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Construction and Use of a Nonradioactive DNA Hybridization Probe for Detection of *Pseudomonas syringae* pv. Tomato on Tomato Plants

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***Pseudomonas syringae* pv. tomato, the causal agent for bacterial speck of tomato, produces the phytotoxin coronatine. A 5.3-kilobase *Xho*I fragment from the chromosomal region controlling toxin production was cloned into the plasmid pGB2, and the resulting recombinant plasmid, pTPR1, was tested for its ability to serve as a diagnostic probe for *P. syringae* pv. tomato. In a survey of 75 plant-associated bacteria, pTPR1 hybridized exclusively to those strains that produced coronatine. The detection limit for this probe, which was labeled with the Chemiprobe nonradioactive reporter system, was approximately 4×10^3 CFU of lesion bacteria. During the 1989 growing season, a total of 258 leaf and fruit lesions from nine tomato fields were screened for *P. syringae* pv. tomato by using pTPR1 and the culture method of detection. The best agreement between the two methods, 90%, occurred early in the season with samples taken from relatively young (5-week-old) plants. Young plants also had a higher percentage of *P. syringae* pv. tomato-positive lesions. *P. syringae* pv. tomato was the only coronatine producer recovered from the nine tomato fields. All 244 *P. syringae* pv. tomato strains isolated during this study reacted strongly with the probe. The *P. syringae* pv. tomato population of healthy field tomato leaves was determined by a pTPR1 colony hybridization procedure. Every probe-positive colony that was isolated and characterized was identified as *P. syringae* pv. tomato. The pTPR1 probe should expedite disease diagnosis and facilitate epidemiological studies of this pathogen. It also should aid in screening transplant seedlings for bacterial speck infestation.**

Pseudomonas syringae pv. tomato causes bacterial speck, one of the major bacterial diseases of tomato plants in Canada. Because bacterial speck lesions on both the leaves and fruit can vary in size, texture, and color, they may be confused with those produced by other tomato pathogens such as *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas syringae* pv. syringae. Accurate diagnosis requires that the pathogen be isolated, purified, and then characterized by a series of biochemical, physiological, and pathogenicity tests. This process is laborious and quite time-consuming. A more rapid means of identification is available through immunoassays and bacteriophage typing (5, 10, 21). Unfortunately, neither procedure is specific enough to serve as a direct diagnostic test for this pathogen.

Nucleic acid hybridization offers another approach to the rapid identification of pathogenic bacteria (9, 13, 27–29, 31). Provided that the appropriate DNA sequence and hybridization conditions are chosen, both the specificity and sensitivity of this method can be quite high (32). Some of the probes that have been developed recognize the coding sequence of virulence factors, while others bind to genes encoding ribosomal RNA or to cryptic chromosomal fragments unique to the pathogen (30). The recent development of highly sensitive hybridization assays that employ stable, safe-to-use non-radioactively labeled DNA probes should lead to the more frequent use of this technique in diagnostic laboratories (19).

Recently, two *Eco*RI restriction fragments of *P. syringae* pv. tomato DNA were combined to make the DNA hybridization probe PST-DNA (7, 8). Although a method was developed whereby PST-DNA could distinguish *P. syringae* pv. tomato from *P. syringae* pv. syringae, this probe is not highly specific. It reacts with several other *P. syringae* pathovars and does not work as well with infected tissue as it does with purified cultures.

In a previous study, we used Tn*5* mutagenesis to identify and characterize a 30-kilobase (kb) region of the *P. syringae* pv. tomato genome involved in the production of the phytotoxin coronatine (26). The only bacteria known to produce this toxin are *P. syringae* pv. tomato, *Pseudomonas syringae* pv. glycinea, *Pseudomonas syringae* pv. maculicola, and *Pseudomonas syringae* pv. atropurpurea (24). A 5.3-kb *Xho*I fragment from this region was tested for sequence homology to genomic DNA from eight *P. syringae* strains; only the known coronatine producers hybridized with the probe. In this study, this *Xho*I fragment, which had been cloned into the plasmid pGB2, was tested for its ability to serve as a diagnostic probe for the bacterial speck pathogen. When tested against bacterial plant pathogens and tomato epiphytes, it hybridized exclusively with the coronatine producers. Procedures were developed for quantifying the pathogen in healthy tomato leaves and for detecting it in leaf and fruit lesions. Every *P. syringae* pv. tomato strain isolated from field tomatoes reacted with the probe. None of the other coronatine producers were recovered from the nine fields screened.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *P. syringae* pathovars were grown on nutrient broth-yeast extract (NBY) agar as previously described (6). *Escherichia coli* strains were grown in Luria-Bertani medium (23) at 37°C. When required, the media were supplemented with one or more of the following

