IN PROCEDURE

1) Start from a well-grown culture 24-48 hrs old on an agar plate.
Note: Use a media that does not allow copious production of polysaccharides for bacteria such as xanthomonads.
2) Pipet 500 ml (1000 ml for bacteria like xanthomonads) resuspension buffer in numbered 1.5 ml micro centrifuge tubes.
3) Collect cells with sterile 10 ,ulloop (one loop full).
4) Dislodge bacteria by vigorous swirling of loop in resuspension buffer.
5) Homogenize cells using a 1000 ml tips (blue tips). (Draw bacterial suspension up and down several times).

Cell Washing

6) Centrifuge 3 min. at maximum rpm (table-top micro centrifuge -13 -16 K rpm)
Note: Centrifuge longer (e.g. 10 min or more) when necessary for bacteria such as xanthomonads that have excess polysaccharides.
7) Remove and discard supernatant using 1000 ,ul tips (blue)
Note: if cells are packed well at the bottom, simply pour off the supernatant)
8) Again centrifuge 3 min at maximum rpm
9) Remove and discard supernatant using 200,ul tips (yellow)
10) Add 100 ,ull x TE (Tris-EDT~ pH 8.0) and homogenize using 1000 ml tips.
(Note: Use "cut off" tips)

Cell Lysis

11) Add 500 ,ul GES
12) Gently invert suspension in tubes
13) Incubate on ice for approximately 5 min
14) Add 250,ul cold (-20°C) ammonium acetate 7.5 M
15) Gently invert tubes
16) Incubate on ice for approximately 5 min
17) Add 500 ,ul chloroformliso-amyl-alcohol (4/1)
18) Shake vigorously until solution is homogenous milky
19) Centrifuge approximately 10 min until upper phase is clear

DNA precipitation

20) Have numbered tubes prepared containing 378 ,ul cold isopropanol (-20°C)
21) Carefully collect 700 (-20°C) of the upper phase DNA solution using 1000 ml tips
Note: Avoid collecting any precipitate at the interface. Also, blue tip ends can be cut to better allow collection of viscous DNA suspension and avoid lower phase. Another trick is to flame Pasteur pipettes so they have a shepherd's crook, then scoop out the DN~ dip in 70% ETOH and then dip in TE to dissolve. It goes faster but only do this if you see a DNA pellet. Otherwise, centrifuge and you will have enough DNA for rep-PCR.
22) Add DNA solution to tubes containing isopropanol.
23) Invert tubes gently. A white viscous cloud of DNA will form.
Note: Optional step - Tubes can be safely stored at -20°C ifnecessary.
24) Centrifuge DNA suspension for approximately 10 min; have tube hinges pointed outward.

Note: Turning the tubes all in the same direction will precipate the DNA in a predictable spot in cases where it cannot be visualized. Sometimes we find it necessary to centrifuge for up to 45 min.

25) Use 100 ill tips to carefully remove supernatant
26) Centrifuge briefly
27) Remove remaining supernatant using 200 ill tips

WasbDNA

28) Add 150 ill 70% ethanol; DO NOT MIX
29) Centrifuge briefly
30) Remove ethanol with 200 ill tips
31) Centrifuge briefly and remove remaining ethanol
32) Air dry DNA in laminar flow hood 5-10 min (or vacuum centrifuge) until pellet is clear
Note: Do not dry too long and dehydrate pellets; it may be difficult to resuspend DNA.
33) Add 100 zzl I x TE, pH 8.0; shake gently; (resuspension in 200 ill is alright and gives more solution to work with)
34) Incubate at room temperature or 4 DC overnight to dissolve DNA
Note: DNA can be heated to 37^{D}C to facilitate dissolving process.
35) Spin down water that condensed on sides of tubes
36) Add 25 j.ll RNAse (50 ilglml stock solution) and mix gently
37) Incubate 1 hat 37^{D}C
38) Store DNA at 4 DC or at _20^{D}C for long-term storage
Note: The purity of the DNA can be checked on a 1% agarose gel using 1 x T AE buffer and run at 3 volts/em for 30 min. Quantify the DNA and label each tube with appropriate code name and DNA concentration prior to storage.

f. QUANTIFY AND DILUTE DNA FOR peR

1) To ensure stock DNA is adequately dissolved heat for 1 min at 65^{D}C and mix
2) Take 10 j.ll and add to 390 j.ll water in numbered 1.5 ml microcentrifuge tubes (This results in a dilution factor [OF] of 40).
3) Measure optical density (OD) at 260 nm (ensure spectrophotometer UV bulb is prewarmed for - 15 min).
4) Calculate concentration of purified DNA (assume OD 1 = 50 ug/ml DNA; use the following formula: OD x 50 g x DF = original DNA concentration in ug/ml.) Dilute to working concentrations.

APPENDIX

B. SEROLOGICAL TECHNIQUES

A. Alvarez

1. ll\'IMUNODIAGNOSTIC METHODS

Antibodies have been used extensively and in many test formats to detect and identify bacteria. The most popular and successful formats include agglutination, the enzyme-linked irnmunosorbent assay (ELISA), immunofluorescence (IF), lateral flow strip tests and flowthrough assays. Formats using immunoblots and magnetic beads are used in research but are not widely used for routine detection of bacterial pathogens in seed, propagative materials, or field samples.

Agglutination methods use antibodies which recognize bacterial cell antigens to aggregate the bacteria directly (10). The antibodies are often bound to particles, such as latex or *Staphylococcus aureus,* to enhance test convenience, sensitivity and stability. These methods are sometimes used to confirm the identity of bacteria from culture or detect bacteria in symptomatic plant samples.

ELISA uses enzyme-labeled antibodies to detect bacteria or bacterial secretions and extracts that are bound to a solid support (9). The enzyme label signals that the antibody has become attached by changing the color of its substrate solution. The method is popular and practical in the laboratory. Under favorable conditions, ELISA can be used to identify bacteria in symptomatic plants because symptomatic tissues often contain high populations of the pathogen *(>10^5 CFU/ml)* (2). Various ELISA protocols have been optimized to detect target bacteria at concentrations as low as 10^3 *CFU/ml* (8) but such sensitivity is not typical of most ELISA bacterial assays. At bacterial concentrations below 10^5, bacteria should first be cultured. ELISA is a useful tool for rapid, specific identification of target colonies in mixed cultures of bacteria.

Immunoblots are a modification of the ELISA format using a nitrocellulose membrane or filter as the solid support. Dot immunobinding assays (DIA) are generally less sensitive than ELISA, but their convenience often warrants use when the pathogen population is sufficiently high (> 1 0^5 *CFU/ml)* for detection (7). Different formats have been used to trap bacteria on membranes, followed by fluorescent or DNA-based methods (4, 7). Immunocapture followed by polymerase chain reaction assays also enhances sensitivity of detection (6).

IF uses antibodies with fluorescent labels to identify bacteria. These methods use antibodies that react with bacterial cell antigens which are visualized in microscopes by their fluorescent labels. It is the only method that combines irnmuno-recognition with direct observation of cell properties. IF is often used to confirm identity of known bacteria in mixed cultures. Although the method is time-consuming and requires extensive training, it can produce sensitive assays suitable for screening negative plant samples.

IF can be applied to colonies as well as individual cells. In the immunofluorescence colony-staining (IFC) protocol, melted agar is mixed with a sample containing live cells and allowed to solidify (11, 12). Microcolonies form after 24 to 72 h incubation at which time the agar is dried to a film in a current of warm air (37°C). The agar film is rehydrated with a specific antibody conjugate, washed and observed using an epifluorescence microscope. Positive colonies are identified by observing fluorescence under low magnification (40X). Sensitivity is greatly enhanced by the enrichment step, attaining detection levels as low as 10^2 CFU/ml (1, 11). IFC has the advantage of differentiating live from dead cells as only the live cells form microcolonies and at the low magnification used, the dead cells are invisible.

