Techniques

Differentiation of *Pseudomonas syringae* pv. *tomato* from *P. s. syringae* with a DNA Hybridization Probe

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ABSTRACT

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A DNA hybridization probe was developed that hybridized strongly to *Pseudomonas syringae* pv. *tomato* DNA but not to *P. s. syringae* DNA on Southern blots and dot blots. This "PST-DNA probe" consisted of cloned 3.5 and 3.6 kilobase *Eco*RI fragments of *P. s. tomato* that were ^{32}P-labeled. The PST-DNA probe detection limits were 2 ng of purified *P. s. tomato* DNA and DNA released in situ from 1×10^5 cells of *P. s. tomato*. Similar amounts of *P. s. syringae* DNA retained about 4% as much of the PST-DNA probe. Sixty-eight strains of *P. s. syringae*, isolated from a variety of plants including tomato, were not recognized by the PST-DNA probe. The PST-DNA probe also did not hybridize to selected strains of *P. aeruginosa*, *P. cichorii*, *P. fluorescens*, *P. viridiflava*, or *Xanthomonas campestris* pv. *vesicatoria*. The PST-DNA probe hybridized to one or more strains of half of the 20 additional pathovars of *P. syringae* examined. Cells released from individual *P. s. tomato* lesions were reliably detected by the PST-DNA probe, even though the amount of DNA recovered varied. A method was developed for using the PST-DNA probe when the amount of DNA on a blot is unknown, and a preliminary test indicated that *P. s. tomato* and *P. s. syringae* lesions could be differentiated. The PST-DNA probe has the potential to give definitive results within one day after a fresh tissue sample is brought to the laboratory.

The production of tomato transplants is an important industry in parts of Florida and southern Georgia. Most of the crop is produced under certification programs to ensure that it is free of important diseases. Accurate identification of pathogenic organisms is essential to prevent epiphytotics in northern tomato-growing areas and to avoid unwarranted loss of certification. With tomato transplants worth over $9,000 per hectare (10), a quarantine results in serious loss of revenue for a producer. At the same time, however, a rapid diagnosis is often important to avoid costly shipping delays.

Two fluorescent pseudomonads are common foliar pathogens of tomatoes in the South (10,13). *Pseudomonas syringae* pv. *tomato* (Okabe) Young et al, the cause of bacterial speck, reduces both fruit quality and yield (9,27,30). Although found infrequently (10), *P. s. tomato* results in the loss of certification because this pathogen can survive epiphytically on tomatoes and nonhost plants for extended periods of time (3,27) and can reach epidemic proportions in fruit production fields (3,15,22). *P. s. syringae* van Hall is the most frequently isolated phytopathogenic bacterium (10), but because it causes a disease that regresses as plants mature, infected plants retain certification. *Xanthomonas campestris* pv. *campestris* (Doidge) Dye, the cause of bacterial spot, is the only other plant pathogenic bacterium routinely isolated from tomato transplants in Georgia (10).

One problem experienced by those responsible for the certification program is that symptoms caused by *P. s. tomato* and *P. s. syringae* are often indistinguishable. These two pathovars can be reliably separated based on several phenotypic characteristics (6,12), but the necessary tests may take up to 1 wk to complete. Attempts to find more rapid methods of differentiating these pathovars have included serological and bacteriophage typing (5,11), but neither procedure is sufficiently specific. DNA hybridization probes, which are rapid and sensitive, have proven their usefulness in detecting viruses and viroids (see 28). There are also several examples where DNA probes have been developed for bacteria (8,19,23,24). The genetic dissimilarity of *P. s. tomato* and *P. s. syringae* (7,20,21) suggested that a probe might clearly differentiate these two pathovars. This possibility was strengthened by restriction fragment length polymorphism (RFLP) studies, which identified two cosmid clones that hybridized to many restriction fragments of *P. s. tomato* DNA but to few fragments of *P. s. syringae* DNA (7). This paper reports the successful development of a differential DNA hybridization probe

that performs well with both purified and crude DNA and has the potential to detect bacteria directly from disease lesions.

