APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1994, p. 1068–1077
0099-2240/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 60, No. 4

Detection and Identification of Phytopathogenic *Xanthomonas* Strains by Amplification of DNA Sequences Related to the *hrp* Genes of *Xanthomonas campestris* pv. vesicatoria†

RUI P. LEITE, JR.,[1] GERALD V. MINSAVAGE,[1] ULLA BONAS,[2] AND ROBERT E. STALL[1]*

Department of Plant Pathology, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida 32611,[1] and Institut des Sciences Végétales, Centre National de la Recherche Scientifique, F-91198 Gif-sur-Yvette, France[2]

Received 18 October 1993/Accepted 7 January 1994

Three pairs of oligonucleotide primers specific for different regions of the *hrp* gene (hypersensitive reaction and pathogenicity) cluster of *Xanthomonas campestris* pv. vesicatoria were designed and tested for amplification of DNA isolated from a large number of different bacteria. DNA sequences related to the *hrp* genes were successfully amplified from *X. fragariae* and from 28 pathovars of *X. campestris*. No DNA amplification occurred with genomic DNA from phytopathogenic strains of *X. campestris* pv. secalis, *X. campestris* pv. translucens, and *X. albilineans* or from nonpathogenic opportunistic xanthomonads and phytopathogenic strains of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xylella*. The DNA from those bacteria also failed to hybridize to *hrp*-specific fragments in Southern blot analysis. DNA fragments amplified with a particular primer pair were of identical size from each of the different phytopathogenic xanthomonads. However, restriction analysis of these fragments by using frequently cutting endonucleases revealed variation in the pattern for these *hrp*-related fragments amplified from the different *Xanthomonas* strains. The restriction patterns generated for the different fragments allowed distinction of the strains representing a pathovar or species of phytopathogenic xanthomonads. We believe that DNA amplification with *hrp*-specific oligonucleotide primers is a highly sensitive and specific method that can be applied for detection and identification of phytopathogenic xanthomonads.

The genus *Xanthomonas* Dowson 1939 contains gram-negative, usually yellow-pigmented bacteria that occur worldwide and cause plant diseases. Over 124 monocotyledonous and 268 dicotyledonous plant species are hosts of *Xanthomonas* strains (37). Among the *Xanthomonas* species, *X. campestris* comprises at least 125 different pathovars that are distinguished by the diseases they cause (13). The genus *Xanthomonas* also includes strains which may be associated with plant material but are not pathogenic to the plants from which they were isolated (3, 22, 38, 40). These opportunistic bacteria can be identified as xanthomonads by the presence of xanthomonadins and by unique fatty acid profiles. Although the identification of bacteria as members of the genus *Xanthomonas* presents no great problem, subgeneric identification of xanthomonads is still difficult.

Traditional methods for the detection and identification of phytopathogenic xanthomonads rely on isolating the organism of interest in pure culture and performing predetermined biochemical, serological, and pathological tests (46, 48). Sometimes, nonselective or selective enrichments are required to increase the sensitivity of the isolation, which may be complicated by the presence of fast-growing contaminating bacteria associated with plant tissue (46). More recently, methods based on metabolic and protein profiling (14, 57–59) and on fatty acid analysis (14, 61) have been used for identification, but isolation and purification of the bacterial strain are still required. Polyclonal or monoclonal antisera produced against strains of *X. campestris* have been used for detection and identification, but they have provided variable results. Polyclonal antisera may cross-react with other bacteria and may be unable to differentiate specific strains or pathovars of *X. campestris* (2). Several monoclonal antisera were produced which reacted specifically with all strains of some pathovars of *X. campestris* that infect relatively few genera of hosts (1, 7). However, for certain *X. campestris* pathovars, mainly those that infect several host genera, no pathovar-specific monoclonal antisera that react with all strains of the respective pathovar have been found (1, 30).

Nucleic acid-based techniques have also been applied to the detection and identification of phytopathogenic bacteria (8, 41, 49, 51), including some members of the xanthomonads (20, 21, 25, 26, 32, 33). The techniques developed for detection and identification of xanthomonads were based on random probes (33) or on plasmid DNA fragments specific for a few pathovars of *X. campestris* (21, 25, 26, 32) or even for a group of strains (20). Highly conserved regions in the bacterial genome of phytopathogenic bacteria could be more useful for the selection of specific DNA probes for detection and identification of a larger number of *Xanthomonas* strains, pathovars, or species.

The *hrp* gene clusters that determine hypersensitivity and pathogenicity may be appropriate for selection of probes for detection and identification of phytopathogenic bacteria. The *hrp* gene cluster is required by bacterial plant pathogens to produce symptoms on susceptible hosts and a hypersensitive reaction on resistant hosts or on nonhosts (60) and has been found in several phytopathogenic bacteria, such as *Erwinia amylovora* (6), *Pseudomonas solanacearum* (12), *P. syringae* pv. phaseolicola (39), and *X. campestris* pv. vesicatoria (11). Furthermore, *hrp* functions seem to be highly conserved among a number of phytopathogenic bacteria (10, 18, 23, 29). The *hrp*

* Corresponding author. Mailing address: Plant Pathology Department, University of Florida, P.O. Box 110680, Gainesville, FL 32611-0680. Phone: (904) 392-7244. Fax: (904) 392-6532. Electronic mail address: REST@gnv.ifas.ufl.edu.

† Florida Agricultural Experimental Station Journal Series R-03428.

genes of phytopathogenic bacteria are also very similar at the protein level to genes that are involved in the secretion of pathogenicity factors by bacterial pathogens of mammals (18, 23). By contrast, nonpathogenic bacteria are unable to produce symptoms on susceptible hosts and hypersensitive reaction on nonhosts, and apparently they do not possess DNA sequences similar to *hrp* genes (39, 53). Physically and functionally similar *hrp* sequences occur among several pathovars of *X. campestris* but not in opportunistic xanthomonads (11, 53).

The objective of this study was to examine sequences of the *hrp* genes of *X. campestris* pv. vesicatoria for their use in detection and identification of phytopathogenic xanthomonads. Oligonucleotide primers specific for *hrp* genes were tested for their suitability for identification of these xanthomonads by PCR. Further, the reliability of identification of the xanthomonads to subgeneric classification was assessed by restriction analysis of amplified DNA fragments. A preliminary report of this work has been presented elsewhere (36).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study and their sources are listed in Tables 1 and 2, respectively. The identity of bacterial strains used here was confirmed by fatty acid analysis (28). All strains of *Acidovorax avenae*, *Agrobacterium tumefaciens*, *Erwinia* spp., *Clavibacter michiganense*, *Pseudomonas* spp., *X. campestris*, and *X. maltophilia* were grown on nutrient agar (Becton Dickinson, Cockeysville, Md.). Nutrient broth cultures were grown for 24 h on a rotary shaker (150 rpm) at 28°C. Strains of *X. albilineans* and *X. fragariae* were cultivated on Wilbrink's medium (31). Strains of *Xylella fastidiosa* were grown on PW medium (15). Strains of *Escherichia coli* were cultivated on Luria-Bertani medium at 37°C (42). All strains were stored in sterile tap water at room temperature or in 30% glycerol at −70°C, or both. Antibiotics were used to maintain selection for resistance markers at the following final concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; rifampin, 100 μg/ml; and spectinomycin, 50 μg/ml.

Plant material and plant inoculations. All plants were maintained in a growth chamber at 28 to 30°C during inoculation and incubation. The pepper cultivar Early Calwonder (ECW) and the isogenic lines ECW-10R, ECW-20R, and ECW-30R have been described elsewhere (44). These lines provided a susceptible reaction or a hypersensitive reaction, depending on the strain used.