Lateral flow strip tests use antibodies labeled with particles, such as gold or colored latex or dyes, to detect bacteria in a sample as it flows along a paper/membrane composite device [strip]. The strip includes capture and control lines where colored particles will aggregate if the test is positive. These tests can be inexpensive, stable, and especially simple to perform. Flowthrough assays are similar and use porous matrices, such as cylinders or elements in syringes, to carry out important steps in the assay. All of these tests have the same limitations of ELISA but can be developed to a very useful assay for specific applications.

Assay sensitivity varies with the specific format and the suitability of the antibody for the target cells or cell components. Direct comparisons of assay sensitivity have been made for a number of immunodiagnostic and DNA-based assays (1,4, 8). (For further details on methods please refer to the APS laboratory manual, *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens [7]).*

A key to successful diagnosis of a bacterial strain using immunodiagnostic methods is a thorough knowledge of the specific antibody used in the test. Certain plant pathogens are more heterogeneous than others with respect to detectable antigens displayed in the natural pathogen population. A few bacterial pathogens can be reliably identified with polyclonal antibodies, but in most cases, the greater specificity of monoclonal antibodies is required for a reliable diagnosis. One must first consult relevant literature to determine the characteristics of the antibody as well as the serological composition of the bacterial population to be detected or identified.

2. LITERATURE CITED

1. Alvarez, A. M., F. -u., Rehman, and 1. E. Leach. 1998. Comparison of serological and molecular methods for detection of *Xanthomonas oryzae* pv. *oryzae* in rice seed. Pages 175-183 in: Seed Health Testing: Progress towards the 21st Century. 1. D. Hutchins and J. Reeves, eds., CAB International, Wallingford, U'K,

2. Alvarez, A. M. and K. Lou. 1985. Rapid identification of *Xanthomonas campestris* pv. *campestris* by an enzyme-linked immunosorbent assay ELISA. Plant Dis. 69: 1022-1026.

3. Anderson, M. 1. and S. T. Nameth. 1990. Development ofa polyclonal antibody-based

serodiagnostic assay for the detection of *Xanthomonas campestris* pv. *pelargonii* in geranium plants. Phytopathology 80:357-360.

4. Brlansky, R. H. R. F. and 1. -M. Lee. 1990. Detection *ofXanthomonas campestris* pv. *citrumelo* and *X citri* from citrus using membrane entrapment immunofluorescence. Plant Dis 74:863-868.

5. Duveiller, E. and C. Bragard. 1992. Comparison ofirnmunofluorescence and two assays for detection of *Xanthomonas campestris* pv. *undulosa* in seeds of small grains. Plant Dis. 76:999-1003.

6. Hartung, 1. S., O. P., Pruvost, 1. Villemot, and A. M. Alvarez. 1996. Rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by irnmunocapture and a nested-polymerase chain reaction assay. Phytopathology 86:95-101.

7. Hampton, R., E. Ball, and S. De Boer (eds.). 1990. Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens, a Laboratory Manual. Hampton, R. Ball E., De Boer, S., eds., APS Press, S1, Paul, Minnesota, USA.

8. Jones, 1. B., G. C., Somodi, and 1. W. Scott. 1997. Increased ELISA sensitivity using a modified extraction buffer for detection of *Xanthomonas campestris* pv. *vesicatoria* in leaf tissue. J. Appl. Microbiol. 83:397-401.

9. McLaughlin, R. and T. Chen. 1990. ELISA methods for plant pathogenic prokaryotes. Pages 197-209 in: *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens, a Laboratory Manual.* Hampton, R., E. Ball, S. De Boer, eds., APS Press, S1, Paul, Minnesota, USA.

10. Slack, S. A. and E. M. Ball. 1990. Latex agglutination for viruses and bacteria. Pages 307-312 in: Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens, a Laboratory Manual Hampton, R., E. Ball, S. De Boer, eds., APS Press, S1, Paul, Minnesota, USA.

11. Van Vuurde, 1. W. L. 1990. Immunofluorescence colony staining. Pages 299-305 in: Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens, a Laboratory Manual Hampton, R., E. Ball, S. De Boer, eds., APS Press, S1, Paul, Minnesota, USA.

12. Van Vuurde, 1. W. L., P. Kastelein, and 1. M. van der Wolf, 1995. Immunofluorescence colony-staining (IFC) as a concept for bacterial detection in quality testing of plant materials and ecological research. Bulletin OEPPIEPPO 25: 157-162.

13. Wang, Z. K., 1. C. Comstock, and N. W. Schaad. 1999. Comparison ofPCR, Bio-PCR,

DIA, ELISA and isolation on semiselective medium for detection of *Xanthomonas a/bilineans,* the causal agent ofleaf scald of sugarcane. Plant Pathol. 48:245-252.

3. DIAGNOSTIC COMPANIES — DIAGNOSTIC RESOURCES

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Plant Research International Public Relations and Information P.O. Box 16 6700 AA VV ageningen The Netherlands Telephone: +31 3174770 17 Facsimile: +31 31741 8094 E-mail: info@plant.wag-ur.nl	Research and Development, diagnostic reagents

APPENDIX

C. AUTOMATED TECHNIQUES

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1. PHYSIOLOGICAL AND BIOCHEMICAL METHODS

a. Fatty Acid Analysis

Fatty acids in bacteria occur primarily in the cell membranes and the specific fatty acid composition of membranes affects their fluidity (8). Fatty acids are associated with the cytoplamic and outer membrane (lipopolysaccharide) in Gram-negative bacteria and with the cytoplasmic membrane in Gram positive bacteria. The role of fatty acids in bacterial classification has been recognized for over 35 years (1) and important taxonomic information can be acquired by examining the fatty acid profile of a bacterial strain. Several hundred fatty acids have been recorded in the prokaryotes. This number and diversity of fatty acids allows for many possible combinations. Most bacteria contain 5-30 individual fatty acids, which are generally 8-20 carbons in length. Many scientists have used fatty acid analysis for identification of unknown bacteria. There are several major classes offatty acids in bacteria - primarily, straight chain saturated (16:0), straight chain mono-unsaturated (16: 1 w7cis), cyc1opropanes (17:0 cyclopropane), isobranched (15:0 iso), anteiso-branched (15:0 anteiso), hydroxy (12:0 20B, 12:0 30H) and mixed (13:0 iso 30H, 17: 1 anteiso A). More details of the types of fatty acid occurring in plant pathogenic bacteria are given in Stead (20).

Gram-negative bacteria usually contain straight chain saturated, mono-unsaturated, cyclopropane and most importantly from a taxonomic point, the 3-hydroxy fatty acids. These short chain 3-hydroxy fatty acids occur in the lipopolysaccharide portion of the membrane and are diagnostic for the group. Branched chain acids or cyc1opropanes are uncommon although branched chain acids do occur in *Xanthomonas* spp. which also contain hydroxy fatty acids (11: 0 iso 30H, 13:0 iso 30R). In contrast hydroxy fatty acids or cyclopropanes occur less frequently in Gram-positive bacteria. However, Gram-positive bacteria are especially rich in iso- and anteiso-branched fatty acids together with straight chain saturated fatty acids. Some also contain mono-unsaturated fatty acids.

Specific fatty acid compounds are useful chemotaxonomic markers. Tables 1 and 2 show how the presence of certain fatty acids may be used to predict genus for the Gram-negative and Gram-positive plant pathogenic genera, respectively.

In addition to the presence of specific chemotaxonomic markers, identification to specific and sometimes to intraspecific levels can be made.

Identification is made by comparing the types and relative amounts of the fatty acids in a profile of an unknown with profiles derived from a wide range of strains in a library. For example, two closely related species may contain the same ten fatty acids but in significantly different proportions. Further information is available from lanse (6), Sasser (15), Stead (19, 20), Stead et al. (21), and Wells (23).