MATERIALS AND METHODS

Bacterial strains. The strains of bacteria used in this research are given in Table 1. The identity of each of the strains of *P. s. tomato* listed has been confirmed (6), but few of the other phytopathogenic bacteria were characterized before being used. Twelve additional strains of *P. syringae*, which were provisionally designated as pathovar *tomato*, were received from P. C. Fahy (Dept. of Agriculture, New South Wales, Australia) and used without further characterization. The *P. s. syringae* strains were isolated from the following plants (the number of strains is given in parentheses): almond (1), apple (2), French bean (4), cauliflower (1), cherry (2), citrus (6), corn (3), foxtail (2), millet (3), okra (1), peach or apricot (5), pear (3), pepper (3), plum (1), sorghum (2), soybean (4), tomato (7), walnut (2), wheat (4), and wild rice (1). The 13 strains of *P. s. syringae* from J. Olive were isolated from washes of nonsymptomatic apple and peach leaves collected in Georgia. Many strains of the additional pathovars of *P. syringae* received from the Plant Diseases Division Culture Collection (PDDCC) were the type strains; however, these and the strains from other sources may not be representative of a particular pathovar. Pseudomonads were grown for 24 hr on King's medium B (16), *X. campestris* and *Agrobacterium tumefaciens* were grown on Nutrient-agar (Difco), and *Escherichia coli* was grown on LB medium (17). The medium to test for utilization of organic substrates was that of Misaghi and Grogan (18) modified to contain a final concentration of 50 mM phosphate buffer, pH 7.0. All strains were stored in 15% glycerol at −80 C (25).

DNA isolation. Total DNA was prepared by the method of Silhavy et al (25) with the modifications described elsewhere (7). Briefly, the procedure entails lysis of bacterial cells with sodium dodecyl sulfate (SDS) in the presence of proteinase K, removal of protein with phenol, and spooling-out high molecular weight DNA after ethanol precipitation. Plasmid DNA was prepared from *E. coli* by an alkaline lysis technique (2). DNA restriction fragments for radioisotope labeling were electroeluted from agarose gels (17). The procedure was modified by using 0.1× TBE buffer (8.9 mM Tris-borate, 8.9 mM boric acid, 0.2 mM EDTA), with elution proceeding at 500 V for 15–30 min. The eluted DNA was concentrated by butanol extraction, purified by phenol extraction, precipitated with ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0).

Subcloning of *P. s. tomato* DNA. Three cosmid clones

TABLE 1. Strains used in the process of developing a hybridization probe for *Pseudomonas syringae* pv. *tomato*