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TABLE 1. Specificity of the pTPR1 probe

Bacterial species	Strain	Source (geographic origin)
Probe-positive strains (*P. syringae* pathovars)		
atropurpurea	NK340	T. Denny (California)
	1304	C. Bender (Japan)
glycinea	F111	E. Ward (Ontario)
	B3	E. Ward (Ontario)
maculicola	438	T. Denny (northern California)
tomato	DC3000	D. Cuppels (Ontario)
	DC84-1	D. Cuppels (Ontario)
	DCT6D1	D. Cuppels (Ontario)
	DC834	D. Cuppels (Ontario)
	G13	B. MacNeill (Ontario)
	188B	B. MacNeill (Ontario)
	208B	B. MacNeill (Ontario)
	JL1035	J. Lindemann (California)
	SM78-1	S. McCarter (Georgia)
	AV80	A. Vidaver (Nebraska)
	OH314	D. Coplin (Ohio)
	NCPPB 2424	NCPPB[a] (Switzerland)
	NCPPB 1108	NCPPB (United Kingdom)
	CNBP 1318	CNBP[b] (Switzerland)
	CNBP 1323	CNBP (France)
	PDDCC 3357	PDDCC[c] (New Zealand)
Probe-negative strains (*P. syringae* pathovars)		
antirrhini	PDDCC 2738	PDDCC (United Kingdom)
coronafaciens	345	T. Denny (Georgia)
lachrymans	419	T. Denny (Ohio)
maculicola	437	T. Denny (northern California)
morsprunorum	436	T. Denny (Pennsylvania)
	PDDCC 567	T. Denny (United Kingdom)
papulans	H82	G. Bonn (Ontario)
	PSP1	N. Gibbins (Ontario)
	NYPSP14	T. Burr (New York)
	NYPSP25	T. Burr (New York)
	4040	G. Bonn (Ontario)
persicae	PDDCC 5846	T. Denny (France)
phaseolicola	HB10Y	A. Vidaver (Nebraska)
	HB6	A. Vidaver (Nebraska)
	GB1	G. Bonn (Ontario)
pisi	150	T. Denny (Washington)
syringae	LG1	G. Bonn (Ontario)
	84-15	T. Denny (Georgia)
	132	T. Denny (Georgia)
	B78	S. McCarter (Georgia)
	PT80-4	S. McCarter (Georgia)
	SMRI-8B	S. McCarter (Georgia)
	5D19	A. Vidaver (California)
	NCPPB 2747	NCPPB (New Zealand)
	NCPPB 2748	NCPPB (New Zealand)
	NCPPB 2750	NCPPB (Australia)
	NCPPB 2749	NCPPB (Australia)
	NCPPB 268	A. Kelman (United Kingdom)
	NCPPB 281	NCPPB (United Kingdom)
	NCPPB 1038	A. Kelman (Brazil)
	PDDCC 3906	PDDCC (Greece)
tabaci	GB1	G. Bonn (Ontario)
tomato	NCPPB 2563	NCPPB (United Kingdom)
	NCPPB 1008	NCPPB (United States)
	NCPPB 880	NCPPB (Yugoslavia)
Xanthomonas campestris pv. vesicatoria	XV21	J. Jones (Florida)
	XV34	J. Jones (Florida)
	XV72	J. Jones (Florida)
	XV79	J. Jones (Florida)
Pseudomonas solanacearum	K60	A. Kelman (North Carolina)
Clavibacter michiganense subsp. *michiganense*	JC83-1	J. Dick (Ontario)
Erwinia carotovora subsp. *atroseptica*	SR8	A. Kelman (Wisconsin)
P. marginalis	DC83-1	D. Cuppels (Ontario)
P. viridiflava	T9B1	D. Cuppels (Ontario)
	MF-2	D. Cuppels (Ontario)
	Ap-1	D. Cuppels (Ontario)
	MM-1	D. Cuppels (Ontario)
P. fluorescens-P. putida group	9A2	D. Cuppels (Ontario)
	8B2	D. Cuppels (Ontario)
	10A3	D. Cuppels (Ontario)
	9B3	D. Cuppels (Ontario)
	1A3	D. Cuppels (Ontario)
	4A3	D. Cuppels (Ontario)
	5A2	D. Cuppels (Ontario)

[a] NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England.
[b] CNBP, Collection Nationale de Bactéries Phytopathogénes, Angers, France.
[c] PDDCC, Plant Diseases Division Culture Collection, Auckland, New Zealand.

filter-sterilized antibiotics: kanamycin (50 μg/ml), tetracycline (25 μg/ml), rifampin (50 μg/ml), or streptomycin (100 μg/ml). All strains were stored at −73°C in NBY broth containing 15% glycerol.