Several individuals have developed intelligence systems to process fatty acid data (2, 23). The most sophisticated and widely used automated system for fatty acid analysis is the Microbial Identification System available through MIDI, Inc. (Newark, DE 19713). This software system identifies and quantifies fatty acids from bacteria using gas chromatography. The resulting fatty acid profile is compared to the designated database(s) and a match is made to the most closely related entry in each database. A similarity index is calculated for each match as an indication of how closely the unknown matches to the library entry. Databases are available from MIDI for bacteria (aerobes and anaerobes), fungi, yeast, and actinomycetes. The user can also generate custom libraries and compare the unknown to as many as four different libraries at once.

In order to obtain reproducible results, bacteria must be grown under uniform conditions of time, temperature and nutrients. The extraction procedure used for fatty acids is completed in four steps. Bacterial cells are harvested in a growth stage that approximates the late log phase. A standard quantity of cells are harvested and total cellular fatty acids are extracted and saponified by adding a solution of methanol and sodium hydroxide and heating it to 100°C. Fatty acids are converted to their fatty acid methyl esters (FAMEs) with the addition of hydrochloric acid and methanol heated to 80°C. The FAMEs are then transferred from the aqueous phase to the organic phase using a mixture of hexane and methyl tertiary butyl ether. The organic phase is then treated with a base wash of sodium hydroxide. This removes acidic residues that were added during the methylation step and thus extends the life of the column in the gas chromatograph (GC). The organic phase is removed and then injected into the GC for analysis (15). The MIDI software is only compatible with Hewlett-Packard GCs. An ffiM compatible PC runs the GC and analyzes the data.

The database that is most useful to plant bacteriologists is the TSBA40 library. For most of the entries in the TSBA40 library, bacteria are grown on TSBA (Trypticase Soy Broth and Agar [BBLD, for 24 hr at 28°C. However, accommodations are made within the database for bacteria that will not grow under the standard conditions. For example, *Acidovorax facilis, Hydrogenophaga palleronii, Xanthomonas albilineans,* and *X fragariae* are all slow growing, so library entries were developed using an incubation time of 48 h. There are other strains in the library which were also produced under various incubation times and

nutritional conditions.

Fastidious pathogens such as *Xylella* spp., which have special growth requirements and which are not in the library, can be characterized using the library generation software. With this software, the user can obtain known isolates of the organism, grow them under the required conditions, and produce custom libraries that can then be used to identify suspect unknowns. The MIDI system is particularly useful for identification of many plant pathogens and saprophytes that are present as epiphytes on plant material and in soil.

The MIDI system includes software for dendrograms (or trees) and 2-D plots. The program for 2-D plots compares samples using principle component analysis. The dendrogram program uses cluster analysis to produce unweighted pair matchings based on the fatty acid profile. Unknowns can be compared to known strains and/or to each other, using the 2-D plot and dendrogram programs. Both of these programs allow the user to compare fatty acid profiles of any organisms or any material containing fatty acids (for example: plant and animal fats, nematodes, synthetic diets, etc.).

b. Substrate Utilization

Substrate utilization has been a traditional method of categorizing and identifying bacterial unknowns. Another microbial identification system which may be useful to plant bacteriologists is Biolog (Hayward, CA 94545). Biolog is an automated system that utilizes 96 well microplates containing 95 substrates. Bacterial cells are harvested and suspended in an inoculating fluid. The cell concentration in the suspension is standardized using a spectrophotometer and inoculated into each well. Substrate utilization is detected by the presence of a purple color resulting from the redox reaction oftetrazolium violet. The result is a metabolic fingerprint of the unknown.

The Biolog system uses different microplates for Gram-negative and Gram-positive bacteria; therefore, that determination must be made before initiating the test. Prior to inoculation into the test wells, bacteria must be grown under standard conditions of nutrients, time and temperature. Cells are grown at 30^{D}C for 24 h on BUG agar (available from Biolog). The plates are read at 4 hand/or 24 h. Plates can be read and the data entered into the system manually, or with an automatic plate reader.

The Biolog software searches the library database, chooses the best match library entry, and calculates a similarity index value. The user can develop custom libraries and programs are also available for dendrograms, 2-D plots, and 3-D plots.

There are many other commercially available test kits that employ substrate

utilization. Systems such as Enterotube, Vitek, and BBL Crystal Microbial ill Kit, are designed for use in medical labs and have limited databases that do not include most plant pathogenic bacteria.

c. Biolog and MIDI: Data application and interpretation

Both MIDI and Biolog can be useful for identification of bacterial unknowns. In addition to the use of chemotaxonomic markers for taxonomic determination, comparisons with libraries of profiles can be made. Calculations for the identification of bacteria to genus, species and other taxonomic units are based on the similarity index. Similarity index is the measure used by both systems when comparing unknowns to the best match library entry. Similarity index is the numerical value that expresses how closely the data for the unknown compares with the mean fatty acid composition or substrate utilization pattern of the strains used to create the library entry. This is not a probability or a percentage, but an expression of the relative distance of the unknown from the population mean of the best match library entry. *As* each value varies from the mean percentage, the similarity index will decrease in proportion to the cumulative variance between the composition of the unknown and the library entry (12).

Therefore, a low similarity index value could mean: 1) the unknown belongs in another, closely related genus or species that was not included in the library; 2) the sample that was analyzed contained more than one bacterial strain 3) the strain was not processed according to the standard protocol; or 4) the identification is accurate, but, due to normal strain variability, the fatty acid composition of the unknown differs greatly from that of the library entry.

Identification to the species level can be quite accurate. Identification of bacteria to the pathovar level can be more problematic. MIDI usually provides the best three matches for pathovar. One of the three is usually correct, although not necessarily the first choice. Biolog provides approximately 10 matches. It is essential to perform classical phenotypic tests as described in this manual and plant inoculations in conjunction with fatty acid analysis or Biolog to confirm pathovar designation. Other researchers (17, 24) have found that using the MIDI library generation software, pathovars of interest can be distinguished. Vemiere et al. (22) have suggested that this strategy will work with the Biolog system as well. Yang (24) has also suggested that fatty acid profiles for *Xanthomonas,* a genus with many pathovars, are more stable when cells are incubated for 48 h instead of the standard 24 h.

Sometimes these systems may have difficulty distinguishing between two closely related species such as *Pseudomonas syringae* vs. *P. cichorii.* In this

instance, ancillary tests such as oxidase must be performed. It should be noted that these problems are, to a large extent, due to problems inherent in bacterial taxonomy and strain variability, rather than to deficiencies in these identification systems. An excellent discussion on *Pseudomonas* taxonomy and fatty acids is presented by Janse et al. (6) and Stead (19). It is important to remember that these tests do not replace the plant pathologist, but rather they increase the confidence with which a diagnosis can be made (19). Furthermore, no single test should be relied upon for critical identification.

Fatty acid analysis using the MIDI system and Biolog may not always eliminate the need for other phenotypic tests to confirm a bacterial identification. However, they do provide a quick answer regarding the taxonomic affiliation of an unknown. Some scientists may want to utilize these technologies infrequently, and therefore do not want to purchase an entire system. There are several private companies as well as university and government laboratories that will run these tests on a fee for service basis. Many of these laboratories have web sites and can be located via the internet.

2. MOLECULAR METHODS

8. 16S rRNA Gene Sequencing

Sequence analysis of the 16S rRNA has become a standard technique for identification of unknown bacteria. Ribosomal RNAs occur in all organisms and are good target molecules. There are several advantages to using genotypic information for bacterial identification. The results are not influenced by environmental conditions such as nutrition, temperature, and age. Therefore, this information is less variable and can be interpreted more precisely than physiological or biochemical tests. All cells contain rRNA. In bacteria, the 16S rRNA gene is highly conserved with minor variations. This combination of similar and variable sequences is useful in the identification of bacterial genera and species (14).