Bacterium	Strain designation	Source or reference[a] (no. of strains)	Bacterium	Strain designation	Source or reference (no. of strains)
P. s. tomato	B118, B88, B120, RG4;	S. McCarter (4)	*coronafaciens*	B142;	S. McCarter (1)
	JL1031;	J. Lindeman (1)		BK374;	B. Kennedy (1)
	PST6, NCPPB 880;	D. Cuppels (2)		5-IT-281;	S. Hirano (1)
	T1;	G. Bonn (1)	*delphinii*	8719;	ATCC (1)
	2844;	PDDCC (1)		529;	PDDCC (1)
	10862;	ATCC (1)	*glycinea*	NCPPB 2159 (race 1),	
P. s. syringae	B3A, B15(+), B301, B362, B359, B368, B382, B407, B427, B452, B455, B458,			J3-20-4A (race 4), A-29-2 (race 4), J3-17-2 (race 5),	
	B460;	J. DeVay (13)		R6 (race 6);	W. Fett (5)
	PS-1, PS-14, PS-18;	D. Ritchie (3)		BK380;	B. Kennedy (1)
	B-301D, 464, 480,		*lachrymans*	PL785;	W. Fett (1)
	W4N-15, W4N-27, W4N-43, W4N-108, HS191,			NK344;	N. Keen (1)
			maculicola	#1, #5, #10;	N. Keen (3)
	NCPPB 1053, SD19,		*mori*	4331;	PDDCC (1)
	Ps-col, 5D417, 5D4198,			NCPPB 1413;	D. Cooksey (1)
	PS1-Bean, PS6-SB;	D. Gross (15)	*morsprunorum*	486;	W. Fett (1)
	BK034, BK035, BK036;	B. Kennedy (3)		5795, 567;	PDDCC (2)
	B5, B6;	E. W. B. Ward (2)	*papulans*	B60-1, B138;	S. McCarter (2)
	1083-3, 0485-10, 0682-8;	D. Cooksey (3)	*passiflorae*	129;	PDDCC (1)
	B61, B64, B76, B78, B84;	S. McCarter (5)	*persicae*	5846;	PDDCC (1)
	IIBCh, 84-15, 82-17, 84-47, 84-61, 84-90, 85-262,		*phaseolicola*	Mex1, NK343;	N. Keen (2)
				B130;	S. McCarter (1)
				BK378;	B. Kennedy (1)
	85-267, 85-274;	R. Gitaitis (9)	*pisi*	NK372;	N. Keen (1)
	DH015;	D. J. Hagedorn (1)		BK373;	B. Kennedy (1)
	PS61;	A. Collmer (1)		LH150 (race 1), LH151 (race 2), LH 152 (race 3);	L. Hadwiger (3)
	PSC-1B;	D. Cuppels (1)			
	SSP106-1, SSP106-16, SSP108-15, SSP108-16, 59WP, 22WP, 45BA, 101WA, 107WA, 116WA, SSA112-1, SSA103-1,		*savastanoi*	4352;	PDDCC (1)
			striafaciens	BK375;	B. Kennedy (1)
			tabaci	2835, 4410;	PDDCC (2)
			tagetis	BK376, BK377;	B. Kennedy (2)
			P. viridiflava	PV1;	J. Jones (1)
	SS13-8;	J. Olive (13)	*P. cichorii*	CUCPB 1125;	R. Dickey (1)
P. s. pathovars				B57;	S. McCarter (1)
antirrhini	19871;	ATCC (1)	*P. fluorescens*	13527;	ATCC (1)
	4303;	PDDCC (1)	*Xanthomonas campestris* pv. *vesicatoria*	83-38, 81-11;	R. Gitaitis (2)
atrofaciens	B143;	S. McCarter (1)	*Escherichia coli*	HB101;	reference 17
atropurpurea	NK340;	N. Keen (1)	*Agrobacterium tumefaciens*	A6;	A. Binns (1)
berberidis	13454;	ATCC (1)			
	4116;	PDDCC (1)			

[a]Source information: ATCC = American Type Culture Collection, Rockville, MD. PDDCC = Plant Diseases Division Culture Collection, Auckland, NZ. Binns, Univ. of Pennsylvania; Bonn, Agriculture Canada, Harrow Research Stn.; Collmer, Univ. of Maryland; Cooksey and Keen, Univ. of California, Riverside; Cuppels and Ward, Agriculture Canada Research Center, London; DeVay, Univ. of California, Davis; Dickey, Cornell Univ.; Fett, USDA Eastern Regional Research Center, Philadelphia; Gitaitis, Univ. of Georgia Coastal Plain Research Stn.; Gross and Hadwiger, Washington St. Univ.; Hagedorn and Hirano, Univ. of Wisconsin; Jones, Univ. of Florida Gulf Coast Research Center; Kennedy, Univ. of Minnesota; Lindeman, Advanced Genetic Sciences, Oakland; McCarter and Olive, Univ. of Georgia; Ritchie, North Carolina St. Univ.

containing *P. s. tomato* genomic DNA were produced during previous research (7). DNA from the cosmids pB88-4 and pB88-6 was digested with *Eco*RI and ligated to DNA from the plasmid pUC9 (29). Products of the ligation reaction were transformed into competent cells of *E. coli* strain TB1 and recombinants selected on LB medium containing 100 μg of ampicillin per milliliter, 40 μg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-glacatoside) per milliliter, and 40 μM IPTG (isopropyl β-D-galactoside) (17). Plasmid DNA prepared from individual clones was digested with *Eco*RI and examined by agarose gel electrophoresis. Four clones with single fragments of *P. s. tomato* DNA were selected for further use.