E. coli HB101 was the host strain for recombinant plasmid pEC18 (Tc^r), which consisted of a 30-kb fragment of *P. syringae* pv. tomato genomic DNA inserted into the cloning vector pLAFR1 (11, 26).

Isolation and manipulation of DNA. Bacterial genomic DNA was isolated and purified as described previously (6). Plasmids were purified by centrifugation (296,000 × *g*) in a two-step cesium chloride gradient (12). A 5.3-kb *Xho*I fragment was purified from pEC18 by using GeneClean (Bio 101, Inc., La Jolla, Calif.) and was subcloned into pGB2(Sm^r Sp^r), a 4-kb derivative of pSC101 (3). The resulting recombinant plasmid, pTPR1, was amplified in *E. coli* DH5.

All DNA manipulations were performed by standard methods (23, 26) with enzymes purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or Pharmacia, Inc. (Piscataway, N.J.) and used according to the recommendations of the manufacturer.

Preparation of filters. For the probe specificity assays, bacteria grown overnight on NBY agar were suspended in sterile, distilled water to a cell density of approximately 10^6 CFU/ml. One-half milliliter of this suspension was placed in the well of a hybri-slot manifold apparatus (Betheseda Research Laboratories, Inc.) containing a 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-soaked nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.). The bacteria were deposited on the filter by applying a vacuum of −65 kPa for not more than 4 min. The bacteria were lysed,

and the liberated DNA was bound to the filter by a procedure described by Maniatis et al. for the in situ hybridization of bacterial colonies (procedure I [23]). Our denaturing solution, however, did not contain NaCl, and the air-dried filter went directly from the neutralizing solution to the vacuum oven.

For probe sensitivity assays, a *P. syringae* pv. tomato DCT6D1 leaf lesion was excised from the plant, cut into quarters, and incubated in 1 ml of sterile, distilled water for 60 min at 4°C. The eluate was serially diluted 1:1 with sterile, distilled water to give a range of bacterial concentrations from 10^5 to 10^2 CFU/ml. The bacteria from each dilution were deposited on a nitrocellulose filter, and the blot was processed as described above. The appropriate dilutions were plated on NBY agar to obtain the exact viable count. For probe sensitivity assays with purified bacterial DNA, the DNA was denatured and processed for filtration by the procedure described by Denny et al. (8). Before denaturation, the DNA was serially diluted 1:1 in TE buffer (10 mM Tris hydrochloride–1 mM EDTA; pH 8.0) to give a range of concentrations from 140 to 0.07 ng. After the DNA samples were deposited on a nitrocellulose filter by using the hybri-slot manifold, the blot was air dried and baked for 2 h at 80°C in a vacuum oven.

Hybridization procedures. The pTPR1 probe was labeled with ^{32}P, as described previously (26), or by Chemiprobe, a nonradioactive DNA labeling kit (ChemiProbe, FMC Bio-Products, Rockland, Maine). The Chemiprobe kit inserts antigenic sulfone groups into the cytosine residues of probe DNA. After hybridization to homologous DNA, the modified probe is located by using a sandwich immunoenzymatic reaction. Monoclonal antibody binds to the sulfone residues of the modified DNA and then to an alkaline phosphatase–anti-immunoglobulin conjugate. Addition of a chromogenic alkaline phosphatase substrate colors the hybridized probe blue. Hybridization and development of the DNA blots were performed according to the high-sensitivity protocol of the manufacturer, except that the hybridization solution was modified to 3× SSC (0.45 M NaCl and 0.045 M sodium citrate), 50% formamide, 1× Denhardt solution (0.02% each Ficoll, polyvinyl pyrrolidone, and bovine serum albumin), 5% dextran sulfate, 200 μg of yeast RNA per ml (Sigma Chemical Co., St. Louis, Mo.), and 20 μg of heat-denatured, sonicated salmon sperm DNA per ml. Optimal color development occurred in 30 to 60 min. The same hybridization solution was used with the ^{32}P-labeled probe. The washing and development of radioactive blots has been described previously (23).

Inoculation of tomato plants. The leaves of 2- to 4-week-old tomato seedlings (*Lycopersicon esculentum* Mill. 'Bonny Best') were infected with *P. syringae* pv. tomato and incubated in a growth chamber under conditions that have already been described (6). Lesions developed in 3 to 5 days.

Tomato leaf bioassay for coronatine. A dried ethyl acetate extract of bacterial culture supernatant was redissolved in sterile, distilled water and applied to the surface of a tomato leaf. If the supernatant contained coronatine, the leaf tissue around the injection site became chlorotic within 5 days. The details of this procedure have already been presented (26).