Sequence analysis of 16S rRNA gene has excellent powers of discrimination from the level of domain (starting at about 55% homology) to moderately related species (below 97.5% similarity). The 16S rRNA gene is useful because at the species level, the information will help the investigator decide if DNA re-association needs to be performed (18). Sequences of 16S rRNA genes are often species specific and are present in multiple copies in microbial genomes. Therefore, they make excellent targets for identification of bacteria at the species level (16). Ribosomal RNA is naturally amplified. When bacterial cells are grown, usually more than 10^3 ribosomes, and as many copies of the 5S, 16S, and 23S rRNA genes are found.

1) Sequencing of the 16S rRNA gene

The 16S rRNA portion of DNA is amplified by PCR. Below are universal primers that can be used (11).

FGPS6-63 - GGA GAG TTA GAT CTT GGC TCA G
FGPL132-38 - CCG GGT TTC CCC ATT CGG

PCR amplified products can be sequenced directly, or for further amplification the PCR products are then purified and ligated into vectors. *E. coli* cells are transformed with plasmids containing the insert. Cells are grown in liquid culture and then plasmids are isolated and purified. Cloned and amplified products can be sequenced using commercially available sequencing kits or the automated sequencing systems. Due to the highly conserved nature of the 16S region, universal primers can be used for both the PCR and the sequencing reactions.

2) Sequence analysis

a) Internet databases

Once sequencing has been completed, the method of data analysis depends upon the desired application. For bacterial identification to genus and species, sequences are submitted via the Internet to BLAST (http://www.ncbi.nIm.nih.govfBLAST) for comparison with other databases. The BLAST program is available from the National Center for Biotechnology Information (http://www.ncbi.nIm.nih.gov/index.html) and provides access to a variety of DNA sequence databases

b) Commercial system

PE Applied Biosystems (Foster City, CA 94404) has an automated system for 16S rRNA sequencing, called the MicroSeq 16S rRNA Gene Kit. The system is available for 500 base pair sequences or for full gene sequences. Genomic DNA is extracted from cells, purified, then added to the PCR module and amplified. The PCR product is sequenced and analyzed. MicroSeq Microbial Identification and Analysis Software automatically compares the unknown sequence to the database and makes the identification.

The current database provided by the manufacturer consists of over 1000 bacterial strains. Some plant pathogens are included in those entries. Tree based software tools are provided that enable phylogenetic analysis and similarity comparisons. The software also allows users to generate

their own sequence databases.

c) Phylogenetic comparisons

For strain analysis and comparison, 16S rDNA sequences must be aligned using a program such as Clustal W. Similarity values or genetic distances are calculated and evolutionary trees or dendrograms are generated. There are numerous published methods and software packages that can be used for this analysis. Trees and dendrograms can be produced by several different methods ilncluding the neighbor joining method (13), the bootstrap method (5), and parsimony. Pairwise evolutionary distances, expressed as estimated changes per 100 nucleotides can be computed from percent similarities (7). Software packages, which provide user access to several of these methods include PHYLIP, PAUP, MEGA, and ARB. For more detailed information on these as well as numerous other available software packages for phylogenetic analysis we suggest the web site http://evolution.genetics.washington.edu/phylip.html.

b. Ribotyping

Another technique that is used for bacterial identification and strain analysis is ribotyping. Ribotyping refers to the use of nucleic acid probes to recognize ribosomal genes. With this method the DNA fingerprint of the ribosomal RNA genes are used to characterize the organism (9). Genes coding for rRNA are highly conserved and the rRNA genes in many bacteria are very similar. The conserved nature ofrRNA genes allows the use of a single probe to characterize phylogenetically distinct bacteria (3). Probing is done with labeled DNA derived from sequences of an *E. coli* 23 S, 16S, and 5S rRNA operon. All bacteria contain this operon and therefore can be characterized by ribotyping (9).

DNA is extracted, purified, and cut with restriction enzymes. Choice of restriction enzyme is particularly important for strain differentiation. Brosch et a1. (4), looked at ribotypes of *Pseudomonas* and some related species and observed that *Sma!* generated more ribotypes than *Hind!* and therefore, was more discriminative. The digested DNA is then run on agarose gel to separate the resulting fragments by size. The bands of DNA on the gel are transferred to a nylon membrane and probed with labeled rRNA operon. Radioactive or non-radioactive methods of detection can be used. The labeled DNA fragments from each strain produces a unique pattern of bands known as *s fingerprint.* Approximately, fifteen or less bands is the desired number per fingerprint.

Automated systems are available that will scan gels and record the data. Analysis can be completed using various software packages and statistical programs. Several programs such as Taxtron are used for lane and band detection, calculations and

dendrograms. A fully automated system for ribotyping has been developed and is sold by Qualicon, Inc. (Wilmington, DE 19880). A database for bacterial identification is included. Currently the number of entries in the database are relatively few, with an emphasis on medical microbiology. As with any automated identification system, the powers of identification are limited by the size of the database. The software does provide the technology for users to create their own database and share entries with other users of the system.

Gels can also be scored manually. Each band is considered a separate locus or unit character, and migration distances are measured. Another method of analysis is to score each band and convert this information to binary data. The resulting matrix can then be analyzed by phenetic and cladistic methods. Pairwise distances of similarity coefficients are calculated. Strains can be clustered by simple matching using UPGMA (unweighted pair group using arithmetic averages) program or SIMQUAL from NTSYS-pc version 1.80 (Exeter Software, Setauket, NY).

Ribotyping is a rapid way of comparing the genetic relationships among different bacteria. When ribotyping is applied to a large number of strains, this analysis technique appears to be an accurate and useful tool for taxonomic and epidemiological studies (10).

3. COMMERCIAL LABORATORIES

F or a list of some commercial laboratories that provide these analytical services, see Figure 1 and Table 3 and p. 355. This listing does not constitute an endorsement or recommendation of these laboratories.

4. LITERATURE CITED

1. Abel, K., H. deSchmertzing, and 1. 1. Peterson. 1963. Classification of microorganism by analysis of chemical composition. 1. Feasibility of utilizing gas chromatography. 1. Bacteriol. 85: 1039-1044.

2. Behme, R. 1., Shuttleworth, R. McNab and A. Colby. 1996. Identification of staphlococci with a self educating system using fatty acid analysis and biochemical tests. 1. Clin. Microbiol. 34:3075-3084.

3. Berthier, Y, V. Verdier, J.-L. Guesdon, D. Chevrier, 1. L. Denis, G. Decoux, and M. Lemattre. 1993. Characterization of *Xanthomonas campestris* pathovars by rRNA gene restriction patterns. Appl. Environ. Microbiol. 59:851-859.

4. Brosch, R., M. Lefevre, F. Grimont, and P. A. D. Grimint. 1996. Taxonomic diversity of pseudomonads revealed by computer interpretation of ribotyping data. Syst. Appl. Microbiol. 19:541-55.

5. Felsenstein, J. 1985. Confidence limits on phylogenies: An approach to using the bootstrap. Evolution 39:783-91.

6. Janse,1. D., 1. H. 1. Derks, B. E. Spit, and W. R. van der Tuin. 1992. Classification of fluorescent soft rot *Pseudomonas* bacteria, including *P. marginalis* strains, using whole cell fatty acid analysis. Syst. Appl. Microbiol. 15:538-553.

7. Jukes, T. H., and C. R Cantor. 1969. Evolution of protein molocules. Pages 121-132 in: H. N. Munro, ed., Mammalian Protein Metabolism v 3. Academic Press, New York.

8. Kaneda, T. 1991. Iso- and anteiso fatty acids in bacteria: Biosynthesis, function, and taxonomic significance. Microbiol. Rev. 55:288-302.

9. Maslow, J. N., M. E. Mulligan, and R. D. Arbeit. 1993. Molecular epidemiology: Application of contemporary techniques to the typing of microorganisms. Clin. Infec. Dis. 17:153-164.

