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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

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A subtraction hybridization technique was employed to make a library enriched for *Pseudomonas solanacearum*-specific sequences. One cloned fragment, PS2096, hybridized under stringent conditions to DNA of 82 *P. solanacearum* strains representing all subgroups of the species. Other plant-associated bacteria, including closely related species such as *Pseudomonas cepacia*, *Pseudomonas pickettii*, or *Pseudomonas syzygii*, did not hybridize to PS2096. A minimum number of between 4×10^5 and 4×10^6 *P. solanacearum* cells could routinely be detected with PS2096 labelled either with [^{32}P]dCTP or with digoxigenin-11-dUTP. To improve the sensitivity of detection, PS2096 was sequenced to allow the construction of specific oligonucleotide primers to be used for polymerase chain reaction (PCR) amplification. After 50 cycles of amplification, 5 to 116 cells, depending on the strain, could reproducibly be detected by visualization of a 148-bp PCR product on an agarose gel. A preliminary field trial in Burundi with the probe and PCR primers has confirmed that they are sensitive tools for specifically detecting low-level infections of *P. solanacearum* in potato tubers.

Pseudomonas solanacearum E. F. Smith causes bacterial wilt, which is one of the most important and widely spread bacterial diseases of crops in the tropics, subtropics, and warm temperate regions of the world. The disease has been recorded on several hundred species representing 44 families of plants (23). Strains of *P. solanacearum* compose a complex taxonomic group, with subspecific groupings consisting of either races, based on host range (10), or biovars, based on the catabolism of certain sugars and sugar alcohols (22).

There are many reports of bacterial wilt on potatoes (e.g., 9, 11, 31, 32) and bananas (e.g., 1, 8, 27) having been spread within and between countries in latently infected planting material. Other crops such as tomatoes have also been shown to be capable of carrying latent infections of *P. solanacearum* (35). These examples emphasize the need for effective quarantine measures against *P. solanacearum*, not only for tropical and subtropical countries but also for temperate-zone countries where race 3 could pose a threat to potato crops, illustrated by the experience in Sweden (32). The success of quarantine procedures relies on the use of simple yet sensitive detection techniques. Current identification methods rely on a series of biochemical tests on purified single colonies which requires 1 to 2 weeks before a species identification is possible. More recently, methods such as metabolic profiling (5) or computer-assisted fatty acid profiling (43), although speeding up the process, still require purification of a single *P. solanacearum* colony, which may be obscured by the more-rapid growth of other plant-associated microorganisms. Specific monoclonal or polyclonal antisera to *P. solanacearum* that do not react with closely related species, such as *Pseudomonas pickettii* or *Pseudomonas syzygii*, have not become available to date.

DNA probes have been developed and applied successfully in the detection and identification of human pathogens (18, 21, 25, 46) and plant-pathogenic bacteria (20, 29, 37, 40, 45). Development of a DNA probe specific for *P. solanacearum* is complicated by the genetic diversity of this species (12, 42) as well as a high degree of DNA homology to *P. pickettii* (36) and the clove disease bacterium *P. syzygii* (4, 38). *P. solanacearum*, *P. syzygii*, and *P. pickettii* are found within the same DNA-DNA homology group of the *Pseudomonas* rRNA homology group II (33, 38). In addition, a closely related pseudomonad "*Pseudomonas celebense*" (19), which causes blood disease of members of the family *Musaceae* in Indonesia, reacts with some *P. solanacearum* DNA probes (13, 14, 42) but shows cultural properties (17) different from those of *P. solanacearum* strains.

In order to avoid the laborious screening of randomly cloned DNA fragments for their specificity for *P. solanacearum*, we utilized a simple subtraction hybridization method to enrich for such sequences. A *P. solanacearum*-specific DNA fragment was isolated and tested for its suitability as a nonradioactive diagnostic probe which could be used as a substitute for current detection and identification procedures. The *P. solanacearum*-specific DNA was sequenced, allowing the construction of specific oligonucleotide primers for detection of lower numbers of bacteria by polymerase chain reaction (PCR) amplification.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. The characteristics and sources of the strains tested in this study are shown in Table 1. *P. solanacearum* was routinely cultured at 28°C in CPG broth (24) or on CPG plates containing 15 g of agar per liter and 0.1 g of 2,3,5,-triphenyltetrazolium chloride per liter to ensure that the fluidal (virulent) morphology was selected during subculturing (26). *Escherichia coli* strains were grown at 37°C in LB medium (30). All other bacterial species used were grown in NYG