Fully expanded leaves of plants were inoculated with bacterial suspensions by infiltrating the bacteria into the intercellular spaces by using a 1-ml plastic syringe with a 27-gauge needle. The concentration of the inoculum was approximately 5×10^8 CFU/ml in sterile tap water, as determined by measuring the optical density in a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.). Plant reactions were scored over a period of several days.

DNA manipulations. Total genomic DNA was isolated by phenol extraction and ethanol precipitation essentially as described by Ausubel et al. (4). Plasmid miniprep, preparation of competent cells, ligation, and transformation of *E. coli* cells were performed by standard procedures (4, 47). For complementation analysis, the helper plasmid pRK2013 was used in triparental mating to mobilize pLAFR3 clones from *E. coli* into *Xanthomonas* cells (16, 19).

Hybridization analysis. Total genomic DNA and amplified DNA fragments were electrophoresed in 0.7% agarose by standard procedures (47). The DNA was then denatured in 0.4 N NaOH–0.6 M NaCl for 30 min, neutralized in 0.5 M Tris · Cl–1.5 M NaCl (pH 7.5) for 30 min, and transferred by the procedure of Southern (52) to a nylon membrane (Schleicher & Schuell, Keene, N.H.). Southern hybridization and detection of the hybridized DNA were carried out by using the Genius Nonradioactive DNA Labeling and Detection kit (Boehringer Mannheim, Indianapolis, Ind.) as specified by the manufacturer. Clones containing the desired insert of the *hrp* gene cluster of *X. campestris* pv. vesicatoria, or in vitro amplified *hrp* fragments used as probes, were labeled by random-primer (17) incorporation of digoxigenin-labeled dUTP. Before use, the probes were denatured by boiling for 10 min followed by chilling in an ice-ethanol slurry. Hybridization was carried out at 68°C with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) sodium dodecyl sulfate (SDS). The membranes were prewashed twice at room temperature for 5 min each in 1× SSC–0.1% (wt/vol) SDS. Two final washes were completed at 65°C for 15 min each in 0.5× SSC–0.1% (wt/vol) SDS.

DNA amplification. Three sets of oligonucleotide primers were selected from the nucleotide sequence of the *hrp* region of *X. campestris* pv. vesicatoria (10). Primers RST2 (5′AGGCC CTGGAAGGTGCCCTGGA3′) and RST3 (5′ATCGCACTG CGTACCGCGCGCGA3′) delineated an 840-bp fragment, RST9 (5′GGCACTATGCAATGACTG3′) and RST10 (5′A ATACGCTGGAACTGCTG3′) delineated a 355-bp fragment, and RST21 (5′GCACGCTCCAGATCAGCATCG AGG3′) and RST22 (5′GGCATCTGCATGCGTGCTCTCC GA3′) delineated a 1,075-bp fragment. The primers map to the complementation groups *hrpB*, *hrpC*, and *hrpD* of *X. campestris* pv. vesicatoria (Fig. 1). Furthermore, the sequences of the oligonucleotide primers RST3 and RST9 originate from *hrpB6*, a gene for a putative ATPase that seems to be highly conserved among different bacteria at the protein sequence level (18). Oligonucleotide primers were synthesized with a model 394 DNA Synthesizer (Applied Biosystems, Foster City, Calif.) by the DNA Synthesis Laboratory, University of Florida, Gainesville.

DNA was amplified in a total volume of 50 μl. The reaction mixture contained 5 μl of 10× buffer (500 mM KCl, 100 mM Tris · Cl [pH 9.0 at 25°C], 1% Triton X-100), 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (Boehringer Mannheim), 25 pmol of each primer, and 1.25 U of *Taq* polymerase (Promega, Madison, Wis.). The amount of template DNA added was 100 ng of purified total bacterial DNA or 25 ng of a plasmid preparation, unless otherwise stated. The reaction mixture was covered with 50 μl of light mineral oil. A total of 30 amplification cycles were performed in an automated thermocycler (PT-100-60; MJ Research, Watertown, Mass.). Each cycle consisted of 30 s of denaturation at 95°C, 30 s of annealing at 62°C, and 45 s of extension at 72°C for primers RST2 and RST3; 30 s at 95°C, 30 s at 52°C, and 45 s at 72°C for primers RST9 and RST10; and 30 s at 95°C, 40 s at 61°C, and 45 s at 72°C for primers RST21 and RST22. The last extension step was extended to 5 min. Amplified DNAs were detected by electrophoresis in 0.9% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.2]) at 5 V/cm of gel (47). The gel was stained with 0.5 μg of ethidium bromide per ml and then photographed over a UV transilluminator (Fotodyne Inc., New Berlin, Wis.) with type 55 Polaroid film (Polaroid, Cambridge, Mass.).

Restriction endonuclease analysis. The DNA fragments amplified from different bacterial strains were restricted with the frequently cutting endonucleases *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I under conditions specified by the manufacturer (Promega). The restricted fragments were separated by electrophoresis in 4% agarose gels (3% NuSieve, 1% SeaKem GTG

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source or reference[a]
X. campestris		
pv. alfalfae KS		DLS
pv. armoraciae 63-27		JEH
pv. begoniae XCB9		JBJ
pv. bilvae XCB		ELC
pv. campestris 33913		ATCC
pv. carotae 13		RES
pv. citri 9771		DPI
pv. citrumelo F1		DPI
pv. dieffenbachiae 729		RES
pv. fici X151		ARC
pv. gardneri XG101		DCH
pv. glycines 87-2		RES
pv. holcicola G-23		JEL
pv. incanae 9561-1		RES
pv. maculifoliigardeniae X22j		DPI
pv. malvacearum RIATC		RES
pv. manihotis Xm125D		RES
pv. papavericola XP5		DCH
pv. pelargonii XCP58		JBJ
pv. phaseoli 85-6		RES
pv. phaseoli "fuscans" XP163A		DCH
pv. physalidicola XP172		DCH
pv. poinsettiicola 071-424		RES
pv. pruni		
X1219L		ARC
FLA1		RES
pv. raphani 69-2		RES
pv. secalis XC129C		RES
pv. taraxaci XT11A		DCH
pv. translucens 80-1		RES
pv. vesicatoria		
75-3	Group A	RES
85-10	Group A	RES
XV56	Group B	RES
85-10::*hrpA22*	*hrpA* Tn*3-gus* insertion mutant of strain 85-10	11
85-10::*hrpC44*	*hrpC* Tn*3-gus* insertion mutant of strain 85-10	11
85-10::*hrpE75*	*hrpE* Tn*3-gus* insertion mutant of strain 85-10	11
85-10::*hrpB85*	*hrpB* Tn*3-gus* insertion mutant of strain 85-10	11
85-10::*hrpD137*	*hrpD* Tn*3-gus* insertion mutant of strain 85-10	11
85-10::*hrpF318*	*hrpF* Tn*3-gus* insertion mutant of strain 85-10	11
pv. vignicola 81-30		RES
pv. vitians XVIT		RES
X198	Isolated from *Strelitzia reginae*	ARC
XCF	Isolated from *Feronia* sp.	ELC
T-55	Opportunistic	RES
INA	Opportunistic	RES
X. albilineans 91-065		JCC
X. fragariae X1297		ARC
X. maltophilia		RES
Acidovorax avenae		
subsp. *avenae* UK142-A		JBJ
subsp. *citrulli* UK20		JBJ
Agrobacterium tumefaciens LBA1050		BJS
Clavibacter michiganense subsp. *michiganense* 69-1		RES
Erwinia carotovora subsp. *carotovora*		
K-SR-347		JAB
B-SR38		JAB
Erwinia herbicola NF-33		RES
Erwinia stewartii SW2		DLC
Pseudomonas solanacearum K60		AK
Pseudomonas syringae		
pv. syringae INB		RES
pv. tomato 987		RES
Xylella fastidiosa 89-1		DLH
Escherichia coli		
DH5α	F⁻ *recA 80dlacZM15*	BRL
HB101	F⁻ *recA*	BRL