10. Nassar, A., Y. Bertheau, C. Dervin, J-P. Narcy, and M. Lemattre. 1994. Ribotyping of *Erwinia chrysanthemi* strains in relation to their pathogenic and geographical distribution. Appl. Environ. Micro. 60:3781-3789.

11. Normand, P., B. Cournoyer, P. Simonet, and S. Nazaret. 1992. Analysis ofa ribosomal RNA operon in the actinomycete *Frankia.* Gene. 111: 119-124.

12. Paisley, Ralph (ed). 1997. Training manual. MIS whole cell fatty acid analysis by gas chromatography. MIDI Labs, Inc., Newark, DE.

13. Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. BioI. Evolu. 4:406-425.

14. Sasaki, T., T. Nishiyama, M. Shintan, and T. Kenri. 1997. Evaluation ofa new method for identification of bacteria based on sequence homology of the 16S rRNA gene. PDA 1. Pharm. Sci. & Tech. 51:242-247.

IS. Sasser, M. 1990. Identification of bacteria through fatty acid analysis. Pages 200-204 in: z. Klement, K. Rudolph, and D. C. Sands, eds., Methods in Phytobacteriology. Akademiai Kiado, Budapest.

16. Seal, S. E., L. A. Jackson, J. P. W. Young, and M. J. Daniels. 1993. Differentiation of *Pseudomonas solanacearum, Pseudomonas syzygii, Pseudomonas pickettii* and the blood disease bacterium by partial16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J. Gen. Micro. 139: 15871594.

17. Sletten, A. 1996. Rapid identification of *Xanthomonas campestris* pv. *graminis* and *X* c. pv. *phlei* by fatty acid profiling. J. Phytopathol. 144: 321-325.

18. Stakenbrandt, E. and B. M. Goebel. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44: 846-849.

19. Stead, D. E. 1992. Grouping of plant pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. Int. J. Syst. Bacteriol. 42:281-295.

20. Stead, D. E. 1991. Techniques for detecting and identifying plant pathogenic bacteria. Pages 76-111 in: Techniques for rapid diagnosis of plant pathogens, J. Duncan and L. Torrance, eds. Blackwell Scientific Publications, Oxford

21. Stead, D. E., J. E. Sellwood, J. Wilson, and I. Viney. 1992. Evaluation ofa commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. J. Appl. Bacteriol. 72:315-321.

22. Verniere, C., O. Provost, E. L. Civerolo, O. Gambin, J. P. Jacquemoud-Collet, and J. Luisette. 1993. Evaluation of the Biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri.* Appl. Environ. Microbiol. 59:243-249.

23. Wells, J. M., J. E. Butterfield, and L. G. Revear. 1993. Identification of bacteria associated with postharvest diseases of fruits and vegetables by cellular fatt . composition: An expert system for personal computers. Phytop th.ot0gy:83:zJ4-s::4S5.

24. Yang, P., L. Vauterin, M. Vancanneyt, Jean Swings, and K. ~ers. 1993. Applicati9 offatty acid methyl esters for taxonomic analysis of the us *Xanthomonas.* Syst. A pl. Microbiol. 16:47-71.

Table 1 **Prediction of Gram-negative genera of plant pathogenic bacteria and other plant associated bacteria based on hydroxy fatty acids**

					+	+	+	+						
		+												
			+	+		+								
						-		+		2			+	
						-	+	+	+	+				
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		2	+			+								
						+		+					+	+
						+								
				+							+	+		
		+		+							+	+		

+ = thought to occur in all strains
(+) = occurs in some taxa/strains

Table 2 Prediction of Gram positive genera of plant pathogenic bacteria based on fatty acids*

	[illegible]	[illegible] ANTEISO	ANTEISO A	ANTEISO	[illegible]
Bacillus	+	+			
Clavibacter	+	+	+	+	
Curtobacterium	+	+		+	
Rathayibacter	+	+	(+)	+	
Rhodococcus					+

*Many Gram positive plant associated bacteria such as [illegible]*throbacter* spp. and *Curtobacterium* spp. have very similar fatty acids in their profiles

+ = thought to occur in all strains

(+) = occurs in some taxa/strains

TABLE 3 List of analytical laboratories and some bacterial identification services provided

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Abbreviations: B, Biolog; M, MIDI technique; NA, not available

5. DIAGNOSTIC COMPANIES

AgResearch NZ Ltd ..
Ruckura Research Center
Private Bag 3123
East Street
Hamilton
New Zealand
64-7 -838-6169
http://www.agreaserach.cri.nz

Analytical Services, Inc.
P.O. Box 515
130 Allen Brook Lane
Williston, VT 05495
(802) 878-513 8
http://www.asimicro.com

Bacterial Identification and Fatty Acid Analysis Laboratory
Plant Pathology Department
P.O. Box 110680
University of Florida
Gainesville, FL 32611
(352) 392-7244
http://plantpath.ifas. ufl. edu/fame

Baron Consulting
P.O. Box 13
Milford, CT 06460
(203) 877-4284
http://www.baronconsulting.com

BCCM™ILMG Bacteria Collection
Laboratorium voor Microbiologie - Universiteit Gent
K. L. Ledeganckstraat 35
B-9000 Gent
Belgium
Phone +32-9-2645108
http://www.belspo.be/bccmllmg.htm

BSI-MAS
Plant Pathology Department
209 Life Sciences Bldg.

Auburn University
Auburn, AL 36849
(334) 844-1982
http://www.ag.auburn.edu/dept/ent/services/bacterid.htm
CCUG, Department of Clinical Bacteriology
University of Goteborg, Mikrobiologen
Guldhedsgatan 10, 6tr
S-413 46 Goteborg, Sweden
46-31-342-4625
http://www.ccug.gu.se

Cytoculture Environmental Biotechnology
249 Tewksbury Ave.
Pt. Richmond, CA 94801
(510) 233-0102
http://www.cytoculture.com

Microbial Insights
2340 Stock Creek Blvd
Rockford, TN 37853-3044
(423) 573-8188
http://www.microbe.com

Microbial ill and MIDI
Labs 125 Sandy Drive
Newark, DE 19713
(302) 737-4297
http://www.microbialid.com

National Collection of Plant Pathogenic Bacteria
Central Science Laboratory
Sand Hutton, York
United Kingdom
Y0411LZ
44-1904-462272
http://www. csl. gov. uk

NCIMBLtd.
23 St. Marcher Drive
Aberdeen AB24 3R Y
Scotland
United Kingdom
44-1224-273332

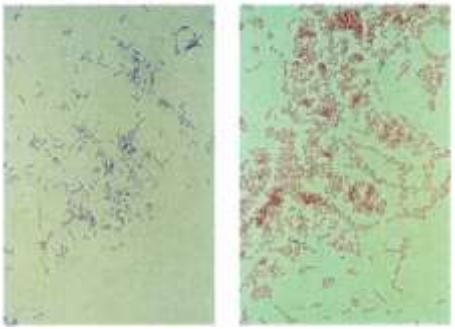
http://www.ncimb.co.uk

Nelson Labs
6280 South Redwood Road Salt
Lake City, Utah 84123-660 (801)
963-2600
http://www.nelsonlabs.com

Plant Pathology & Microbiology Diagnostic Lab
Texas A & M University
RM 101 Peterson Building
College Station, Texas 77843
(409) 845-8033
http://plantpathology. tamu. edu/index4. html

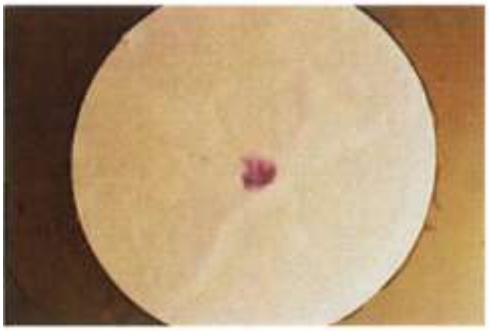
Qualicon, Inc.
Wilmington, DE
(800) 863-6842
http://www.qualicon.com