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TABLE 1. Bacterial strains

Organism and strain	Race	Biovar	Origin[a]	Source[b]
Pseudomonas solanacearum				
GMI8131	1	1	Tobacco, Colombia, Granada S247	A
UW25	1	1	Tomato, United States, Kelman K60	W
UW26	1	1	Tomato, United States, Kelman K74	W
UW30	1	1	Tomato, Trinidad, Dudman K136	W
UW90	1[c]	1	Tobacco, Brazil, Robbs ENA521	W
UW256	1	1	Potato, Costa Rica, Gonzalez G-7	W
UW275	1	1	*Melampodium perfoliatum*, Costa Rica, Sequeira	W
UW278	1	1	Tobacco, Mexico, Fucikovsky	W
R563	1	1	Potato, Peru, Pinedo CIP120	W
R142		2	Clove, Indonesia, Eden-Green S710	R
R330		N2[d]	Potato, Brazil, Reifschneider 68	R
R361		N2	Potato, Peru, Turkensteen CIP61	R
R568		N2	Potato, Brazil, Neto 172, CIP226	R
R573		N2	Soil, Peru, Martin CIP162	R
R578		N2	Eggplant, Peru, Aley SR130	R
R583		N2	Potato, Peru, Martin CIP172	R
R132	1	3	Potato, Fiji, Hayward B2122	R
R306	1	3	Potato, Brazil, Reifschneider 52	R
R314	1	3	Potato, Cameroun, Pirkko Hay CIP292	R
R374	1	3	*Heliconia* sp., Costa Rica, Sequeira S118, UW6	R
S9	1	3	Peanut, Malaysia, Seal	D
S729	1	3	Clove, Indonesia, Eden-Green	R
S825	1	3	Clove, Indonesia, Eden-Green	R
T456	1	3	Castor bean, Indonesia, Supriadi	B
T494	1	3	Peanut, Indonesia, Subandiah 1105B	B
UW8	1	3	*Eupatorium odoratum*, Costa Rica, Sequeira K201	W
UW119	1[c]	3	Potato, Costa Rica, Gonzalez S213	W
UW130	1	3	Tomato, Peru, Sequeira S225	W
UW147	1	3	Tobacco, Australia, Hayward S240	W
UW255	1	3	Pepper, Costa Rica, Gonzalez G-1	W
UW380	1	3	Olive, China, He OPS2	W
GMI1000	1	4	Tomato, Guyana (3)	A
GMI1336	1[c]	4	*hrp* mutant of GMI1000	A
PD1682	1	4	Ginger, Thailand, Sardsud	J
R27	1	4	Potato, Indonesia, Hayward B050	R
TomM	1	4	Tomato, Malaysia, Seal	D
UW27	1	4	Tobacco, United States, Kelman K105	W
UW74	1	4	Potato, Sri Lanka, CMI no. B2861	W
UW151	1	4	Ginger, Australia, Hayward S244	W
UW359	1	4	Ginger, The People's Republic of China, He ZPS1	W
UW360	1	4	Mulberry, The People's Republic of China, He MPS5	W
UW369	1	4	Peanut, The People's Republic of China, He PPS14	W
UW378	1	4	Olive, The People's Republic of China, He OPS1	W
UW361	1	5	Mulberry, The People's Republic of China, He MPS4	W
UW373	1[c]	5	Mulberry, The People's Republic of China, He MPS2	W
UW160	2	1	Plantain, Peru, Sequeira S253	W
UW167	2	1	Banana, Costa Rica, Sequeira K160	W
GMI8133	2	1	Plantain, Colombia, Thurston S210	A
JEBUG1	2A[d]	1	Banana, The Philippines, Elphinstone	R
JEBUG2	2A	1	Banana, The Philippines, Elphinstone	R
JEBUG4	2A	1	Banana, The Philippines, Elphinstone	R
P13	2A	1	Banana, The Philippines, Seal	D
P23	2A	1	Banana, The Philippines, Seal	D
R150	2	1	Banana, Costa Rica, Buddenhagen BUD100	R
R152	2	1	*Heliconia* sp., Colombia, Buddenhagen H249	R
R155a	2	1	Banana, Grenada, Eden-Green M107a	R
R161	2	1	Banana, Grenada, Eden-Green G1	R
R203	2	1	Plantain, Guyana, McDonald, CMI no. B8365	R
R367	2	1	Plantain, Colombia, Kelman, UW181	R
R372	2	1	Plantain, Costa Rica, Sequeira S232, UW139	R
R481	2A	1	Banana, The Philippines, Eden-Green P10	R
R484	2A	1	Banana, The Philippines, Eden-Green P14	R
R497	2A	1	Banana, The Philippines, Eden-Green P12	R
R368	2	1	Plantain, Colombia, Kelman K254	R
R570	2	1	Plantain, Costa Rica, CIP19, UW155	R
R579	2	1	Plantain, Costa Rica, Sequeira S233, UW140	R
R589	2	1	Plantain, Colombia, Granada G28	R
R598	2	1	Banana, Grenada, Hunt S101ci	R