[a] AK, A. Kelman, University of Wisconsin, Madison; ARC, A. R. Chase, University of Florida, Apopka; ATCC, American Type Culture Collection, Rockville, Md.; BJS, B. J. Staskawicz, University of California, Berkeley; BRL, Bethesda Research Laboratories, Gaithersburg, Md.; DCH, D. C. Hildebrand, University of California, Berkeley; DLC, D. L. Coplin, Ohio State University, Columbus; DLH, D. L. Hopkins, University of Florida, Leesburg; DLS, D. L. Stuteville, Kansas State University, Manhattan; DPI, Department of Plant Industry, Gainesville, Fla.; ELC, E. L. Civerolo, U.S. Department of Agriculture, Beltsville, Md.; JAB, J. A. Bartz, University of Florida, Gainesville; JBJ, J. B. Jones, University of Florida, Bradenton; JCC, J. C. Comstock, U.S. Department of Agriculture, Canal Point, Fla.; JEH, J. E. Hunter, Cornell University, Geneva, N.Y.; JEL, J. E. Leach, Kansas State University, Manhattan; RES, R. E. Stall, University of Florida, Gainesville.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pLAFR3	Tet^r, *rlx*$^+$, RK2 replicon	54
pBluescript II KS +/−	Amp^r, Bluescript	Stratagene
pRK2013	Km^r, TraRK2$^+$, Mob$^+$, ColE1 replicon	19
pXV9	pLAFR3 clone from *X. campestris* pv. vesicatoria 75-3 containing *hrp* genes	11
pXV840	pBluescript II KS +/− clone containing 271-bp *Sau*3AI *hrp* fragment amplified from *X. campestris* pv. vesicatoria 75-3	This study
pXV1075	pBluescript II KS +/− clone containing 335-bp *Sau*3AI *hrp* fragment amplified from *X. campestris* pv. vesicatoria 75-3	This study
pXV5.1	pLAFR3 clone containing 5.1-kb *Eco*RI fragment from pXV9	This study
pXV5.5	pLAFR3 clone containing 5.5-kb *Eco*RI fragment from pXV9	This study
pXV56/3-48	pLARF3 clone from *X. campestris* pv. vesicatoria XV56 containing *hrp* genes	This study
pXCP58/2	pLARF3 clone from *X. campestris* pv. pelargonii XCP58 containing *hrp* genes	This study

[FMC BioProducts, Rockland, Maine]) in TAE buffer at 8 V/cm. Phage λ *Pst*I-restricted DNA fragments were used as molecular standards. The gel was stained with 0.5 μg of ethidium bromide per ml for 40 min and then destained in 1 mM $MgSO_4$ for 1 h and photographed over a UV transilluminator with type 55 Polaroid film.

RESULTS

Specificity of the oligonucleotide primers to the *hrp* gene cluster. Three pairs of oligonucleotide primers were tested for amplification of fragments from genomic DNA of *X. campestris* pv. vesicatoria 75-3 and from plasmids that contain cloned parts of the *hrp* region from strain 75-3. Plasmid pXV9 harbors a fragment of approximately 27 kb of strain 75-3 containing almost the entire *hrp* region (11). Plasmid pXV5.5 harbors a 5.5-kb *Eco*RI fragment containing part of the *hrp* complementation groups *hrpA* and *hrpB* (Fig. 1). The 5.1-kb *Eco*RI insert

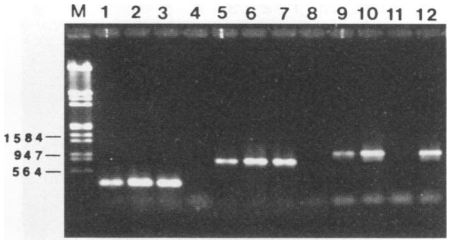


FIG. 2. Amplification of fragments of the *hrp* gene cluster from *X. campestris* pv. vesicatoria 75-3. The following DNAs were used: 75-3 (lanes 1, 5, and 9), plasmid pXV9 (lanes 2, 6, and 10), plasmid pXV5.5 (lanes 3, 7, and 11), and plasmid pXV5.1 (lanes 4, 8, and 12). Lanes: M, phage λ restricted with *Eco*RI and *Hin*dIII; 1 to 4, amplification with primers RST9 and RST10; 5 to 8, amplification with primers RST2 and RST3; 9 to 12, amplification with primers RST21 and RST22. Molecular sizes are given in base pairs.

of plasmid pXV5.1 maps to the complementation groups *hrpC* and *hrpD* (Fig. 1). We amplified fragments of the expected 355, 840, and 1,075 bp in length from total genomic DNA of strain 75-3 by using primers RST9 and RST10, RST2 and RST3, and RST21 and RST22, respectively (Fig. 2). The 355- and 840-bp fragments were also amplified from plasmids pXV9 and pXV5.5 (Fig. 2), whereas the 1,075-bp fragment was amplified from plasmids pXV9 and pXV5.1 (Fig. 2), thus confirming the sizes and locations of fragments predicted by the DNA sequence analysis (10). The 355- and 840-bp sequences correspond to genes in the *hrpB* operon, whereas the 1,075-bp fragment occurs in the *hrpC-hrpD* region (Fig. 1). Except for *hrpB6*, which encodes a putative ATPase (18), the functions of these genes are unknown. The *hrpD1* gene is highly similar to a sequence of pathogenicity genes in *X. campestris* pv. glycines (10, 29).

The identity of the amplified fragments was further confirmed by restriction enzyme analysis. The 355-, 840-, and 1,075-bp fragments amplified both from total DNA of strain 75-3 and from DNA of pXV9, pXV5.1, and pXV5.5 were digested with *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I. The banding patterns were identical for each of the three sets of fragments amplified from strain 75-3 and plasmids containing cloned parts of the *hrp* region (Fig. 3) and matched the restriction map generated from the DNA sequence of the *hrp* gene cluster of strain 75-3 (10).

Similarity of the *hrp* fragments amplified from *X. campestris* pv. vesicatoria to DNA of other bacteria. Total genomic DNA of strains of different pathovars of *X. campestris* and of

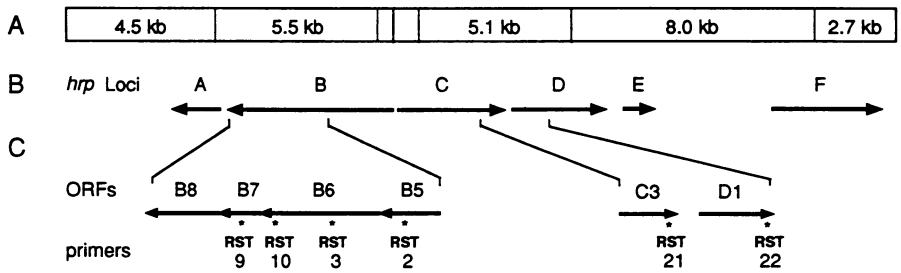


FIG. 1. Structural organization of the *hrp* region in *X. campestris* pv. vesicatoria. (A) *Eco*RI fragments of the *hrp* region. (B) Position and orientation of the *hrp* loci, designated *hrpA* to *hrpF* (11, 50). (C) Position and orientation of the open reading frames (ORFs). The sizes of the loci are based on a combination of genetic and sequence analysis from which possible open reading frames are predicted (10). Only the open reading frames relevant for this study, *hrpB5* to *hrpB8*, *hrpC3*, and *hrpD*, are shown here. For each RST oligonucleotide primer used for DNA amplification, its position in the DNA sequence is indicated by an asterisk (*).