Preparation of DNA blots. Southern blots of total DNA digests were prepared by the method of Smith and Summers (26). A modification of the method described by Kafatos et al (14) was used to prepare dot blots of purified DNA. Each sample was diluted with TE buffer so that the desired amount of DNA (most often 200 ng) was contained in a volume of 25 μl. The DNA was denatured by adding an equal volume of 0.4 N NaOH and incubating at room temperature for approximately 10 min. The reaction tube was transferred to an ice bath and diluted with nine volumes of 1.1 M ammonium acetate (pH 7.0). A 0.5-ml sample was drawn through a nitrocellulose membrane (prewetted for 30 min in 6× SSC [1× SSC is 0.15 M NaCl and 15 mM sodium citrate]) held in a commercial dot blot apparatus. Each sample dot was washed with 0.5 ml of ammonium acetate, and the entire membrane was immersed briefly in 6× SSC after removal from the apparatus. The membrane was dried at room temperature and baked for 1.5 hr at 80 C in a vacuum oven.

Dot blots of DNA from bacterial cells were made with a procedure similar to that described for colony hybridization (17). Bacteria usually were grown on agar medium and suspended in 10 mM $MgCl_2$ to give an $A_{600nm} = 0.1$ (approximately 1×10^8 colony-forming units [cfu]/ml). Bacteria were also recovered from disease lesions. Greenhouse-grown tomato plants were infected with *P. s. tomato* as described elsewhere (6), and lesions allowed to develop for 7–10 days. Leaves were harvested, rinsed briefly in water, and individual lesions were cut out with a #1 cork borer (4 mm diameter). Leaf disks were finely chopped with a sterile razor blade, and the cells released by shaking for 1 hr in 13- × 150-mm glass tubes (held vertically) containing 1.0 ml of 10 mM $MgCl_2$. When samples were taken from this suspension, most of the plant debris was avoided by pushing a plastic pipette tip firmly against the bottom of the tube. Aliquots of the cell suspensions (0.1 ml of suspended cells, 0.4 ml of tissue extract) were filtered through a nitrocellulose membrane held in a dot blot apparatus. The membrane was placed (with cells up) on filter paper saturated with 0.5 M NaOH and 1.5 M NaCl for 5 min. The membrane was transferred, for 5 min each, to a filter saturated with 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl and then to a filter saturated with 2× SSC. The membrane was rinsed in 6× SSC (when present, contaminating plant material was gently scrubbed off using a gloved finger), dried, and baked as above.

Hybridization. Samples of electoeluted DNA (200 ng) to be radioisotope labeled were applied to 0.025-μm VS filters (Millipore Corp., Bedford, MA) and dialyzed against TE buffer for 1 hr at room temperature; ^{32}P-dATP was then incorporated in a nick-translation reaction (17). Hybridization was performed in 50% formamide, 10% dextran sulfate as described elsewhere (6). Filters were washed five times in 0.1× SSC plus 0.1% SDS, dried, and autoradiographed using Kodak XAR-5 film and a Cronex Lightning Plus intensifying screen (Du Pont). Southern blots and dot blots hybridized to labeled *P. s. syringae* DNA were washed at 52 C, whereas all other dot blots were washed at 65 C. The radioactivity in each dot was quantified by removing the dot with a #4 cork borer (7 mm diameter) and depositing it in liquid scintillation fluid (4 g of Omnifluor [NEN-Du Pont]/ L of toluene). Each dot was counted for 5 min in a liquid scintillation spectrometer, nonspecific background counts were subtracted, and the values reported as counts per minute (CPM).