Continued on following page

TABLE 1—*Continued*

Organism and strain	Race	Biovar	Origin[a]	Source[b]
SB	2A	1	Banana, The Philippines, Soguilon SB1	P
BUR1		2	Potato, Burundi, Seal	D
BUR2		2	Potato, Burundi, Seal	D
BUR3		2	Potato, Burundi, Seal	D
BUR4		2	Potato, Burundi, Seal	D
E/225a		2	Potato, Ethiopia, Wondimagegne	E
E/301	3	2	Potato, Ethiopia, Wondimagegne	E
E/310		2	Potato, Ethiopia, Wondimagegne	E
E/N35-89	3	2	Potato, Ethiopia, Wondimagegne	E
E/1677A	3	2	Potato, Ethiopia, Wondimagegne	E
GMI8141	3	2	Potato, Colombia, Thurston S206	A
K46	3	2	Potato, Kenya, Forde	R
P2	3	2	Potato, The Philippines, Seal	D
R39	3	2	Potato, Egypt, Lelliott NCP909	R
UW19	3	2	Potato, Colombia, Thurston S205	W
UW23	3	2	Potato, Israel, Volcani K56	W
UW73	3	2	Potato, Sri Lanka, CMI no. B2768	W
Pseudomonas celebense (blood disease bacterium)				
PO2			Plantain, Indonesia, Baharuddin	G
R229			Banana, Indonesia, Eden-Green T389	R
R230			Banana, Indonesia, Eden-Green T334	R
R234			Banana, Indonesia, Eden-Green T391	R
R604			Banana, Indonesia, Hartati t509	R
T340			Banana, Indonesia, Eden-Green	R
Pseudomonas pickettii				
E1625			Soil/potato, Ethiopia, Wondimagegne	E
NC11149				N
Pseudomonas syzygii				
R001			Clove, Indonesia, Eden-Green	R
R004			*Hindola fulva*, Indonesia, Eden-Green	R
Pseudomonas cepacia GMI8101				A
Pseudomonas syringae pv. tabaci				E
Alcaligenes eutrophus GMI8105				A
Alcaligenes faecalis E/1643				E
Erwinia carotovora E/412				E
Erwinia chrysanthemi (NCPPB 2030)				E
Erwinia herbicola (NCPPB 2971)				E
Erwinia sp. E/422b				E
Escherichia coli ED8767				D
Klebsiella pneumoniae E/316				E
Klebsiella sp. E/1626a				E
Xanthomonas campestris pv. campestris 8004				D
Xanthomonas campestris pv. campestris 45				D
Xanthomonas campestris pv. graminis				D
Xanthomonas campestris pv. holcicola				D
Xanthomonas campestris pv. malvacearum				D
Xanthomonas campestris pv. vesicatoria NCPPB 2595				D

[a] Host, country, isolator, and alternative strain designations (where applicable) are given.

[b] Strains were contributed by the sources designated as follows: A, M. Arlat and P. Barberis, CNRS-INRA, Auzeville, Castanet-Tolosan Cedex, France; B, Supriadi, Balai Penelitian Tanaman Rempah dan Obat, Jl. Cimanggu 3, Bogor, Indonesia; D, M. Daniels and S. Seal, The Sainsbury Laboratory, Norwich, United Kingdom; E, E. Wondimagegne and J. Turner, University of East Anglia, Norwich, United Kingdom; G, B. Baharuddin and K. Rudolph, Institut für Pflanzenpathologie oder Pflanzenschutz der Universität, Gottingen Universität, D-3400 Göttingen, Germany; J, J. Janse, Plant Protection Service, Bacteriology Department, 6700 HC Wageningen, The Netherlands; N, National Collection of Type Cultures, London, United Kingdom; P, C. Soguilon and A. Quimio, University of The Phillipines at Los Baños, College, Laguna, The Philippines; R, S. Eden-Green, J. Elphinstone, and S. Forde, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, United Kingdom; W, D. Cook and L. Sequeira, Department of Plant Pathology University of Wisconsin-Madison, Madison, Wis.

[c] Nonpathogenic strains.