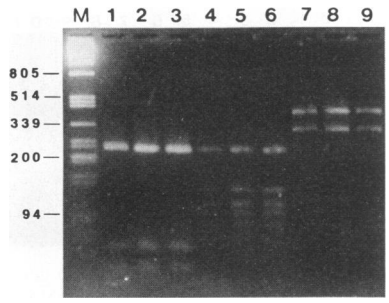


FIG. 3. *Taq*I restriction endonuclease analysis of fragments of the *hrp* gene cluster amplified from total genomic DNA from *X. campestris* pv. vesicatoria 75-3 and from plasmids containing the *hrp* region. Lanes: M, phage λ restricted with *Pst*I; 1 to 3, the 355-bp fragments from *X. campestris* pv. vesicatoria 75-3 and plasmids pXV9 and pXV5.5, respectively; 4 to 6, the 840-bp fragments from *X. campestris* pv. vesicatoria 75-3 and plasmids pXV9 and pXV5.5, respectively; 7 to 9, the 1,075-bp fragments from *X. campestris* pv. vesicatoria 75-3 and plasmids pXV9 and pXV5.1, respectively. Molecular sizes are given in base pairs.

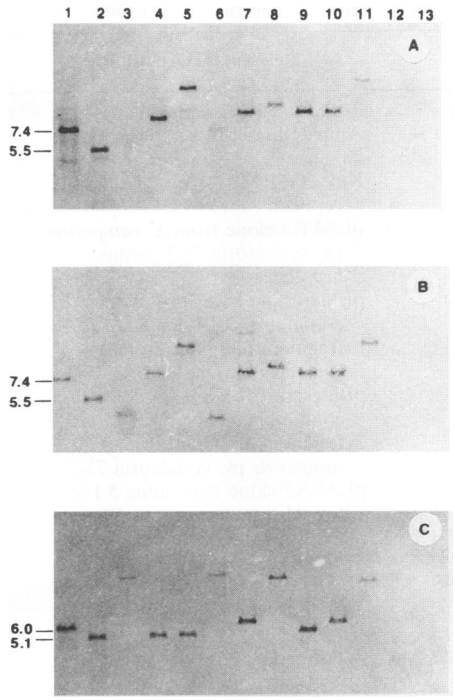


FIG. 4. Hybridization of the *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 to total genomic DNA of strains of *X. campestris*. Approximately 3 μg of *Eco*RI-digested DNA was loaded per lane. The blots were probed with the labeled 355-bp (A), 840-bp (B), and 1,075-bp (C) fragments amplified from *X. campestris* pv. vesicatoria 75-3. Lanes: 1, *X. campestris* pv. vesicatoria 75-3, *Bam*HI digested; 2, *X. campestris* pv. vesicatoria 75-3; 3, *X. campestris* pv. vesicatoria XV56; 4, *X. campestris* pv. alfalfae KS; 5, *X. campestris* pv. begoniae XCB9; 6, *X. campestris* pv. campestris 33913; 7, *X. campestris* pv. glycines 87-2; 8, *X. campestris* pv. holcicola G-23; 9, *X. campestris* pv. malvacearum RIATC; 10, *X. campestris* pv. phaseoli 85-6; 11, *X. campestris* pv. pruni FLA1; 12, *X. campestris* pv. secalis XC129C; 13, *X. campestris* pv. translucens 80-1. Molecular sizes are given in kilobase pairs.

Xanthomonas spp. was digested with *Eco*RI, separated in agarose gels, blotted, and probed with each of the three fragments amplified from DNA of *X. campestris* pv. vesicatoria 75-3. The hybridization signals in the genomic DNA of strain 75-3 corresponded to the predicted 7.4-kb *Bam*HI and 5.5-kb *Eco*RI fragments (355- and 840-bp probes) and to the 6.0-kb *Bam*HI and 5.1-kb *Eco*RI fragments (1,075-bp probe) (Fig. 4). Homology to these three *hrp* fragments of strain 75-3 was detected in strains of *X. fragariae* and of 28 different pathovars of *X. campestris* (Fig. 4; Table 3). However, polymorphisms were observed in the DNA from these different pathovars (Fig. 4). Total genomic DNA of *X. albilineans*, *X. campestris* pv. secalis, and *X. campestris* pv. translucens, which are pathogens of monocotyledonous plants, as well as DNA of nonphytopathogenic *X. maltophilia* and the opportunistic strains of *X. campestris* T-55 and INA, did not hybridize to any of the three *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 (Fig. 4; Table 3).

DNA from strains of the phytopathogens of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xylella* failed to hybridize to the three *hrp* fragments (Table 3). Further, total genomic DNA of *Erwinia herbicola*, a bacterium commonly associated with plant tissue, also did not hybridize to any of the *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 under the conditions used (Table 3).

Amplification of the *hrp* fragments from other *X. campestris* pathovars and related *Xanthomonas* spp. Primer pairs RST2 and RST3 and RST21 and RST22 were used to amplify DNA sequences from strains representing 28 different pathovars of *X. campestris*, as well as from *X. fragariae*. In all cases with both sets of primers, fragments of identical sizes were amplified from different pathovars of *X. campestris* and related *Xanthomonas* spp. (Fig. 5; Table 3). However, amplification with primers RST2 and RST3 usually gave a low yield of DNA for strains of *X. campestris* pv. carotae, *X. campestris* pv. gardneri, *X. campestris* pv. papavericola, *X. campestris* pv. pelargonii, and *X. campestris* pv. taraxaci (Fig. 5; Table 3). Although DNA isolated from *X. campestris* pv. holcicola hybridized to the 840-bp *hrp* fragment from *X. campestris* pv. vesicatoria 75-3 (Fig. 4), primers RST2 and RST3 did not amplify the DNA fragment from this pathovar (Table 3). No amplification occurred with purified total genomic DNA from a number of bacteria, including phytopathogenic strains of the xanthomonads *X. albilineans*, *X. campestris* pv. secalis, and *X. campestris* pv. translucens and of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xylella*, the nonphytopathogens *Erwinia herbicola* and *X. maltophilia*, and the opportunistic strains of *X. campestris* T-55 and INA, when either RST2 plus RST3 or RST21 plus RST22 was used (Table 3). The failure to amplify the DNA fragments from all these bacterial strains was expected because of the lack of hybridization to the three *hrp* fragments from *X. campestris* pv. vesicatoria 75-3 (Table 3).

DNA fragments delineated by the primers RST9 and RST10 were consistently amplified only from strains of *X. campestris* pv. fici, *X. campestris* pv. physalidicola, *X. campestris* pv. vesicatoria, and *X. campestris* X198 (Fig. 5; Table 3). However, some pathovars of *X. campestris*, including pathovars alfalfae, citrumelo, maculifoliigardinae, and manihotis, as well as strain XCF, sometimes produced low yields in the amplification of the 355-bp fragment (Fig. 5; Table 3).