Warren Labs
650 "0" Street
Greely, CO 80632-0350 (970)
351-6344
httpv/www.warrenlaba.corn



Positive Negative

Fig. 1. Gram stain



Positive

Fig. 2. Oxidase test

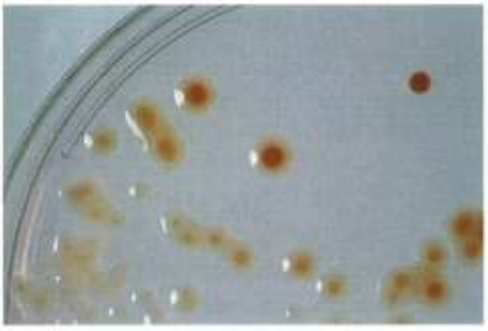


Fig. 3. Range of colony types of *Agrobacterium tumefaciens* on Brisbane and Kerr medium 1A

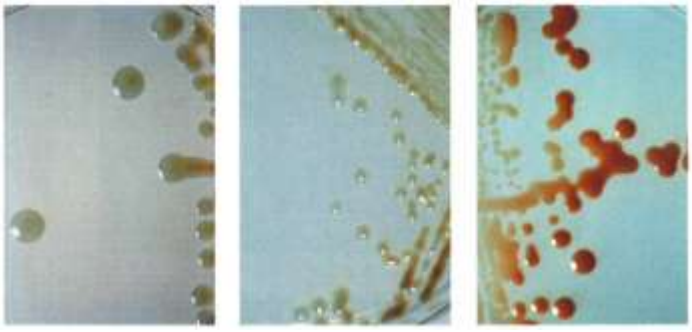


Fig. 4. Range of colony types of *Agrobacterium rhizogenes* on Brisbane and Kerr medium 2E

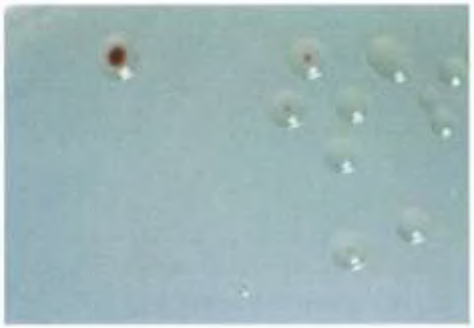
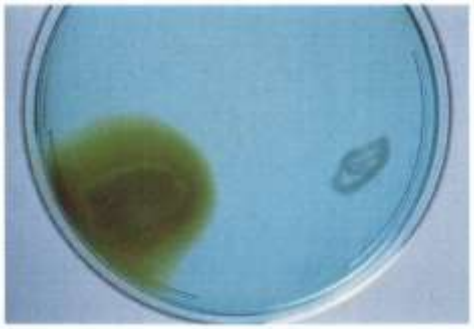
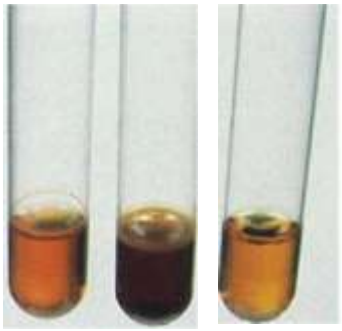


Fig. 5. Colonies of *Agrobacterium vitis* on Roy and Sasser medium



Positive Negative

Fig. 6. 3-Ketolactose test



Negative Positive Negative

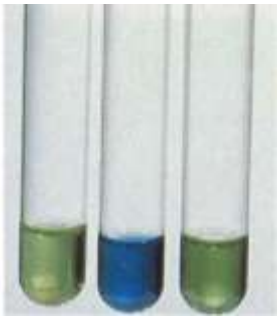
Fig. 7. Ferric ammonium citrate

Positive Negative

Fig. 8. Citrate utilization

Acid Alkaline Uninoculated

Fig. 9. Action on litmus milk



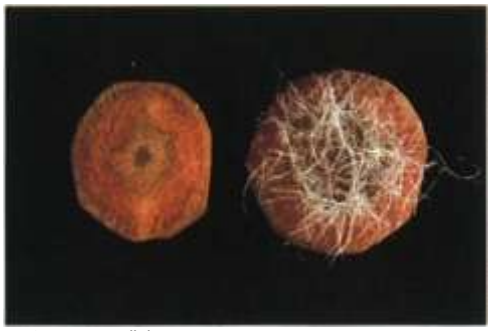
Negative Positive Negative

Fig. 10. Alkali from malonic acid

Photo credits: Fig. 1, S. Poe. Figs. 2-10, L. M. Moore.

PLATE 1

A. Tomato



B. Carrot root disk

Fig. 1. Pathogenicity tests for *Agrobacterium tumefaciens* (A) and A. *rhizogenes* (B)

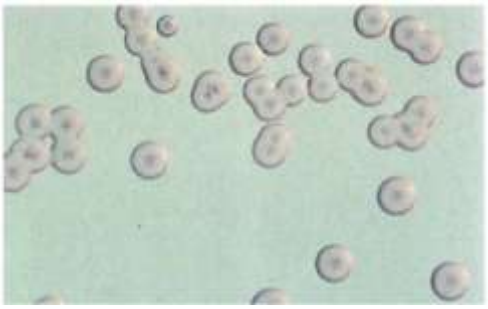


Fig. 2. Colonies of *Erwinia amylovora* on CCT medium

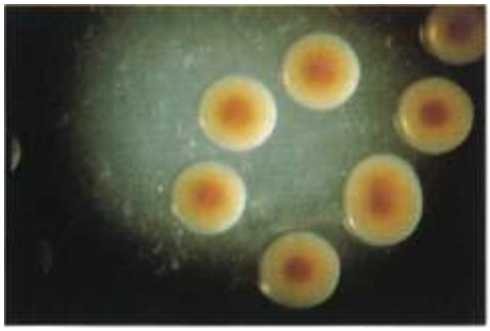
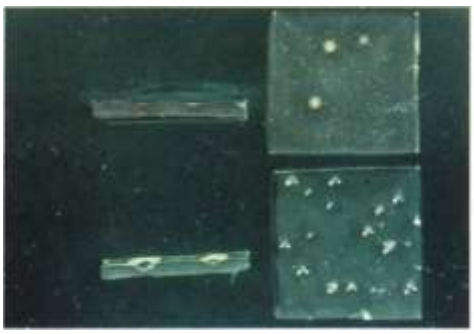


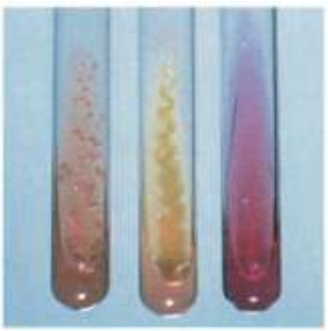
Fig. 3. Colonies of *Erwinia amylovora* on MS medium

Fig. 4. Colonies of *Erwinia amylovora* (rows 1 and 2), *Pantoea herbicola* (row 3, two colonies on the left), *E. carotovora* subsp. *atroseptica* (row 3, three colonies on the right), *Agrobacterium tumefaciens* (row 4, three colonies on the left), and *Pseudomonas syringae* pv. *syringae* (row 4, two colonies on the right) on MM2Cu medium after 4 days at 28 C



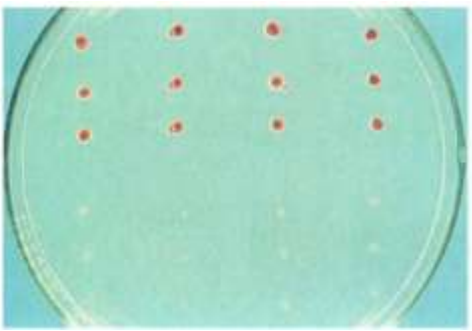
Pseudomonas spp. (top) *Erwinia carotovora* (bottom)