[d] N2, new biovar 2 (lowland strains); 2A, banana "bugtok" strains.

medium (15). Whole *P. solanacearum* cells to be used for providing template for PCR amplifications were grown in MM minimal medium (6) to ensure adequate lysis of the cells. Lysis was performed by boiling 100 μl of an overnight MM culture (or a loopful of bacteria from a colony resuspended in 100 μl of sterile distilled water) for 5 min. After being cooled to room temperature, 1 to 5 μl of the boiled culture was used per reaction without further treatment.

DNA manipulations. Bacterial DNA was isolated by the method of Boucher et al. (7). The procedures for agarose gel electrophoresis, Southern blotting, preparation of competent cells, ligation, and transformation were done as described by Maniatis et al. (28). Restriction enzyme digestions were carried out according to the conditions defined by the supplier, using 5 U of enzyme per μg of DNA. Hybridizations were performed at 65°C with probe DNA labelled with either [^{32}P]dCTP or digoxigenin-11–dUTP, which was detected by chemiluminescence, according to the manufacturer's instructions (Boehringer Mannheim). Dot blots were prepared on Boehringer positively charged nylon membranes with the aid of a Hybridot manifold (Bethesda Research Laboratories, Life Technologies, Inc.). Cells were grown in minimal medium and adjusted to an optical density of 0.3 at 600 nm (approximately 4×10^8 CFU/ml). Serial 10-fold dilutions were made with sterile distilled water, and 100-μl aliquots of each dilution were added to the membranes. Cell dots were lysed by placing the membrane on Whatman 3 MM paper soaked in 1.5 M NaCl–0.5 M NaOH for 7 min, neutralized for 5 min in 1.5 M NaCl–0.5 M Tris-Cl (pH 7.2)–1 mM EDTA, and fixed by being boiled for 1 min in sterile distilled water.

Subtraction hybridization. Sequences present in one strain (the target strain) not present in another strain (the driver strain) were enriched by the removal of common DNA. The technique employed was based on increasing the rate of reassociation of DNA molecules by the presence of a high concentration of inorganic phosphate (3). *Xanthomonas campestris* pv. vesicatoria was used as the driver strain, and 250 μg of DNA was sheared by ultrasonication (model Soniprep 150; Medical Scientific Equipment) to a size range of 1 to 3 kb and then mixed with 1 μg of *Mbo*I-digested *P. solanacearum* UW25 DNA. The mixture was denatured at 100°C for 5 min and then allowed to reassociate for 18 h at 86°C in 2.4 M phosphate buffer (pH 6.8). The reassociated DNA mixture was dialyzed extensively against 10 mM Tris-Cl–1 mM EDTA (pH 8.0), precipitated with ethanol, and redissolved in 250 μl of sterile distilled water. Ligations were carried out overnight at 12°C, each reaction mixture containing 5 μl of subtracted mixture and 0.15 μg of phosphatase-treated *Bam*HI-digested pBR322 DNA. Aliquots of the ligation mixture were transformed into competent *E. coli* ED8767 cells, and transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. Transformants containing recombinant plasmids were tetracycline sensitive. Preparation of the insert DNA from the clones was carried out by PCR amplification using oligonucleotide primers corresponding to sequences flanking the *Bam*HI site of pBR322. Sequencing was performed with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

PCR amplifications. PCR amplifications were performed with DNA thermal cyclers (Perkin-Elmer Cetus). Reaction volumes (25 to 50 μl) contained 1× PCR buffer (10 mM Tris-Cl [pH 8.3] at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [wt/vol] gelatin [G-2500; Sigma], 0.05% [vol/vol] Nonidet P-40 [N-3516; Sigma], 0.05% [vol/vol] Tween 20 [P-1379; Sigma]), 0.2 mM (each) dNTP, 1.25 U of Ampli*Taq* polymerase (Perkin-Elmer Cetus) per 50 μl of reaction volume, 1 μM primers, and template DNA. The primers were synthesized with an Applied Biosystems 391 DNA synthesizer. Each reaction mixture was overlaid with 2 drops of light mineral oil (M-3516; Sigma), heated at 96°C for 2 min to ensure complete denaturation of the template DNA, and then cycled through a temperature profile. For amplification of the insert DNA from subtraction hybridization pBR322 clones, the primers constructed were BF (5′ATGCGTCCG GCGTAGA3′) and BR (5′CACTATCGACTACGCGATCA 3′). Bacteria were inoculated into LB containing ampicillin and allowed to grow at 37°C for 1 h or until growth was just visible. Reaction mixtures (25 μl) were set up by using 1 μl of culture as template. After 2 min at 96°C, the reaction mixtures were cycled 30 times through phases of denaturation (94°C for 30 s), annealing (48°C for 30 s), and extension (72°C for 1 min), with a final extension period of 10 min at 72°C to allow all extension products to be completed. Samples (5 μl) of reaction mixtures were electrophoresed in 2% agarose gels (A-6013; Sigma) in 1× TBE (8.9 mM Tris, 0.25 mM Na_2EDTA, 0.89 mM boric acid [pH 8.3]). All products larger than the product from control *E. coli* cells harboring pBR322, i.e., 64 bp, have inserts.