The identity of these *hrp*-related fragments amplified from different strains of phytopathogenic *Xanthomonas* spp. was further confirmed by Southern hybridization analysis. Internal portions of the 840- and 1,075-bp DNA fragments, as well as

TABLE 3. Hybridization of the *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 to total genomic DNA and amplification of *hrp*-related fragments from different bacterial strains

Strain	Southern hybridization of fragment:			DNA amplification[a] of fragment:		
	355 bp	840 bp	1,075 bp	355 bp	840 bp	1,075 bp
X. campestris						
pv. alfalfae KS	+	+	+	(+)	+	+
pv. armoraciae 63-27	+	+	+	−	+	+
pv. bilvae XCB	+	+	+	−	+	+
pv. begoniae XCB9	+	+	+	−	+	+
pv. campestris 33913	+	+	+	−	+	+
pv. carotae 13	+	+	+	−	(+)	+
pv. citri 9771	+	+	+	−	+	+
pv. citrumelo F1	+	+	+	(+)	+	+
pv. dieffenbachiae 729	+	+	+	−	+	+
pv. fici X151	+	+	+	+	+	+
pv. gardneri XG101	+	+	+	−	(+)	+
pv. glycines 87-2	+	+	+	−	+	+
pv. holcicola G-23	+	+	+	−	−	+
pv. incanae 9561-1	+	+	+	−	+	+
pv. maculifoliigardeniae X22j	+	+	+	(+)	+	+
pv. malvacearum RIATC	+	+	+	−	+	+
pv. manihotis Xm125D	+	+	+	(+)	+	+
pv. papavericola XP5	+	+	+	−	(+)	+
pv. phaseoli 85-6	+	+	+	−	+	+
pv. phaseoli "fuscans" XP163A	+	+	+	−	+	+
pv. physalidicola XP172	+	+	+	+	+	+
pv. pelargonii XCP58	+	+	+	−	(+)	+
pv. poinsetiicola 071-424	+	+	+	−	+	+
pv. pruni X1219L	+	+	+	−	+	+
pv. raphani 69-2	+	+	+	−	+	+
pv. secalis XC129C	−	−	−	−	−	−
pv. taraxaci XT11A	+	+	+	−	(+)	+
pv. translucens 80-1	−	−	−	−	−	−
pv. vesicatoria						
75-3	+	+	+	+	+	+
XV56	−	+	+	−	+	+
pv. vignicola 81-30	+	+	+	−	+	+
pv. vitians XVIT	+	+	+	−	+	+
X198	+	+	+	+	+	+
XCF	+	+	+	(+)	+	+
T-55	−	−	−	−	−	−
INA	−	−	−	−	−	−
X. albilineans 91-065	−	−	−	−	−	−
X. fragariae X1297	+	+	+	−	+	+
X. maltophilia	−	−	−	−	−	−
Acidovorax avenae						
subsp. *avenae* UK142-A	−	−	−	−	−	−
subsp. *citrulli* UK20	−	−	−	−	−	−
Agrobacterium tumefaciens LBA 1050	−	−	−	−	−	−
Clavibacter michiganense subsp. *michiganense* 69-1	−	−	−	−	−	−
Erwinia carotovora subsp. *carotovora*						
K-SR-347	−	−	−	−	−	−
B-SR-38	−	−	−	−	−	−
Erwinia herbicola NF-33	−	−	−	−	−	−
Erwinia stewartii SW2	−	−	−	−	−	−
Pseudomonas solanacearum K60	−	−	−	−	−	−
Pseudomonas syringae						
pv. syringae INB	−	−	−	−	−	−
pv. tomato 987	−	−	−	−	−	−
Xylella fastidiosa 89-1	−	−	−	−	−	−

[a] +, positive reaction; −, negative reaction; (+), weak signal.

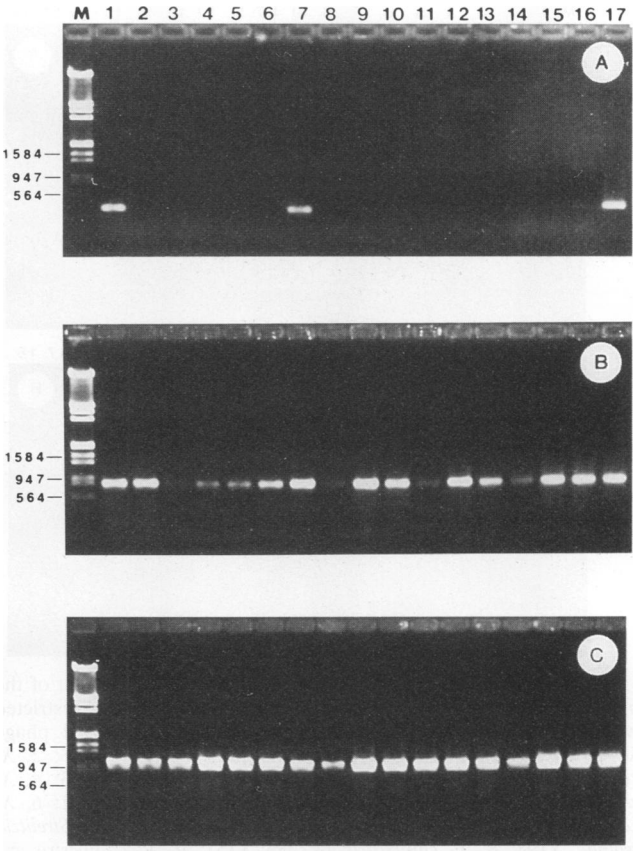


FIG. 5. Amplification of the 355-bp (A), 840-bp (B), and 1,075-bp (C) fragments of the *hrp* gene cluster from strains of *Xanthomonas campestris*. Lanes: M, phage λ restricted with *Eco*RI and *Hin*dIII; 1, *X. campestris* pv. vesicatoria 75-3; 2, *X. campestris* pv. bilvae XCB; 3, *X. campestris* pv. carotae 13; 4, *X. campestris* pv. citri 9771; 5, *X. campestris* pv. citrumelo F1; 6, *X. campestris* pv. dieffenbachiae 729; 7, *X. campestris* pv. fici X151; 8, *X. campestris* pv. gardneri XG101; 9, *X. campestris* pv. maculifoliigardeniae X22j; 10, *X. campestris* pv. manihotis Xm125D; 11, *X. campestris* pv. pelargonii XCP58; 12, *X. campestris* pv. phaseoli "fuscans" XP163A; 13, *X. campestris* pv. poinsettiicola 071-424; 14, *X. campestris* pv. taraxaci XT11A; 15, *X. campestris* pv. vignicola 81-30; 16, *X. campestris* pv. vitians XVIT; 17, *X. campestris* pv. physalidicola XP172. Molecular sizes are given in base pairs.

the entire 355-bp fragment amplified from *X. campestris* pv. vesicatoria 75-3, were used as probes. The internal probe for the 840-bp fragment consisted of a 271-bp insert of plasmid pXV840, and the internal probe for the 1,075-bp fragment consisted of a 335-bp insert of plasmid pXV1075. The inserts were obtained from fragments amplified from DNA of *X. campestris* pv. vesicatoria 75-3 by cloning *Sau*3AI digests into the *Bam*HI site of the vector pBluescript II KS +/− (Stratagene, La Jolla, Calif.). In all cases, the DNA fragments amplified from different *Xanthomonas* strains with each set of primers hybridized with the respective probe (data not shown).

Restriction endonuclease analysis of amplified *hrp*-related DNA fragments. To address the question of degree of sequence conservation among different strains, we examined the 840- and 1,075-bp *hrp* fragments amplified from strains of different pathovars of *X. campestris*, as well as from *X. fragariae*, by restriction endonuclease analysis with the endonucleases *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I. Restriction fragment length polymorphisms were apparent for both fragments.