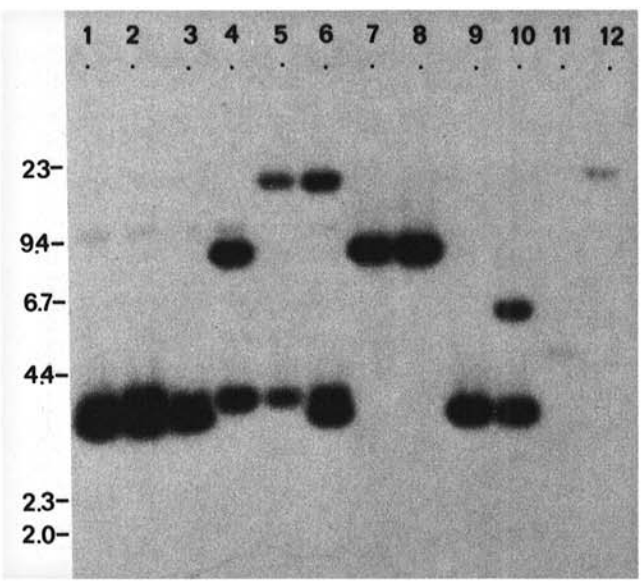

Fig. 1. Autoradiograph of a Southern blot of total DNAs of *Pseudomonas syringae* pv. *tomato* (lanes 1–10) and *P. syringae* pv. *syringae* (lanes 11 and 12) digested with *Eco*RI and hybridized to the PST-DNA probe. Lanes contained: 1, B118; 2, B88; 3, B120; 4, JL1031; 5, 2844; 6, RG4; 7, NCPPB 880; 8, 10862; 9, PST6; 10, T1; 11, B78; 12, B3A. The sizes (in kb) of lambda DNA digested with *Hin*dIII are given at the far left.

RESULTS

Selection of the *P. s. tomato* specific DNA probe. In preliminary experiments four purified fragments of *P. s. tomato* genomic DNA were evaluated individually for their ability to hybridize only to total *P. s. tomato* DNA on Southern blots. Two fragments hybridized strongly to both *P. s. tomato* and *P. s. syringae* DNAs, but two fragments that hybridized strongly only to *P. s. tomato* DNA were selected for further evaluation. One fragment, which came from the cosmid pB88-4 and was 3.6 kilobases (kb) long, was cloned in the plasmid pJCA2. The second fragment, which came from the cosmid pB88-6 and was 3.5 kb long, was cloned in the plasmid pJCA11. The isolated 3.6- and 3.5-kb fragments were mixed in equal proportions by weight and ^{32}P-labeled to make the PST-DNA probe. A nonspecific probe made with total DNA from strain B78 of *P. s. syringae* will be referred to as the B78-DNA probe.

The PST-DNA probe hybridized strongly to Southern blots of DNA from 31 strains of *P. s. tomato*, but hybridized very weakly to DNA from 20 strains of *P. s. syringae* (representative results are shown in Fig. 1). Twenty-one strains of *P. s. tomato* contained only the 3.6- and 3.5-kb fragments of the PST-DNA probe (e.g., strain B118), but the remaining strains were polymorphic. More detailed analysis showed that the 3.5-kb fragment hybridized to itself or to one larger restriction fragment in all strains; the larger fragment was 9 kb for strains JL1031, NCPPB 880, and 10862 and 20 kb for strain 2844. Polymorphisms observed for the 3.6-kb fragment were its hybridization to a 6-kb fragment of strain T1 and to an additional 20-kb fragment of strain RG4. The 3.6-kb fragment did not hybridize to DNA from strains NCPPB 880, 10862, and PST6.

Sensitivity of the PST-DNA probe. The ability of the PST-DNA probe to detect *P. s. tomato* DNA and differentiate it from *P. s. syringae* DNA on dot blots was evaluated. The dot blots were made with solutions of purified DNA or with DNA from cultured cells or cells from disease lesions that had been lysed in situ on the nitrocellulose membrane. The PST-DNA probe detected as little as 2 ng of purified *P. s. tomato* DNA with exposures of several hours, but 0.2 ng of DNA gave a signal barely above background (Fig. 2). In contrast, 200 ng of *P. s. syringae* DNA was required to give a reliable signal. These results were confirmed by quantifying the radioactivity in the dots. Replica filters showed that 200 ng of DNA from the two strains of *P. s. syringae* retained between 1.7 and 4.9% as much of the PST-DNA probe as did the same amount of DNA from the two strains of *P. s. tomato*. The quantitative difference between the two pathovars was slightly less when the

PST-DNA probe was of lower specific activity (results not shown).