Fig. 5. Colonies of soft rotting bacteria on CVP medium



Negative Positive Un inoculated

Fig. 6. Acid production from carbohydrates



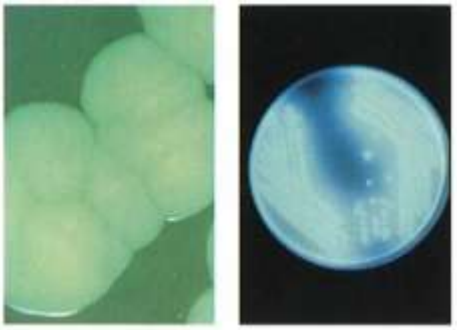
Positive (top) Negative (bottom)

Fig. 7. Utilization of keto-methyl glucoside

Photo credits: Fig. 1, L. M. Moore. Fig. 2, C. A. Ishimaru. Fig. 3, M. N. Schroth. Fig. 4, K. Geider. Figs. 5 and 7, A. Kelman. Fig. 6, D. W. Dye. **PLATE**

ElWinia carotovora subsp, *atroseptica* *Pseudomonas marginalis* 13)

Fig. 1. Potato soft rot



Visible light UV light

Fig. 2. Colonies of *Pseudomonas syringae* on King et al.'s medium B

Negative Positive

Fig. 3. Arginine dihydrolase test

Fig. 4. Tobacco hypersensitive test

Distortion reaction

Fig. 5. Kidney bean inoculated with *Pseudomonas syringae* pv. *pisi*

Angular lesions

Fig. 6. Kidney bean inoculated with *Pseudomonas syringae* pv. *tabaci*

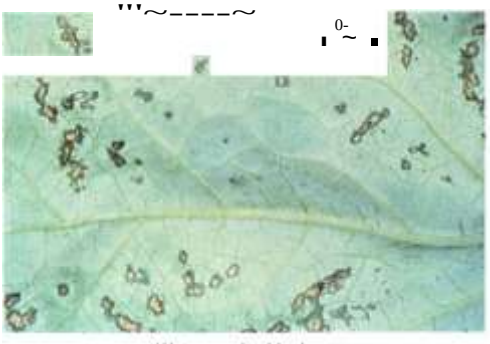


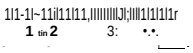
Fig. 7. Lima bean inoculated with *Pseudomonas syringae* pv. *phaseolicoJa*

Fig. 8. Colonies of virulent strains (white colonies) and avirulent strains (red colonies) of *Ralstonia soJanacearum* on TIC medium

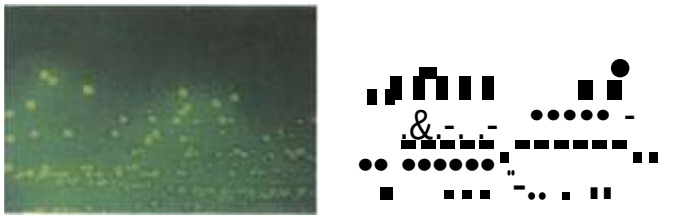
Photo credits: Fig. 1, A. Kelman. Fig. 2, N. W. Schaad and D. C. Sands. Fig. 3, L. M. Moore. Figs. 4-7, D. C. Sands. Fig. 8, A. Kelman.

pv. *cempestris* pv. *citri* type A

Fig. 1. Colonies of *Xanthomonas campestris* on YDC agar



Scale for Fig. 1

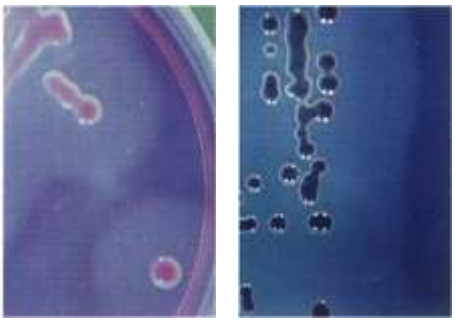


Tween xes

MXP XTS

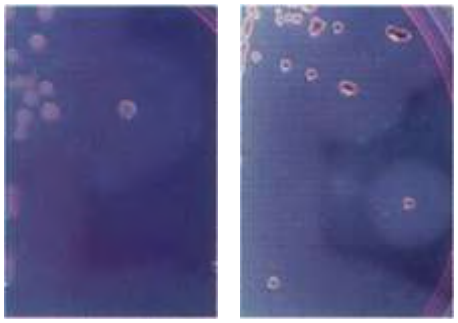
Fig. 2. Colonies of *Xanthomonas campestris* pvs. *vesicatoria, carotae,* and *vignicola* and *X. trenetucens* on Tween, XCS, MXP, and XTS, respectively

Scale for Fig. 2 (em)



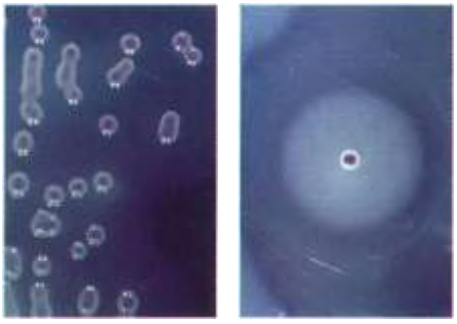
SX SM

pv. *campestris*



SX SM

pv. *begoniae*



SX SX

pv. *glyciniae* pv, *dieffenbachiae*

Fig. 3. Colonies of *Xanthomonas campestris* on SX and SM agars

Scale for Fig. 3 (cm)

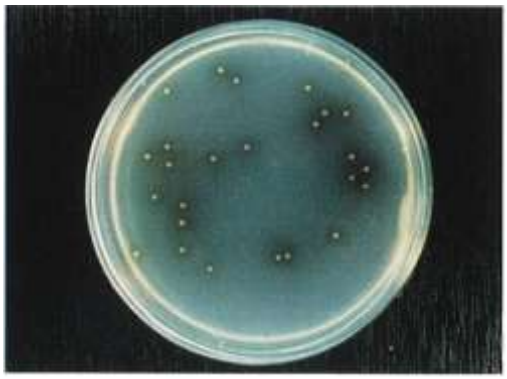


Fig. 4. Colonies of *Xanthomonas campestris* pv. *diet-tenbachiae on* ET medium (also useful for esculin test)

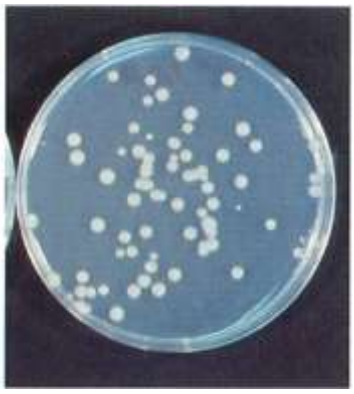


Fig. 5. Colonies of *Xanthomonas csmpestrte* pv. *manihotis* on CTA medium

Photo credits: Fig. 1, N, W. Schaad. Fig. 2, J. Olsen and N. W. Schaad. Fig. 3, J. Olsen, Fig. 4, A. M, Alvarez. Fig. 5, A. Fessehaie.