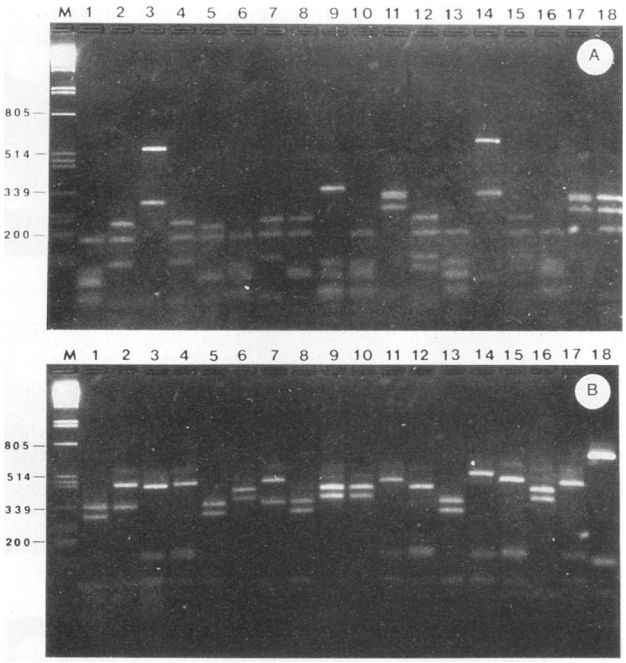


FIG. 6. Restriction analysis of the 1,075-bp DNA fragment of the *hrp* gene cluster amplified from strains of *X. campestris* and restricted with the endonucleases *Hae*III (A) and *Sau*3AI (B). Lanes: M, phage λ restricted with *Pst*I; 1, *X. campestris* pv. vesicatoria 75-3; 2, *X. campestris* pv. bilvae XCB; 3, *X. campestris* pv. carotae 13; 4, *X. campestris* pv. citri 9771; 5, *X. campestris* pv. citrumelo F1; 6, *X. campestris* pv. dieffenbachiae 729; 7, *X. campestris* from *Strelitzia reginae* X198; 8, *X. campestris* pv. fici X151; 9, *X. campestris* pv. maculifoliigardeniae X22j; 10, *X. campestris* pv. manihotis Xm125D; 11, *X. campestris* pv. pelargonii XCP58; 12, *X. campestris* pv. phaseoli "fuscans" XP163A; 13, *X. campestris* pv. poinsettiicola 071-424; 14, *X. campestris* pv. taraxaci XT11A; 15, *X. campestris* pv. vignicola 81-30; 16, *X. campestris* pv. vitians XVIT; 17, *X. campestris* pv. papavericola XP5; 18, *X. campestris* pv. holcicola G-23. Molecular sizes are given in base pairs.

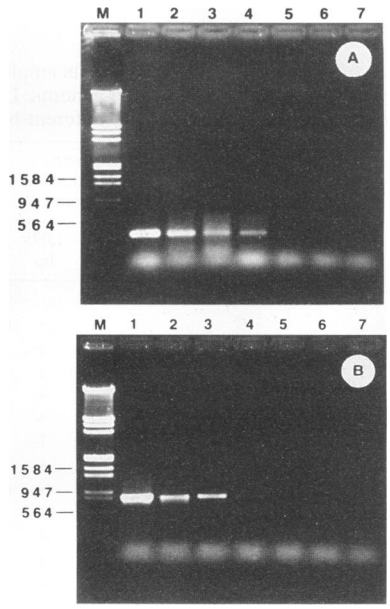


FIG. 7. Amplification of the 355-bp (A) and 840-bp (B) fragments of complementation group B of the *hrp* gene cluster from samples with different amounts of DNA template of *Xanthomonas campestris* pv. vesicatoria 75-3. Lanes: M, phage λ restricted with *Eco*RI and *Hin*dIII; 1, 25 ng of template; 2, 2.5 ng; 3, 0.25 ng; 4, 25 pg; 5, 2.5 pg; 6, 0.25 pg; 7, 0.025 pg. Molecular sizes are given in base pairs.

For example, the 1,075-bp fragment, amplified from strains of different pathovars of *X. campestris* by using primers RST21 and RST22 and then restricted with *Hae*III and *Sau*3AI, yielded different restriction patterns (Fig. 6). Although restriction fragment length polymorphisms were observed with all four endonucleases for both the 840- and 1,075-bp fragments, restriction analysis with *Cfo*I and *Hae*III produced more distinct patterns for differentiation of the groups or pathovars of *X. campestris*.

Pathogenicity function of the DNA sequence of *X. campestris* pathovars from which *hrp*-related fragments were amplified. To determine whether the DNA sequences from which *hrp*-related fragments were amplified have common functions in different strains of *X. campestris*, we investigated the functional homology of these fragments in *X. campestris* pv. vesicatoria group B and *X. campestris* pv. pelargonii. The cosmid clones pXV56/3-48, of a pLAFR3 library of strain XV56 of *X. campestris* pv. vesicatoria group B (5), and pXCP58/2, of a pLAFR3 library of *X. campestris* pv. pelargonii XCP58 (43), were identified by amplification of the 1,075-bp *hrp* fragment with primers RST21 and RST22. Plasmid pXV56/3-48 was transferred into the *X. campestris* pv. vesicatoria mutants 85-10::*hrpA22*, 85-10::*hrpB85*, 85-10::*hrpC44*, 85-10::*hrpD137*, 85-10::*hrpE75*, and 85-10::*hrpF318*, which carry Tn*3-gus* insertions in the different *hrp* complementation groups (11). Plasmid pXV56/3-48 fully restored the pathogenicity of mutants with mutations in *hrpB*, *hrpD*, and *hrpE* and restored the hypersensitive reaction-inducing ability but not pathogenicity to the *hrpC* mutant. However, this plasmid failed to complement the *hrpA* and *hrpF* mutants. Similarly, plasmid pXCP58/2 from *X. campestris* pv. pelargonii was also transferred into the six nonpathogenic Tn*3-gus* mutants of *X. campestris* pv. vesicatoria 85-10. This plasmid fully restored the wild-type phenotype to mutants with mutations in *hrpC*, *hrpD*, and *hrpE* but failed to complement *hrpA*, *hrpB*, and *hrpF* Tn*3-gus* mutants.

Sensitivity of amplification of the *hrp* fragment for detection of *X. campestris* pv. vesicatoria. The sensitivity of the amplification of specific DNA fragments in detection of *X. campestris* was determined by using 10-fold dilutions of purified total bacterial DNA of *X. campestris* pv. vesicatoria 75-3. The oligonucleotide primer pairs RST9 plus RST10 and RST2 plus RST3 were used for amplification of the 355- and 840-bp *hrp*, respectively, from samples containing as little as 0.25 pg of total bacterial DNA after 30 cycles of DNA amplification (Fig. 7). The sensitivity of the DNA amplification of fragments for the detection of *X. campestris* was also tested for plant samples to which known numbers of bacterial cells were added. The presence of *X. campestris* pv. vesicatoria was detected, after DNA extraction and concentration, by DNA amplification with primers RST9 and RST10 in plant samples containing approximately 10 CFU/ml (34).

DISCUSSION

Sequence homology to small *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 was found among phytopathogenic strains of several pathovars of *X. campestris* and related *Xanthomonas* spp. by Southern hybridization analysis. DNA probes representing regions of the *hrpB*, *hrpC*, and *hrpD*

loci hybridized strongly to total DNA from strains of 28 different pathovars of *X. campestris*. These results confirm and extend previous information (11, 53) on the conservation of the *hrp* region among the phytopathogenic xanthomonads. In contrast, the DNA of *X. campestris* pv. secalis and *X. campestris* pv. translucens did not hybridize to the *hrp* fragments. *X. campestris* pv. secalis is genetically only weakly related to a few pathovars of *X. campestris* as shown by DNA-DNA hybridization experiments (27). Previously, *X. campestris* pv. translucens has shown only weak hybridization to DNA probes representing the entire *hrp* gene cluster region of *X. campestris* pv. vesicatoria (11, 53). Although hybridization was not observed to DNA of all strains of *X. campestris* included in this study, the *hrp* region seems useful for detection and identification of a large number of phytopathogenic xanthomonads. A major advantage in using the *hrp* sequence is the lack of homology to DNA from nonphytopathogenic xanthomonads, as observed for *X. maltophilia* and *X. campestris* T-55 and INA, as well as from phytopathogens of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xylella*.