For amplification of the *P. solanacearum*-specific product, primers PS96-H (5′TCACCGAAGCCGAATCCGCGTCCA TCAC3′) and PS96-I (5′AAGGTGTCGTCCAGCTCGAAC CCGCC3′) were used. After 2 min of denaturation at 96°C, 50-μl reaction volumes were cycled 35 to 50 times through phases of denaturation (94°C for 10 s), annealing (64°C for 20 s), and extension (74°C for 20 s), with a final extension for 10 min. The PCR products were resolved by running 15 μl on 2.0% agarose gels and staining with ethidium bromide.

Axenic tomato plants. Axenic tomato plants were grown essentially as described by Boucher et al. (6), with the exception that seedlings were grown in sterilized soil in sterile 100-ml plastic screw-capped containers (Richardson's of Leicester, Leicester, England). Seedlings were inoculated at the two-leaf stage by stabbing ~10^6 *P. solanacearum* cells into the stems with a sharpened tungsten wire. After 2 days at 28°C, the bacteria were reisolated by cutting the stem below the inoculation point and placing the stem in 500 μl of sterile distilled water for 5 min. The stem was subsequently removed, the water was boiled for 5 min, and 10 μl of the reaction mixture was used as a template in PCR procedures.

RESULTS

One hundred twenty-one clones containing subtracted-*P. solanacearum* DNA obtained were obtained, of which 44 had inserts that were large enough (>100 bp) to be labelled adequately with digoxigenin-11–dUTP. Fifteen of these inserts did not hybridize to all *P. solanacearum* strains, and 16 of the inserts hybridized under stringent conditions to some of the closely related species. Twelve of the 44 clones contained moderately to highly repetitive sequences. Although a highly repeated sequence would allow greater sensitivity of detection, the copy number of these sequences was generally extremely variable between *P. solanacearum* strains, and hence, sensitivity of detection would also vary greatly. The insert PS2096 from one plasmid was selected for further study as it showed less variation in copy number between strains and lay within an apparently well-conserved ~0.9-kb *Eco*RI fragment. Probe PS2096 hybridized to 82 of 85 tested *P. solanacearum* strains in Southern (Fig. 1) and cell (Fig. 2) blots. Three strains (UW119, UW373, and GMI1336) did not hybridize to PS2096 under stringent conditions, nor did 27 strains from other bacterial species (Table 1). PS2096 was tested on membranes containing squashes of potato stems and tubers collected at field stations in Burundi. PS2096 hybridized to five of eight samples from wilted plants, but not to the three healthy plant controls for which the results are shown in Fig. 3.

To determine the sensitivity of this probe, dilutions of exponentially growing cultures were dotted onto nylon membranes and probed with PS2096 labelled either with