Fig. 1. Lettuce with symptoms of corky root

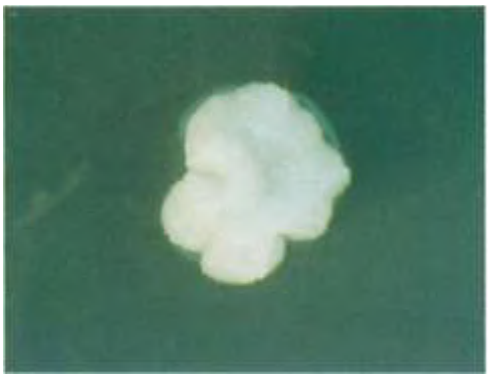
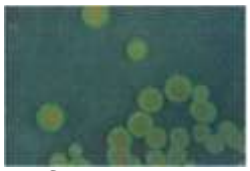
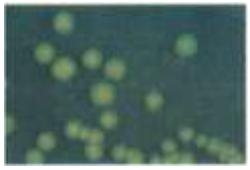


Fig. 2. Colonies of *Rhizomonas suberifaciens* on S-medium

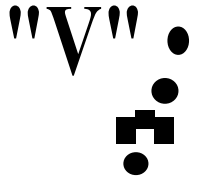


C. *f1accumfaciens* pv. *tteccumteciens*



R. fascians

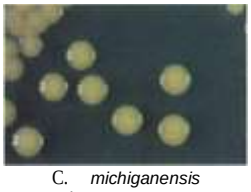
C. *michiganensis* subsp. *insidiosus*



C. *michiganensis* subsp. *michiganensis*

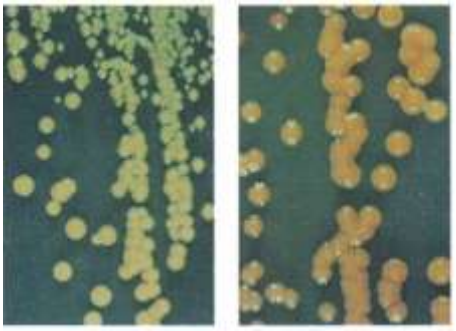
C. *michiganensis* subsp. *nebraskens~*

C. *michiganensis* subsp. *sepedon~us*



C. *michiganensis* subsp. *tesseflarius*

Fig. 3. Colonies of *Curtobacterium f1accumfaciens, Rhodococcus fascians,* and several *Clavibacter michiganensis* subspecies on NBY medium



subsp. *nebraskensis* subsp. *tesseflarius*

Fig. 4. Colonies of *Clavibacter michiganensis* subspecies on CNS medium

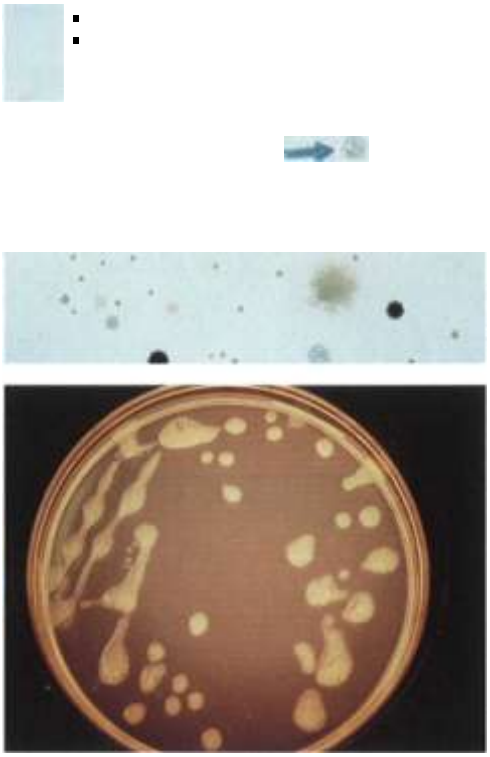


Fig. 5. Colonies of *Clavibacter michiganensis* subsp. *michiganensis* growing on SCM (top) and m SCM (bottom)

Photo credits: Figs. 1 and 2, L. E. Datnoff. Figs. 3 and 4, R. R. Carlson. Fig. 5, M. Fatmi (top) and H. A. Balkan and C. M. Waters (bottom).

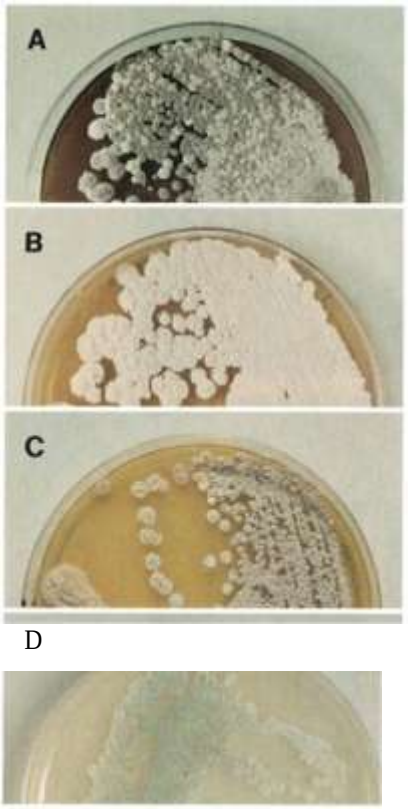


Fig. 1. Sporulating colonies of (A) *Streptomyces scabies,* (8) S. *acidiscabies,* and (e) *S. turgidiscabies* on YME (ISP2) medium and (0) S. *ipomoeae* on SGM

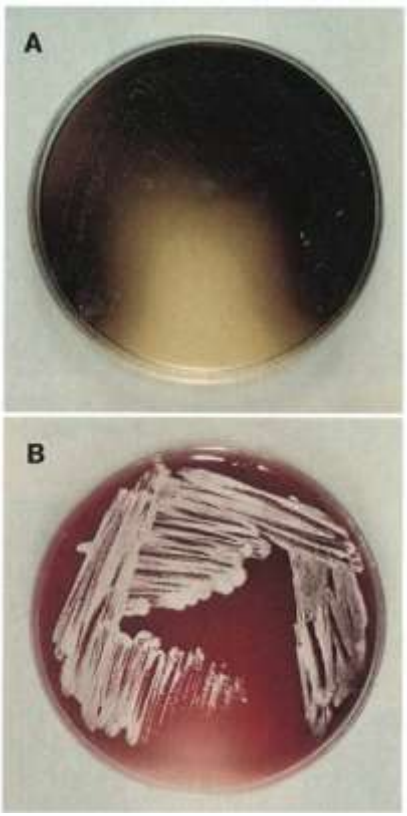


Fig. 2. Pigment production by pathogenic *Streptomyces* spp.: (A) S. *scabies* produces melanin on peptone yeast extract iron medium; (B) S. *acidiscabies* produces a pH-sensitive red-yellow pigment on modified salts starch agar

Fig. 3. Radish seedlings (A) in culture tubes and (B) removed from tubes, 7 days after treatment: seedlings were (a) untreated or treated with (b) 75 nM purified thaxtomin A, (c) filter-sterilized *Streptomyces scabies* culture supernatant (containing thaxtomin A but not the pathogen), causing stunting and radial swelling, and (d) whole S. *scabies* culture, causing necrosis and seedling collapse

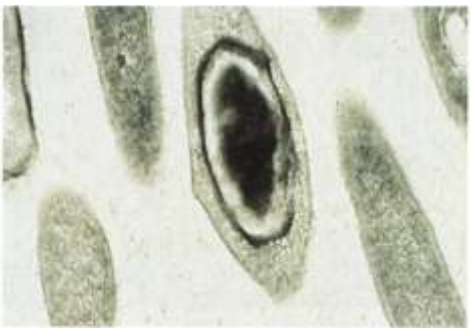
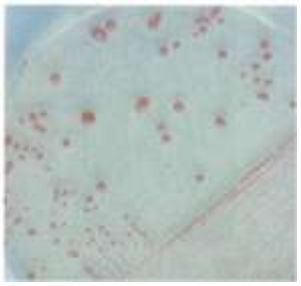


Fig. 4. Endospores of *Bacillus* spp.



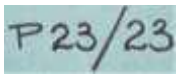


Fig. 5. Growth of *Clostridium puniceum* on potato infusion agar

Fig. 6. Growth of *Clostridium puniceum* on potato slice

Photo credits: Figs. 1-3, R. Loria. Fig. 4, J. Leary. Figs. 5 and 6, A. Kelman.

PLATE 6