The three pairs of oligonucleotide primers described in this study are specific for the *hrpB*, *hrpC*, and *hrpD* regions of *X. campestris* pv. vesicatoria 75-3 and were used to amplify DNA fragments from *X. fragariae* and from 31 of 33 phytopathogenic taxa of *X. campestris* tested, which comprise at least 28 different pathovars of this species. In all cases, each set of primers amplified DNA fragments identical in size, suggesting a high degree of structural conservation between operons, as seen with primers RST21 and RST22. Cloned regions of DNA of *X. campestris* pv. vesicatoria group B and *X. campestris* pv. pelargonii, from which the *hrp*-related fragments were also amplified, fully restored pathogenicity to several nonpathogenic Tn*3-gus* mutants of *X. campestris* pv. vesicatoria 85-10. This supports the contention that these fragments were amplified from DNA sequences which also control the pathogenicity in other xanthomonads.

In contrast to the narrow spectrum of oligonucleotide primers previously used for detection and identification of only certain strains of *X. campestris* (26), the *hrp*-specific primer pairs RST2 plus RST3 and RST21 plus RST22 seem very useful for the identification of a large range of phytopathogenic xanthomonads. This is perhaps not surprising, because the *hrp* region seems very conserved among different phytopathogenic xanthomonads as determined by Southern hybridization experiments in the present and previous studies (11, 53). Further, the nucleotide sequences of primers RST21 and RST22 are identical to corresponding sequences of pathogenicity genes of *X. campestris* pv. glycines (29). In addition, the cloned fragment from *X. campestris* pv. glycines complements *hrpD* mutants of *X. campestris* pv. vesicatoria (10), suggesting functional homology between these regions from both pathovars.

Primers RST9 and RST10, which delineate a fragment of 355 bp, allowed DNA amplification only from a limited number of pathovars of *X. campestris*, despite hybridization of the fragment to the majority of the strains of this species. It should be noted that the sequence of RST9 originates from *hrpB6*, a gene for a putative ATPase that seems to be highly conserved among different bacteria at the protein sequence level (18). These results indicate differences in the DNA sequences of *Xanthomonas* spp. corresponding to one or both primers used. However, this set of primers seems useful for specific detection of strains of *X. campestris* pv. vesicatoria group A, *X. campestris* pv. fici, *X. campestris* pv. physalidicola, and *X. campestris* X198.

The identification of strains of *X. campestris* at the pathovar level is difficult even by using different techniques, such as fatty acid analysis (14, 24), serology (1), metabolic profile (14, 57), and SDS-polyacrylamide gel electrophoresis of proteins (58); thus, the restriction analysis of amplified *hrp*-related fragments may be a valuable tool for identification of subgroups of phytopathogenic strains and pathovars of *X. campestris*. For example, restriction analysis with frequently cutting endonucleases produced a characteristic restriction pattern for the 840- or 1,075-bp DNA fragments amplified from the *Xanthomonas* spp. included in this work, which seem to be highly conserved within each group of phytopathogenic xanthomonads (data not shown). In this way, restriction fragment length polymorphism profiles of a particular *hrp* region could be established for each phytopathogenic group of xanthomonads, thus facilitating the identification and classification of these bacterial strains. In fact, this has already been demonstrated for strains of *X. campestris* causing diseases of citrus (35).

The use of oligonucleotide primers provides a sensitive and specific tool for detection of DNA by amplification. Theoretically, the limit of detection of an amplifiable DNA sequence is estimated to be as low as one single target cell in the reaction mixture (56). In our studies, we were able to find a detectable signal for as little as 0.25 pg of total bacterial DNA. This level of sensitivity is comparable to those obtained by others without the use of any technique to enhance the signal (45, 55). Furthermore, *X. campestris* pv. vesicatoria could be detected in plant samples containing as little as 10 CFU/ml, without prior enrichment or cultivation of the organism. In addition to the sensitivity of the technique, the specificity of the oligonucleotide primers to phytopathogenic xanthomonads certainly ensures selectivity against background nontarget microorganisms, which are always present in the samples.

In conclusion, the results presented here indicate that phytopathogenic strains of *X. campestris* and related xanthomonad species can be detected and may be identified by analysis of DNA fragments amplified with *hrp* gene-specific primers. The conservation of the *hrp* DNA sequence among a large number of pathovars of *X. campestris*, as well as in related *Xanthomonas* spp., but lack of the *hrp* DNA sequence among nonphytopathogenic bacteria, makes this method a useful tool for detection and identification of many plant pathogens. Consequently, *hrp* oligonucleotide primers may be also useful to determine the pathogenic nature of unknown xanthomonads. This is particularly significant for assessing the complex population of phytopathogenic and nonpathogenic xanthomonads associated with plants and plant parts. The presence of phytopathogenic strains in such samples may be determined by amplification of the *hrp* fragments without the need for the troublesome methods of isolation of the organism and inoculation into potential host plants. Moreover, restriction fragment length polymorphisms detected in the genomes of different strains seem valuable for the study of the relatedness of phytopathogenic xanthomonads, particularly among *Xanthomonas* spp. and pathovars of *X. campestris*. Of course, this has to be extended by testing larger numbers of strains for each species, subspecies, or pathovar. The genetic methods of analyzing populations of bacteria will provide valuable additional information for taxonomic, ecological, and epidemiological studies of phytopathogenic xanthomonads.

ACKNOWLEDGMENTS

Financial support was provided to R. P. L., Jr. by IAPAR and CNPq, Brazil. Work in the laboratory of U.B. was supported by grants from the Bundesministerium für Forschung und Technologie (322-4003-0316300A) and the EEC (BIOT-CT90-0168).