Cells from freshly grown pure cultures of bacteria were collected by filtering suspensions onto a nitrocellulose membrane, and releasing and fixing the DNAs to the membrane during treatment with NaOH. The PST-DNA probe detected as little as 1×10^5 cfu of strain B118 *P. s. tomato* (Fig. 3). The dot with 1.2×10^7 cfu of B118 cells had the equivalent of about 40 ng of DNA based on the quantity of PST-DNA probe retained. Counting total cells (viable and dead) in a Petroff-Hausser chamber indicated that colony-forming units underestimated the total cell number by 50%. Nevertheless, the log cfu/dot were directly related to the log CPM/dot as expected ($r = 0.9$). Two strains of *P. s. syringae* barely retained the PST-DNA probe even at greater than 1×10^7 cfu/dot. This weak hybridization was not due to poor lysis of the cells, to poor binding of the DNA to the membrane, or to inability of the DNA to hybridize to the PST-DNA probe, because a replica filter strongly hybridized to the B78-DNA probe (Fig. 3).

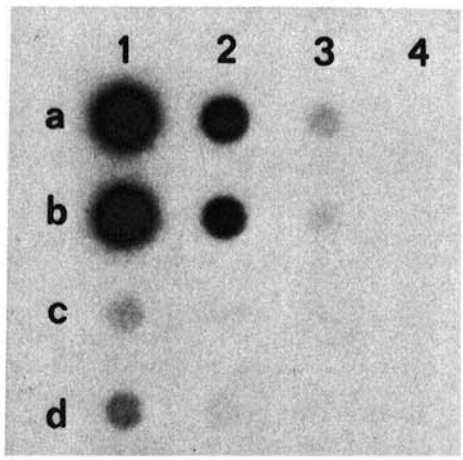

Fig. 2. Autoradiograph of a dot blot of purified DNAs from *Pseudomonas syringae* pv. *tomato* (rows a and b) and *P. s. syringae* (rows c and d) hybridized to the PST-DNA probe. The rows contained 10-fold dilution series of DNA from the following strains: row a, B88; row b, B118; row c, B78; row d, B3A. The columns 1–4 contained, respectively, 200 ng, 20 ng, 2.0 ng, and 0.2 ng DNA. The PST-DNA probe had a specific activity of 5×10^7 CPM/μg of DNA, and the hybridization solution contained 1.6×10^6 CPM/ml. The exposure was for 2 hr.

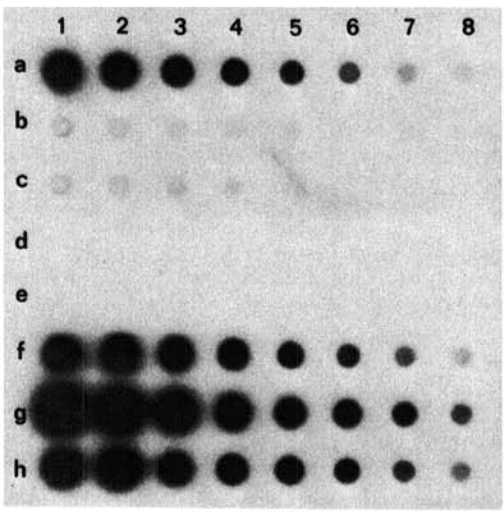

Fig. 3. Autoradiograph of replica dot blots of DNA released in situ from bacterial cells of *Pseudomonas syringae* pv. *tomato* (rows a and f) and *P. s. syringae* (rows b, c, g, and h). The filter containing rows a–c was hybridized to the PST-DNA probe, whereas the filter containing rows f–h was hybridized to total ^{32}P-DNA from strain B78 of *P. s. syringae*. The rows contained twofold dilution series (1/128 endpoint) of cells from the following strains: rows a and f, B118 ($1.2 \times 10^7 - 9.4 \times 10^4$ cfu); rows b and g, B78 ($1.9 \times 10^7 - 1.5 \times 10^5$ cfu; rows c and h, B3A ($2.9 \times 10^7 - 2.3 \times 10^5$ cfu). Rows d and e were blank. The exposure was for 7 hr.