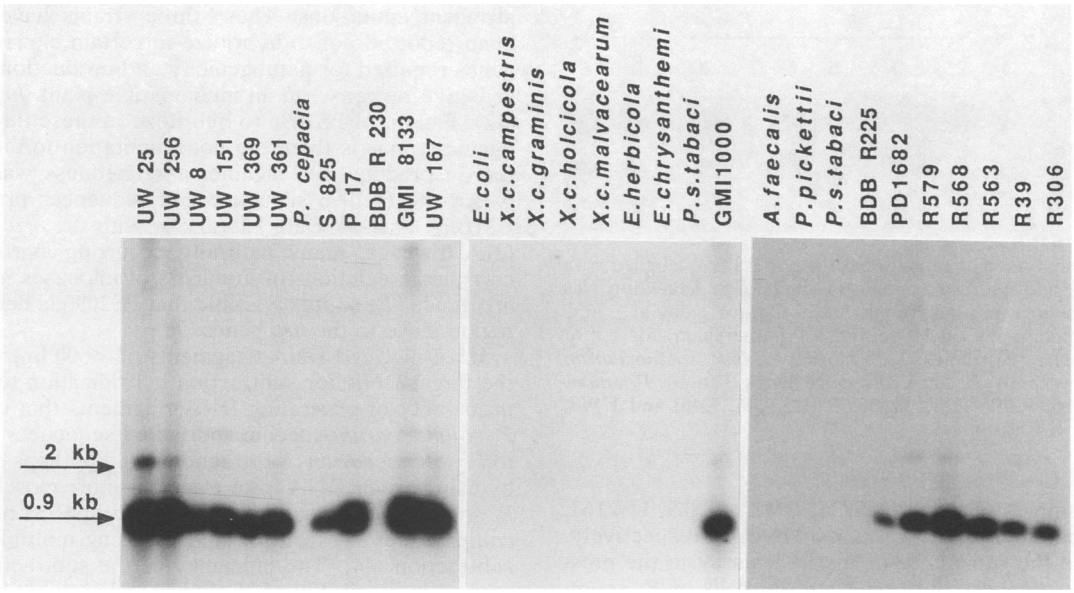

FIG. 1. Southern transfer of ~5 μg of *Eco*RI-restricted DNA of *P. solanacearum* strains and other bacteria, probed with PS2096. Probe PS2096 was labelled with either [^{32}P]dCTP (left and middle panels) or with digoxigenin-11–dUTP (panel on the right). Arrows on the left indicate the approximate sizes (in kilobases) of hybridizing bands. BDB, blood disease bacterium; X.c., *X. campestris*; E. chrysanthemi, *Erwinia chrysanthemi*; P.s., *P. syringae*; *A. faecalis*, *Alcaligenes faecalis*.

[^{32}P]dCTP or with digoxigenin-11–dUTP (Fig. 4). PS2096 hybridized to a greater extent to some strains (K46 and UW26) than to others (R142), with the limit of detection between 4×10^5 and 4×10^6 CFU. No difference in the sensitivity of detection was observed between probe PS2096 labelled with [^{32}P]dCTP or digoxigenin-dUTP when the latter was detected by chemiluminescence. Although this sensitivity allows PS2096 to be used for rapid identification of *P. solanacearum* and diagnosis of moderately infected plant material, the sensitivity is not considered adequate for detection of low-level latent infections or for quarantine purposes.

PCR amplification of DNA using appropriate primers has been shown to permit sensitive and specific detection of bacteria. PS2096 was sequenced and determined to consist of 172 bp of *P. solanacearum* DNA. Synthetic oligonucleotide primers, PS96-F and PS96-R, were constructed so that they amplified a region of 144 bp from PS2096. These primers were found to amplify also a larger fragment from DNA of *P. syringae* pv. tabaci and *P. pickettii*. To improve the selectivity, a pair of longer oligonucleotide primers, PS96-H and PS96-I, was constructed and found to possess the required specificity. Purified DNA or lysed cells of *P. solanacearum* strains (with the exceptions of GMI1336, UW119, and UW373) and extracts of wilt-infected potato plants or tomato seedlings inoculated with *P. solanacearum* gave a characteristic 148-bp band with primers PS96-H and PS96-I (Fig. 5). PCR applied to purified DNA or cells from other species (Fig. 5) or to extracts from healthy field-grown (Burundi) potato stems failed to generate products of any size (41). The PCR technique could detect lower numbers of *P. solanacearum* cells in field samples than detected by probe PS2096; all samples from wilted potato plants, including the three that did not hybridize detectably to probe PS2096 (Fig. 3, lanes 7, 8, and 9), did produce the specific band with primers PS96-H and PS96-I after PCR amplification.

To determine the limit of detection of the PCR assay with primers PS96-H and PS96-I, 5-μl samples of 10-fold dilutions of exponentially growing cultures of five strains were used as template for the PCR. After electrophoresis of 15-μl aliquots of the PCR (50 rounds) mixture, the 148-bp product could be

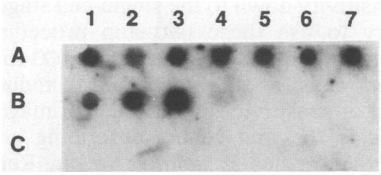

FIG. 2. Cell dot blot of 11 strains of *P. solanacearum* and 8 strains of other bacteria probed with digoxigenin-11–dUTP-labelled PS2096. Approximately 2×10^7 cells were used for each dot. Dots A (lanes 1 to 7) and B (lanes 1 to 4) represent *P. solanacearum* strains as follows: A1, UW25; A2, UW256; A3, R568; A4, R578; A5, UW160; A6, UW167; A7, GMI8133; B1, UW361; B2, UW19; B3, UW23; and B4, UW373. Dots B (lanes 5 to 7) and C (lanes 1 to 7) represent non-*P. solanacearum* strains as follows: B5, PO2; B6, *E. herbicola*; B7, blank; C1, *X. campestris* pv. vesicatoria; C2, *X. campestris* pv. campestris 8004; C3, *Pseudomonas cepacia*; C4, *Alcaligenes eutrophus*; C5, *P. pickettii* E/1625; C6, *P. pickettii* 11149; C7, blank. The blot was exposed to Hyperfilm-MP (Amersham International) for 1 h at room temperature.