Collection of plant samples from tomato fields. Tomato leaves and fruit with bacterial speck-like lesions were collected from nine grower fields in southwestern Ontario. The fields were planted with the fresh market cultivar 'Pik Rite' or the processing cultivar 'Heinz 2653', 'Heinz 722', or 'Ferry Morse 6203'. Because of heavy rains, planting dates varied from 15 May to 6 June 1989. Four fields were assayed on each of the following dates in 1989: 12 July (5 to 9 weeks after planting), 8 August (9 to 13 weeks after planting), 31 August (12 weeks after planting), and 13 September (12 to 14 weeks after planting). Two fruit and two leaf samples were taken from each of five widely spaced locations in a 6,000-m^2 area of each field (10 samples per field). The samples were placed on moistened paper towels in plastic bags and were kept on ice until they were processed.

Isolation of bacteria from infected plant tissue. All plant material was thoroughly washed with tap water before being sampled. Leaf or fruit tissue containing one lesion (approximately 2 mm^2) was excised from the plant, cut into quarters, placed in a test tube containing 0.6 ml of sterile, distilled water, and incubated at 4°C for 60 min. Before being placed in the water, fruit lesions were carefully scraped on the underside to remove any attached pulp. Samples of the eluate were either filtered through the hybri-blot manifold (0.5-ml samples) or streaked on King medium B (22) agar. The plates were incubated at 25°C for 48 h. Nitrocellulose blots of the lesion eluates were processed by the procedure described for the probe specificity assays.

Isolation of bacteria from symptomless field tomato leaves. Two lesion-free leaf samples were collected from each of four southwestern Ontario tomato fields at the end of the 1989 growing season (13 September 1989). Twenty-five grams of leaf tissue from each sample was placed in a 1-liter flask with 250 ml of sterile, distilled water and shaken (150 rpm) at room temperature for 60 min. The wash water was filtered through a grade GF/A glass microfiber filter (Whatman Inc., Clifton, N.J.) and then through a 0.45-μm-pore-size cellulosic filter (Micron Separations, Inc., Westboro, Mass.). The material that collected on the cellulosic filter was washed into 5 ml of sterile water. The suspension was serially diluted with water and plated on NBY agar and Vogel-Bonner–tartrate (VB-tar) agar. VB-tar consisted of VB minimal medium (6) in which 0.3% D-(−)-tartaric acid (Sigma Chemical Co.) replaced the 0.2% citric acid. Colonies appearing on VB-tar plates after a 40-h incubation period (at 25°C) were transferred to Colony/Plaque Screen nylon filters (Dupont, NEN Research Products, Boston, Mass.) and lysed according to the instructions of the filter manufacturer. After neutralization with 1 M Tris hydrochloride (pH 7.5), the filters were air dried and hybridized to the pTPR1 probe as described above.

Characterization of bacteria isolated from infected plant tissue. Fluorescent colonies resembling *P. syringae* pv. tomato (opaque, off-white, and slightly fluidal) were selected from King medium B agar plates that had been streaked with eluate from tomato plant lesions. After purification, they were tested for pathogenicity and the ability to use D-(−)-tartrate, erythritol, or DL-lactate as the sole carbon source (4). The only compound of the three that can be used by *P. syringae* pv. tomato is D-(−)-tartrate.

RESULTS

Construction of a DNA probe specific for coronatine-producing bacteria. In a previous study, a 5.3-kb *Xho*I restriction fragment from the *cor* region of *P. syringae* pv. tomato chromosomal DNA was used as a probe in a sequence homology study of eight different *P. syringae* strains. Only the DNA of coronatine producers hybridized with the probe. In this study, this 5.3-kb *Xho*I fragment was subcloned into the *Sal*I cleavage site of pGB2, a cloning vector with no sequence homology to *P. syringae* pv. tomato (data not shown). The resulting plasmid, pTPR1, was purified, labeled