Preliminary experiments indicated that leaf lesions caused by *P. s. tomato* contained greater than 1×10^6 cfu, which would be within the detectable range of the PST-DNA probe. A total of 42 individual lesions in three separate experiments were used to determine the ability of the PST-DNA probe to detect the presence of *P. s. tomato* cells. The PST-DNA probe hybridized strongly to DNA isolated from 40 lesions and weakly to the other two samples (results not shown). Eleven samples of healthy tomato tissue did not retain the PST-DNA probe, even in one experiment where the samples were heavily contaminated by nonfluorescent bacteria.

Additional tests of the specificity of the PST-DNA probe. The first experiment to evaluate more extensively the specificity of the PST-DNA probe used purified DNA from 69 strains of bacteria received as *P. s. syringae* and a limited number of other bacteria. The PST-DNA probe hybridized strongly only to the *P. s. tomato* control and to strain 1083-3 (Fig. 4). Southern-blot analysis confirmed the positive hybridization of strain 1083-3 DNA (see Fig. 8 below). Strain 1083-3 was isolated in California from a cauliflower leaf with many lesions and was assumed to be *P. s. syringae* without being characterized. Strain 1083-3 used D-tartrate as a sole carbon source but did not use DL-lactate or erythritol. These data suggest that strain 1083-3 is probably *P. syringae* pv. *maculicola* (4). In this experiment the PST-DNA probe did not hybridize to *E. coli*, *A. tumefaciens*, *P. viridiflava*, *P. fluorescens* (but see below), *P. cichorii*, or *X. campestris* pv. *vesicatoria*.

Experiments were conducted to more accurately quantify the average difference in hybridization of the PST-DNA probe to strains of *P. s. tomato* and *P. s. syringae*, information that is required for developing a method to evaluate unknowns. In this experiment replica dot blots of DNA from 12 strains of *P. s. syringae* (seven of which were originally from tomato leaf lesions)

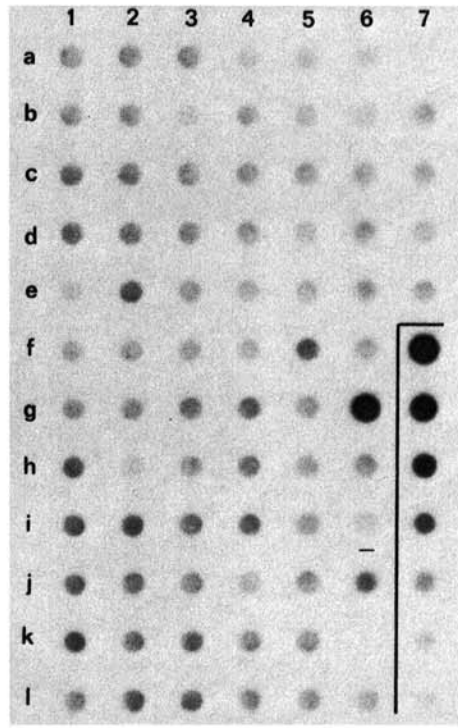

Fig. 4. Autoradiograph of a dot blot of purified DNAs from 69 strains of bacteria received as *Pseudomonas syringae* pv. *syringae* (columns 1–5 and column 6 rows a–i), six miscellaneous species of bacteria (columns 6 rows j–l and 7 rows a–e), and strain B118 of *P. s. tomato* (column 7 rows f–l) hybridized to the PST-DNA probe. Except for the *P. s. tomato* control, each dot received approximately 200 ng of DNA. The putative strains of *P. s. syringae* were those given in Table 1. The other bacteria were (column designation): *Escherichia coli* (6j); *Agrobacterium tumefaciens* (6k), *P. viridiflava* (6l); *P. fluorescens* (7a); *P. cichorii* (7b and 7c); *Xanthomonas campestris* pv. *vesicatoria* (7d and 7e). The *P. s. tomato* DNA was applied as a twofold dilution series from 200 to 3 ng. The exposure was for 6.5 hr.