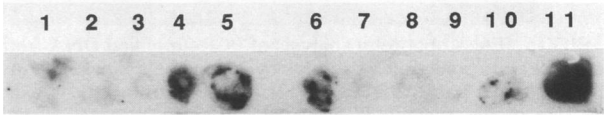

FIG. 3. Potato stem and tuber squashes on positively charged nylon membrane probed with digoxigenin-11–dUTP-labelled PS2096. Lanes: 1 and 3, healthy stem; 2, symptomless tuber; 4, 5, 6, 8, 10, and 11, stem squashes from several wilting potato plants; 7 and 9, tubers from wilting plants.

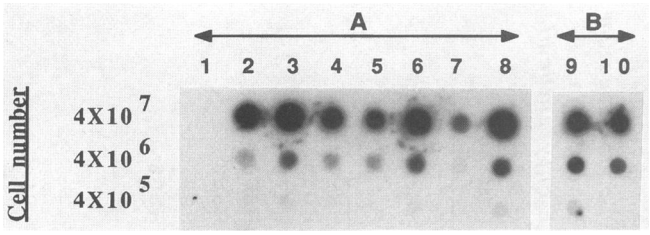

FIG. 4. Comparison of sensitivity of probe PS2096 labelled with [^{32}P]dCTP (A) and with digoxigenin-11-dUTP (B). Detection was done by autoradiography on Kodak XAR5 film for 1 day at −70°C (A) or by luminography on Hyperfilm-MP (Amersham) for 1 h at room temperature (B). Lanes: 1, *E. herbicola*; 2, *P. solanacearum* UW26 mixed with 10^7 *E. herbicola* cells; lanes 3 to 10, *P. solanacearum* UW26, R361, R289, R330, R142, K46, K46, and UW8, respectively.

seen from samples of strains UW25, UW19, S825, UW167, and R142, containing 5, 6, 14, 22, and 116 cells, respectively. To determine the sensitivity of the PCR assay in the presence of large numbers of other bacteria, cells of *P. solanacearum* UW19 and UW25 were mixed with 20-, 200-, 2,000-, and 20,000-fold excesses of either *Erwinia herbicola* or *X. campestris* pv. campestris cells. The sensitivity of the PCR method remained the same when there was a 20- or 200-fold excess of *E. herbicola* or *X. campestris* pv. campestris cells. However, with 2,000- and 20,000-fold excesses, the level of reproducible detection was decreased 10-fold.

DISCUSSION

A 172-bp *P. solanacearum*-specific probe, PS2096, was isolated by screening 44 subtraction hybridization library clones against 85 *P. solanacearum* strains and 27 strains from other bacterial species. The 85 *P. solanacearum* strains originated from 49 different host-country combinations and were therefore considered to be a representative sample of the species. Only three *P. solanacearum* strains, GMI1336, UW119, and UW373, did not hybridize to PS2096 under stringent conditions. These three strains have previously been reported not to hybridize to certain clones containing genes required for pathogenicity and production of a hypersensitive response on an incompatible plant (*hrp* genes [7]) (12). Failure of PS2096 to hybridize to these three nonpathogenic strains is therefore not a limitation to the diagnostic test in practice. No significant relatedness was found between the PS2096 sequence and sequences present in the EMBL/GenBank Data Library or with the *hrp* clone pVir2 (2). However, many naturally occurring avirulent strains carry large deletions of hundreds of kilobases including the *hrp* genes (7), so it is possible that PS2096 is derived from a region close to the *hrp* genes.