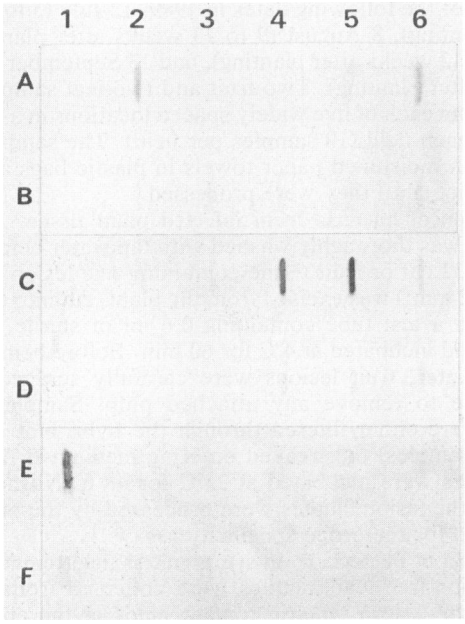

FIG. 1. Slot blot hybridization of DNA released in situ from cultures of plant-associated bacteria with the sulfonated pTPR1 probe. *P. syringae* strains were as follows: A1, tomato DCT6D1.1 (spontaneous Cor$^-$ mutant); A2, atropurpurea 340; A3, coronafaciens 345; A4, lachrymans 419; A5, maculicola 437; A6, maculicola 438; B1, morsprunorum 436; B2, morsprunorum 567; B3, persicae 308; B4, pisi 150; B5, syringae 313; B6, syringae 132; C1 papulans NYPSP25; C2, papulans PSP1; C3, papulans NYPSP14; C4 and C5, tomato 3000; C6, tomato 2563; D1, syringae 281; D2, syringae 2750; D3, syringae 2747; D4, syringae 3906; D5, syringae 1038; D6, antirrhini 2738; E1, tomato DC3000. Other *Pseudomonas* strains were as follows: E2, *P. marginalis* DC83; E3, *P. viridiflava* MM1; E4, *P. viridiflava* AP1; E5, *P. viridiflava* T9B1; E6, *P. viridiflava* MF2. Members of the *P. fluorescens-P. putida* group were as follows: F1, strain 4A3; F2, strain 1A3; F3, strain 9B3; F4, strain 10A3; F5, strain 8B2; F6, strain 9A2.

nonradioactively by using Chemiprobe, and tested for hybridization specificity and sensitivity.

Probe specificity. The specificity of pTPR1 was verified by slot blot hybridization assays with genomic DNA from several different tomato epiphytes, tomato pathogens, and *P. syringae* pathovars (Table 1). Representative blots are shown in Fig. 1. The *P. syringae* pv. tomato strains selected for this study were geographically diverse and included three strains (G13, 188B, and 208B) of Race 1, a group defined by its ability to infect tomato cultivars carrying the bacterial speck resistance gene *Pto*. The epiphytes consisted of *Pseudomonas marginalis*, the *Pseudomonas fluorescens-Pseudomonas putida* group, and *Pseudomonas viridiflava*. Ten of the fifteen *P. syringae* pv. syringae strains which had been isolated from tomato plants caused the tomato disease bacterial fleck. All of the *P. syringae* strains listed in Table 1 were screened by the tomato leaf bioassay for coronatine production. Only the 21 probe-positive strains (Table 1) induced leaf chlorosis. All of the *P. syringae* pv. tomato strains, as well as the pTPR1-positive strains from other pathovars, were tested for pathogenicity on tomato leaves. The three probe-negative *P. syringae* pv. tomato strains, NCPPB 880, NCPPB 1008, and NCPPB 2563, were also nonpathogenic. *P. syringae* pv. maculicola 438 produced typical bacterial speck symptoms, while the *P. syringae* pv. atropurpurea and *P. syringae* pv. glycinea strains induced a

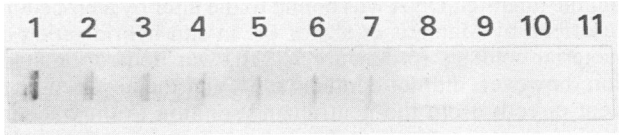

FIG. 2. Slot blot hybridization of DNA released in situ from the bacteria present in a *P. syringae* pv. tomato DCT6D1 leaf lesion with the sulfonated pTPR1 probe. The lesion contained 2.2×10^6 CFU. Lanes 1 to 10, two-fold dilution series of the lesion extract: 2.2×10^5, 1.1×10^5, 5.5×10^4, 2.8×10^4, 1.4×10^4, 6.9×10^3, 3.4×10^3, 1.7×10^3, 8.6×10^2, and 4.3×10^2 CFU, respectively. Lane 11 contained undiluted extract of symptomless leaf tissue.

small amount of chlorosis but no necrotic lesions. *P. syringae* pv. maculicola 437 formed lesions without the chlorotic halos. Recent physiological and restriction fragment length polymorphism studies have shown that the *P. syringae* pv. tomato and the *P. syringae* pv. maculicola strains are indistinguishable (T. Denny, personal communication).

Probe sensitivity. One bacterial speck lesion from a tomato plant infected with strain DCT6D1 contains approximately 3×10^6 CFU of the pathogen (5). The sensitivity of the pTPR1 probe was determined by applying serial (1:1) dilutions of bacterial speck lesion eluate to a nitrocellulose filter by using the hybri-slot manifold. Healthy tissue eluate served as a negative control for each blot. The limit of detection was $(3.8 \pm 0.5) \times 10^3$ CFU per slot (Fig. 2). At lower concentrations, detection was difficult because of the slight amount of nonspecific background present in each slot impression. Background increased significantly if the vacuum filtration time exceeded 4 min (data not shown). The same limit of detection ($[3.8 \pm 0.5] \times 10^3$ CFU per slot) was obtained when serial dilutions of a purified DCT6D1 culture were tested for hybridization to the probe.