REFERENCES

1. **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. c.* pv. citrumelo with monoclonal antibodies. Phytopathology **81:**857–865.
2. **Alvarez, A. M., and K. Lou.** 1982. Rapid field identification of a bacterial pathogen by an enzyme-linked immunosorbent assay (ELISA). Phytopathology **72:**947. (Abstract.)
3. **Angeles-Ramos, R., A. K. Vidaver, and P. Flynn.** 1991. Characterization of epiphytic *Xanthomonas campestris* pv. phaseoli and pectolytic xanthomonads recovered from symptomless weeds in the Dominican Republic. Phytopathology **81:**677–681.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
5. **Beaulieu, C., G. V. Minsavage, B. I. Canteros, and R. E. Stall.** 1991. Biochemical and genetic analysis of a pectate lyase gene from *Xanthomonas campestris* pv. vesicatoria. Mol. Plant Microbe Interact. **4:**446–451.
6. **Beer, S. V., D. W. Bauer, X. H. Jiang, R. J. Laby, B. J. Sneath, Z.-M. Wei, D. A. Wilcox, and C. H. Zumoff.** 1991. The *hrp* gene cluster of *Erwinia amylovora*, p. 53–60. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
7. **Benedict, A. A., A. M. Alvarez, and L. W. Pollard.** 1990. Pathovar-specific antigens of *Xanthomonas campestris* pv. pelargonii. Appl. Environ. Microbiol. **56:**572–574.
8. **Bereswill, S., A. Pahl, P. Bellemann, W. Zeller, and K. Geider.** 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. Appl. Environ. Microbiol. **58:**3522–3526.
10. **Bonas, U.** Unpublished data.
11. **Bonas, U., R. Schulte, S. Fenselau, G. V. Minsavage, B. J. Staskawicz, and R. E. Stall.** 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant Microbe Interact. **4:**81–88.
12. **Boucher, C. A., F. Van Gijsegem, P. A. Barberis, M. Arlat, and C. Zischek.** 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity and hypersensitivity on tobacco are clustered. J. Bacteriol. **169:**5626–5632.
13. **Bradbury, J. F.** 1984. Genus II. *Xanthomonas* Dowson 1939, 187, p. 199–210. *In* N. R. Krieg and J. C. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
14. **Chase, A. R., R. E. Stall, N. C. Hodge, and J. B. Jones.** 1992. Characterization of *Xanthomonas campestris* strains from aroids using physiological, pathological, and fatty acid analyses. Phytopathology **82:**754–759.
15. **Davis, M. J., W. J. French, and N. W. Schaad.** 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. **6:**309–314.
16. **Ditta, G., S. Stanfield, D. Corbin, and D. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
17. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
18. **Fenselau, S., I. Balbo, and U. Bonas.** 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. vesicatoria are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant Microbe Interact. **5:**390–396.
19. **Figurski, D., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
20. **Garde, S., and C. L. Bender.** 1991. DNA probes for detection of copper resistance genes in *Xanthomonas campestris* pv. vesicatoria. Appl. Environ. Microbiol. **57:**2435–2439.
21. **Gilbertson, R. L., D. P. Maxwell, D. J. Hagedorn, and S. A. Leong.** 1989. Development and application of a plasmid DNA probe for detection of bacteria causing common bacterial blight of bean. Phytopathology **79:**518–525.
22. **Gitaitis, R. D., M. J. Sasser, R. W. Beaver, T. B. McInnes, and R. E. Stall.** 1987. Pectolytic xanthomonads in mixed infections with *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tomato*, and *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper transplants. Phytopathology **77:**611–615.
23. **Gough, C. L., S. Genin, C. Zischek, and C. A. Boucher.** 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant Microbe Interact. **5:**384–389.
24. **Graham, J. H., J. S. Hartung, R. E. Stall, and A. R. Chase.** 1990. Pathological, restriction-fragment length polymorphism, and fatty acid profile relationships between *Xanthomonas campestris* from citrus and noncitrus hosts. Phytopathology **80:**829–836.
25. **Hartung, J. S.** 1992. Plasmid-based hybridization probes for detection and identification of *Xanthomonas campestris* pv. citri. Plant Dis. **76:**889–893.
26. **Hartung, J. S., J. F. Daniel, and O. P. Pruvost.** 1993. Detection of *Xanthomonas campestris* pv. citri by polymerase chain reaction method. Appl. Environ. Microbiol. **59:**1143–1148.
27. **Hildebrand, D. C., N. J. Palleroni, and M. N. Schroth.** 1990. Deoxyribonucleic acid relatedness of 24 xanthomonad strains representing 23 *Xanthomonas campestris* pathovars and *Xanthomonas fragariae*. J. Appl. Bacteriol. **68:**263–269.
28. **Hodge, N. C. (University of Florida).** 1991. Personal communication.
29. **Hwang, I., S. M. Lim, and P. D. Shaw.** 1992. Cloning and characterization of pathogenicity genes from *Xanthomonas campestris* pv. glycines. J. Bacteriol. **174:**1923–1931.
30. **Jones, J. B., G. V. Minsavage, R. E. Stall, R. O. Kelly, and H. Bouzar.** 1993. Genetic analysis of a DNA region involved in expression of two epitopes associated with lipopolysaccharide in *Xanthomonas campestris* pv. vesicatoria. Phytopathology **83:**551–556.
31. **Koike, H.** 1965. The aluminum-cap method for testing sugarcane varieties against leaf scald disease. Phytopathology **55:**317–319.
32. **Lazo, G. R., and D. W. Gabriel.** 1987. Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. Phytopathology **77:**448–453.
33. **Lazo, G. R., R. Roffey, and D. W. Gabriel.** 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism. Int. J. Syst. Bacteriol. **37:**214–221.
34. **Leite, R. P., Jr.** Unpublished data.
35. **Leite, R. P., Jr., D. S. Egel, and R. E. Stall.** 1994. Genetic analysis of *hrp*-related DNA sequences of *Xanthomonas campestris* strains causing diseases of citrus. Appl. Environ. Microbiol. **60:**1078–1086.
36. **Leite, R. P., Jr., R. E. Stall, and U. Bonas.** 1992. Identification of plant pathogenic *Xanthomonas* species and pathovars based on amplified DNA fragments related to *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **82:**1100. (Abstract.)
37. **Leyns, F., M. De Cleene, J.-G. Swings, and J. De Ley.** 1984. The host range of the genus *Xanthomonas*. Bot. Rev. **50:**308–356.
38. **Liao, C. H., and J. M. Wells.** 1987. Association of pectolytic strains of *Xanthomonas campestris* with soft rots of fruits and vegetables at retail markets. Phytopathology **77:**418–422.
39. **Lindgren, P. B., R. C. Peet, and N. J. Panopoulos.** 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. **168:**512–522.
40. **Maas, J. L., M. M. Finney, E. L. Civerolo, and M. Sasser.** 1985. Association of an unusual strain of *Xanthomonas campestris* with apple. Phytopathology **75:**438–445.
41. **Manulis, S., Y. Gafni, 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.
42. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Minsavage, G. V.** Unpublished data.
44. **Minsavage, G. V., D. Dahlbeck, M. C. Whalen, B. Kearney, U.**

Bonas, B. J. Staskawicz, and R. E. Stall. 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. vesicatoria-pepper interactions. Mol. Plant Microbe Interact. **3:**41–47.

45. **Pickup, R. W.** 1991. Development of molecular methods for the detection of specific bacteria in the environment. J. Gen. Microbiol. **137:**1009–1019.
46. **Saettler, A. W., N. W. Schaad, and D. A. Roth (ed.).** 1989. Detection of bacteria in seed and other planting material. American Phytopathological Society, St. Paul, Minn.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Schaad, N. W. (ed.).** 1988. Laboratory guide for identification of plant pathogenic bacteria. American Phytopathological Society, St. Paul, Minn.
49. **Schaad, N. W., H. Azad, R. C. Peet, and N. J. Panopoulos.** 1989. Identification of *Pseudomonas syringae* pv. phaseolicola by a DNA hybridization probe. Phytopathology **79:**903–907.
50. **Schulte, R., and U. Bonas.** 1992. Expression of the *Xanthomonas campestris* pv. vesicatoria *hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. J. Bacteriol. **174:**815–823.
51. **Seal, S. E., L. A. Jackson, and M. J. Daniels.** 1992. Isolation of a *Pseudomonas solanacearum*-specific DNA probe by subtraction hybridization and construction of species-specific oligonucleotide primers for sensitive detection by the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3751–3758.
52. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
53. **Stall, R. E., and G. V. Minsavage.** 1990. The use of *hrp* genes to identify opportunistic xanthomonads, p. 369–374. *In* Z. Klement (ed.), Proc. 7th Int. Conf. Plant Pathog. Bacteria 1989. Akadémiai Kiadó, Budapest.
54. **Staskawicz, B. J., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169:**5789–5794.
55. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance the detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54:**2185–2191.
56. **Steffan, R. J., and R. M. Atlas.** 1991. Polymerase chain reaction: applications in environmental microbiology. Annu. Rev. Microbiol. **45:**137–161.
57. **Van den Mooter, M., and J. Swings.** 1990. Numerical analysis of 295 phenotypic features of 266 *Xanthomonas* strains and an improved taxonomy of the genus. Int. J. Syst. Bacteriol. **40:**348–369.
58. **Vauterin, L., J. Swings, and K. Kersters.** 1991. Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. J. Gen. Microbiol. **137:**1677–1687.
59. **Vauterin, L., P. Yang, B. Hoste, M. VanCanneyt, E. L. Civerolo, J. Swings, and K. Kersters.** 1991. Differentiation of *Xanthomonas campestris* pv. *citri* strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA-DNA hybridization. Int. J. Syst. Bacteriol. **41:**535–542.
60. **Willis, K., J. J. Rich, and E. M. Hrabak.** 1990. The *hrp* genes of phytopathogenic bacteria. Mol. Plant Microbe Interact. **4:**132–138.
61. **Yang, P., L. Vauterin, M. VanCanneyt, J. Swings, and K. Kersters.** 1993. Application of fatty acid methyl esters for the taxonomic analysis of the genus *Xanthomonas*. Syst. Appl. Microbiol. **16:**47–71.