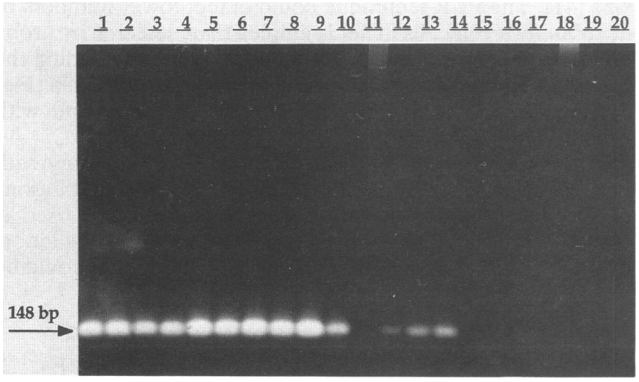

FIG. 5. Electrophoretic analysis of PCR-amplified DNA from *P. solanacearum* and other bacteria by using the *P. solanacearum*-specific primer pair PS96-H and PS96-I. The arrow on the left shows the size of the specific PCR product. Lanes: 1 to 14, *P. solanacearum* UW278, UW73, UW360, R361, R563, UW361, UW160, GMI8133, R142, R289, UW373, UW74, UW378, and PD1682, respectively; 15, *E. herbicola*; 16, *P. cepacia*; 17, *A. eutrophus*; 18, *P. syringae* pv. tabaci; 19, *P. celebense* T340; 20, *P. syzygii* R001.

*Mbo*I-digested DNA (fragments of <600 bp) was used as the target DNA for subtraction hybridization to reduce the probability of generating DNA fragments that contain both *P. solanacearum*-specific and linked sequences not specific to *P. solanacearum*. Subtraction could perhaps be improved by using driver DNA from a species more closely related to *P. solanacearum*, such as *P. pickettii* or *P. syzygii*, than *X. campestis* pv. vesicatoria and by using multiple rounds of subtraction (44). The efficiency of the subtraction was not studied in detail. Six of 25 probes tested did hybridize to *X. campestris* pv. vesicatoria, but the proportion of clones from an unsubtracted library that would cross-hybridize is unknown.

The DNA probe PS2096 offers a rapid and precise identification method but cannot, however, detect less than ~10^5 to 10^6 bacteria and hence is insufficiently sensitive to detect low-level infections. This problem could be overcome by amplification by selective growth of the bacteria on a membrane, followed by lysis and hybridization with PS2096. The alternative approach used was to sequence the DNA probe to allow construction of specific oligonucleotide primers for more-sensitive detection through the application of PCR technology. Between 5 and 116 CFU of *P. solanacearum* could be detected with primers PS96-H and PS96-I and 50 rounds of amplification. As the PCR technique does not distinguish between viable and nonviable organisms, this might be an overestimate of the sensitivity. The sensitivity is comparable, however, to that reported for *Listeria monocytogenes* (16), for which a minimum number of 542 cells (viable and nonviable) could reliably be detected after only 35 PCR amplification rounds. The sensitivity could be improved further by hybridization of the PCR product with an internal part of the amplified fragment (37) or by a second round of amplification (16). Either of these methods should bring the sensitivity down to the single-cell stage. It may also be necessary to use these two-step procedures for field samples that contain a large excess (≥2,000-fold) of non-*P. solanacearum* cells, as this was found to reduce the reproducible level of sensitivity by an order of magnitude.

This work is the first report describing a DNA probe specific for the species *P. solanacearum*. Recently, Cook and Sequeira (14) utilized a subtraction hybridization procedure to enrich for *P. solanacearum* race 3-specific sequences and obtained a probe that reacted with all race 3 strains but with only 5 of 90 non-race 3 strains tested. They did not report whether their probe hybridizes to closely related pathogens such as *P. syzygii*, *P. cepacia*, or *P. pickettii*. Although *P. pickettii* is usually recorded from human infections arising from contaminated water supplies, one isolate, E1625, was supplied to our laboratory as originating from a wilt-infected potato field in Ethiopia. Should *P. pickettii* strains be commonly found in soil, it is clearly important that probes should not cross-react with this species. Eight of 15

subtraction hybridization probes tested did hybridize to *P. pickettii* strains under stringent conditions, confirming the high genetic relatedness previously reported for these two species (36).

The PCR can be used specifically to detect very low numbers of *P. solanacearum* cells, without the need for prior enrichment or cultivation. In many instances, it is quicker and advantageous to use methods that do not require culturing of the organism. Selective media have been shown to reduce the efficiency with which microorganisms are recovered from the environment (39). Moreover, bacteria can enter a nonculturable but apparently viable state (34) and not be detected by traditional isolation procedures.

Many questions regarding the epidemiology of bacterial wilt have remained unanswered to date partly because of the lack of a simple, rapid, and sensitive identification procedure. Seed has been suggested to be a vehicle for the spread of bacterial wilt, but no conclusive data have been reported (23). Although many weeds have been shown to be symptomless carriers of *P. solanacearum* (23), there are probably also many unknown hosts that maintain high levels of inoculum between successive crops. The application of probe PS2096 and PCR primers PS96-H and PS96-I should enable such aspects of the epidemiology to be investigated.

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