The sensitivity of the sulfonated pTPR1 probe was compared with that of a ^{32}P-labeled probe by using serial dilutions (1:1) of purified genomic DNA from *P. syringae* pv. tomato DC3000 (Fig. 3). The sulfonated probe detected 0.25 to 0.5 ng of DNA, while the radiolabeled probe gave a positive signal with approximately 1.0 ng. As with the lesion assays, the background obscured the Chemiprobe color development when the DNA concentration dropped below 0.5 ng.

Detection of *P. syringae* pv. tomato in field tomato plant lesions. Two hundred fifty-eight leaf and fruit lesions were collected from nine tomato fields in southwestern Ontario during the 1989 growing season. Each lesion was screened for *P. syringae* pv. tomato by using the pTPR1 probe and the

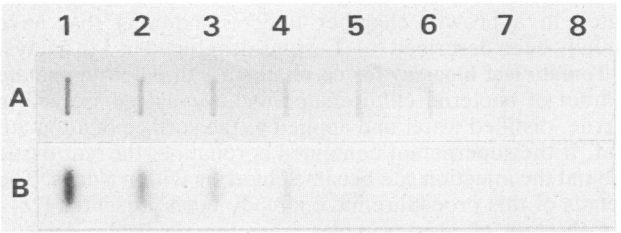

FIG. 3. Slot blot hybridization of purified DNA from *P. syringae* pv. tomato DC3000 with sulfonated (A) and ^{32}P-labeled (B) probe pTPR1. Lanes 1 to 8, two-fold dilution series of the purified DNA: 8.8, 4.4, 2.2, 1.1, 0.55, 0.28, 0.14, and 0.07 μg, respectively. The radiolabeled probe had a specific activity of 8×10^7 cpm/μg and the hybridization buffer contained 2×10^6 cpm/ml. The ^{32}P-labeled blot was exposed to XAR X-ray film for 7 days at −70°C.

TABLE 2. Comparison of the pTPR1 probe method (P) with a culture method (C) for detection of *P. syringae* pv. tomato in necrotic lesions on field tomato plants[a]

Sample date	Lesion no. and source[b]	No. of colonies				
		P+/C+	P−/C−	P+/C−	P−/C+	Agreement (%)[c]
12 July	40 L	33	0	0	7	83
	30 F	19	2	1	8	70
8 August	39 L	8	6	0	25	36
	37 F	18	7	0	12	68
31 August	38 L	9	13	1	15	58
	38 F	20	3	0	15	61
13 September	18 L	1	8	0	9	50
	18 F	8	2	0	8	56
Total	258	116[d]	41	2[d]	99	61

[a] Colonies with typical *P. syringae* pv. tomato morphology were selected from King medium B plates that had been streaked with lesion eluate, purified, and then tested for pathogenicity and the ability to use erythritol, DL-lactate, and D-(−)-tartrate as carbon sources.

[b] L, Leaf; F, fruit.

[c] % Agreement = [(no. of P+/C+ colonies + no. of P−/C− colonies)/no. of lesions] × 100.

[d] The King medium B plates for several of the lesions were heavily overgrown with yellow- and orange-pigmented fluidal colonies. Sixteen of the P+/C+ lesions were initially P+/C−; only after two or three attempts were *P. syringae* pv. tomato colonies isolated from the King medium B plates for these lesions.

culture method of detection (Table 2). The culture method consisted of streaking lesion eluates on King medium B, selecting fluorescent colonies with typical *P. syringae* pv. tomato morphology, and performing pathogenicity and carbon source utilization tests on purified cultures of the isolated bacteria. The pathogen was present in 217 lesions (84%) with younger plants having a significantly ($P = 0.05$) higher level of infestation. Leaf and fruit lesions collected on 12 July from 5- to 9-week-old plants were 100 and 93% positive whereas those collected on 13 September from 14-week-old plants were 56 and 89% positive. The percent agreement between the two detection methods was also significantly higher ($P = 0.05$) with younger plants (Table 2). The best agreement, 90%, occurred with 30 lesion samples collected on 12 July from the two most recently planted (5-week-old) fields. The lowest percent agreement occurred with the leaf lesion samples collected on 8 August. All of the fields in our sampling area were damaged by severe rain storms and flooding in late July. The leaf lesions collected on 8 August contained unusually high numbers of bacteria, the majority of which had a colony morphology significantly different from that of *P. syringae* pv. tomato (data not shown). A total of 18 of the 258 lesions were initially probe positive and culture negative. The pathogen was eventually recovered from 16 of these lesions but only after two or three attempts at isolation from the original King medium B plates. Plates for the two probe-positive, culture-negative lesions (Table 2) were heavily overgrown with large fluidal yellow- and orange-pigmented bacterial colonies. Lack of agreement between the two identification methods was due primarily to culture-positive, probe-negative lesions. However, purified cultures of the *P. syringae* pv. tomato strains isolated from the 99 culture-positive, probe-negative lesions all hybridized strongly to pTPR1.

Screening symptomless field tomato leaves for *P. syringae* pv. tomato. A colony blot hybridization procedure was adapted to detect *P. syringae* pv. tomato on symptomless tomato leaves. Samples of young healthy tomato leaves were collected on 13 September 1989 from 14-week-old plants in four different fields. Leaf wash water from these samples was plated on NBY agar and VB-tar agar. After a 24-h incubation period, the small colonies appearing on VB-tar plates were transferred to nitrocellulose and subsequently hybridized to pTPR1. The total number of bacteria per gram of leaf tissue, as determined on NBY agar, varied from 1.3×10^6 to 1.3×10^7 while the number of *P. syringae* pv. tomato per gram of leaf tissue, as determined by VB-tar colony hybridization, ranged from 2.2×10^5 to 1.2×10^6 (Table 3). Approximately 65% of the bacterial population able to grow on NBY agar could not be recovered on VB-tar. Thirty-two replica-plated colonies from the VB-tar plates (eight from each field) were purified and tested for fluorescence on King medium B, for sugar utilization, and for pathogenicity on tomato plants. Only the 27 probe-positive colonies were identified as *P. syringae* pv. tomato (Fig. 4).

TABLE 3. Populations of *P. syringae* pv. tomato on symptomless leaves in tomato fields infested with the pathogen

Field and sample	Bacterial population (cells/g of leaf tissue, 10^5)			*P. syringae* pv. tomato (%)[a]
	Total on:		pTPR1 positive	
	NBY	VB-tar		
A				
Sample 1	40	17	4.6	11.5
Sample 2	64	38	9.0	14.1
B				
Sample 1	110	23	2.2	2.0
Sample 2	13	4.4	3.6	27.8
C				
Sample 1	19	5.6	3.6	18.9
Sample 2	130	25	2.2	1.7
D				
Sample 1	61	18	6.4	10.5
Sample 2	68	30	12	17.7

[a] % = (no. of pTPR1-positive bacteria per gram of leaf tissue/total no. of bacteria per gram of leaf tissue) × 100.

DISCUSSION

The DNA probe pTPR1 provides an effective means of identifying field isolates of *P. syringae* pv. tomato. All 244 *P. syringae* pv. tomato strains isolated from Ontario tomato fields during the 1989 growing season produced coronatine and reacted with this probe. *P. syringae* pv. tomato was the only coronatine-synthesizing bacterium acquired during the sampling period. Furthermore, every virulent *P. syringae* pv. tomato strain in our culture collection, which contains isolates from Canada, the United States, New Zealand, and Europe, was probe positive. Although coronatine production is not a pathogenicity factor (1, 25, 26), it appears to be an important trait that may give the pathogen a competitive advantage in its natural habitat. Purified coronatine causes not only leaf chlorosis but also plant stunting and hypertrophy of potato tubers (25). Bender et al. have shown, by using Tn*5* mutants, that the toxin plays a significant role in lesion expansion and bacterial multiplication on tomato leaves (1). Growth studies with our own Tn*5*-induced Cor$^-$ mutants have confirmed these findings (unpublished data).

In field studies, the probe and culture methods for detection of *P. syringae* pv. tomato showed the best agreement, 90%, when lesion samples were taken from relatively young plants. For plants over 5 weeks old, lack of agreement was due, in most cases, to probe-negative, culture-positive le-

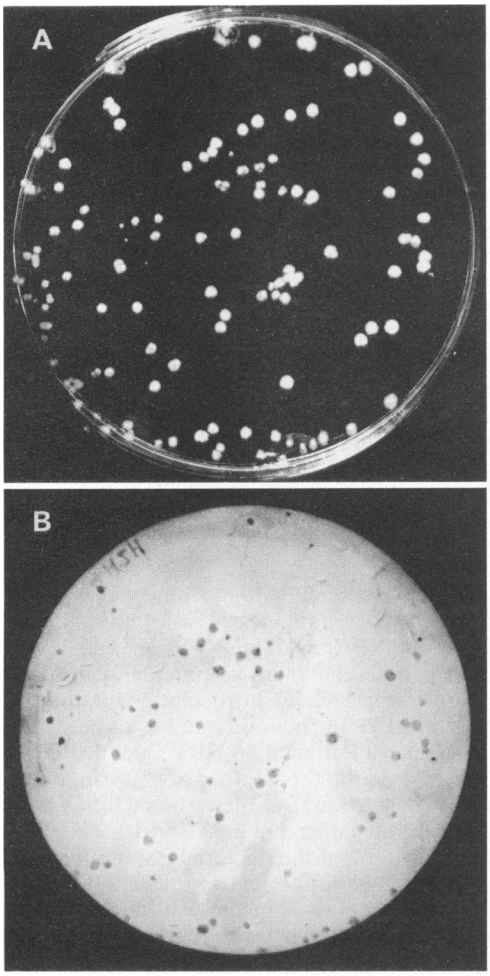

FIG. 4. Colony hybridization analysis of the bacteria recovered on a VB-tar agar plate from the wash water of healthy field tomato leaves. (A) Plate before the colony lift was performed; (B) colony lift after hybridization with the sulfonated pTPR1 probe. Only *P. syringae* pv. tomato colonies gave a positive signal.

sions. Probe-negative lesions were never observed on young plants (3 to 5 weeks old) cultivated and inoculated in growth chambers (Fig. 2). Earlier work has shown that the number of viable *P. syringae* pv. tomato cells in leaf lesions drops significantly as the lesions age (5). Perhaps the probe-negative, culture-positive lesions were formed early in the growing season and, as the *P. syringae* pv. tomato population fell, became overgrown with microbial opportunists. Typical bacterial speck leaf and fruit lesions may contain, in addition to *P. syringae* pv. tomato, pectolytic xanthomonads, *P. syringae* pv. syringae, *P. viridiflava*, *P. marginalis*, *P. fluorescens*, and *P. putida* (2, 5, 15). The leaf lesions with the lowest percent agreement between the two detection methods, those lesions that had been collected after the heavy rains in late July, contained exceptionally high numbers of extraneous microorganisms (as was observed on King medium B plates). Since several pathogens can produce lesions on field tomato plants that resemble bacterial speck (14, 21), our probe-negative, culture-positive lesions may not have been formed by *P. syringae* pv. tomato. The bacterial speck pathogen itself may have been the opportunist in some of these lesions. Schaad et al., who found that *Pseudomonas syringae* pv. phaseolicola colonies less than 96 h old did not always react with their phaseolotoxin DNA probe, suggested that a low *tox* gene-to-total genomic DNA ratio in young colonies may be responsible for a probe-negative reaction (29). Young colonies of *P. syringae* pv. tomato hybridized strongly with pTPR1. However, since coronatine genes were plasmid encoded in most of the *P. syringae* pv. tomato strains we have examined (unpublished data), a low *cor* gene-to-total DNA ratio in field lesion bacteria, which are subjected to harsh environmental conditions, may be possible and may also help explain our results.

The sensitivity of the sulfonated pTPR1 probe, 4,000 CFU or 0.5 to 0.25 ng of purified genomic DNA, was equivalent to that of a ^{32}P-labeled probe. Similar detection limits have been obtained with other sulfonated DNA probes (16, 18) and with ^{32}P-labeled probes for *X. campestris* pv. phaseoli (13) and *Salmonella typhi* (28). This level of sensitivity is more than adequate for enumerating *P. syringae* pv. tomato on field plants by the colony hybridization procedure or for screening young plants for bacterial speck lesions. Although biotinylation, another nonradioactive reporter system, has an equivalent or slightly better level of detection, its widespread use in plant disease diagnosis has been limited by the presence of endogenous biotin in plant material (17).

The pTPR1 probe, coupled with the semiselective medium VB-tar, offers a highly sensitive and specific means of quantifying bacterial speck on tomato plants. Coronatine-producing strains of *P. syringae* pv. tomato, *P. syringae* pv. glycinea, *P. syringae* pv. atropurpurea, and *P. syringae* pv. maculicola were the only bacteria found to react with pTPR1. Of these pathovars, only tomato and maculicola, which, as mentioned earlier, are indistinguishable, were able to use D-(−)-tartrate as a carbon source. Results can be obtained with this procedure within 3 to 4 days of sample collection. The probe should facilitate epidemiological studies of this pathogen and aid in the testing of disease forecasting systems such as the one recently developed by Jardine and Stephens (20). It will provide growers with a rapid means of screening transplant seedlings before planting and early in the growing season, when detection of bacterial speck-infested plants is crucial. It also should benefit the tomato transplant industry of Florida and Georgia, whose plants must be certified free of *P. syringae* pv. tomato before they can be shipped to the northern tomato-growing regions.

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