and 10 strains of *P. s. tomato* were separately hybridized to the PST-DNA probe and to the B78-DNA probe (Fig. 5). After autoradiography, the radioactivity in each dot was quantified and the average CPM retained calculated (Table 2). Strains of *P. s. tomato* retained about 29 times more of the PST-DNA probe than did strains of *P. s. syringae*. The DNA sample of *E. coli* retained about 25% as much of the PST-DNA probe; this may have been due to contamination, since DNA from cells of *E. coli* did not hybridize to the PST-DNA probe (see Fig. 6). DNA from both *P. s. tomato* and *P. s. syringae* hybridized strongly to the B78-DNA probe, but the former retained about one half as much of this probe as did the latter. The effectiveness of the PST-DNA probe in differentiating *P. s. tomato* from *P. s. syringae* could be enhanced by dividing the ratio from the PST-DNA probe (ratio A) by the ratio from the B78-DNA probe (ratio B). Even without this enhancement, the differences between the two pathovars were highly significant for both probes ($P \leq 0.001$). The B78-DNA probe also hybridized moderately to the DNAs from the strain of *P. viridiflava* and the two strains of *P. cichorii* (Fig. 5).

Use of the PST-DNA probe would be significantly simplified if the specificity demonstrated when using purified DNA was also observed when DNA is released from cells lysed in situ on a nitrocellulose membrane. Preliminary experiments showed that colony blots (17) of all 31 strains of *P. s. tomato* given in Table 1 hybridized to the PST-DNA probe. For the experiment shown in Figure 6, 12 strains tentatively identified as *P. s. tomato* by P. Fahy were examined along with representative strains of *P. s. syringae*, strains of controls, and six strains of *P. aeruginosa*. The strains of *P. aeruginosa* were isolated from washes of tomato leaves taken from fields in south Georgia in April 1987 (R. Gitaitis and T. Denny, *unpublished data*). Other than the *P. s. tomato* control, the only other samples that hybridized to the PST-DNA probe were 11 of the 12 putative strains of *P. s. tomato* (Fig. 6). The results from the B78-DNA probe demonstrated that hybridizable DNA was released from all of the pseudomonads. The strain from P. Fahy that did not hybridize to the PST-DNA probe (DAR 35664b) was isolated from pepper, which is (at best) an uncommon host for *P. s. tomato* ((4); S. M. McCarter, *personal communication*). In addition, because this strain used DL-lactate and erythritol but not D-tartrate (a pattern typical of *P. s. syringae*), it is very unlikely to be *P. s. tomato*. The hybridization of DNA from cells of *P. fluorescens* to the B78-DNA probe was unexpected in light of the results when using purified DNA and is unexplained at this time. The average radioactivity in the dots was determined (Table 2), and, except for the generally lower degree of hybridization, the results were very similar to those obtained when

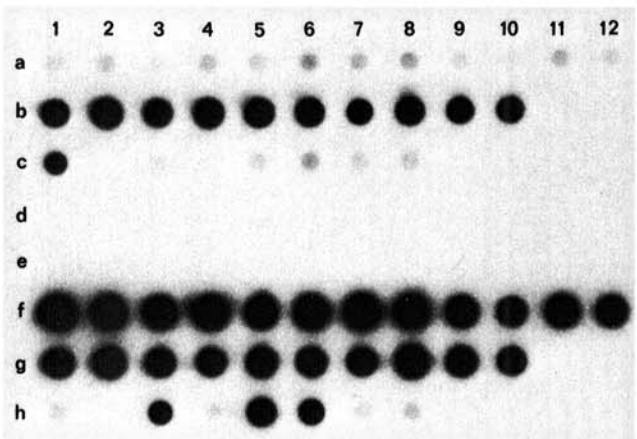

Fig. 5. Autoradiograph of replica dot blots of purified DNA from *Pseudomonas syringae* pv. *syringae* (rows a and f), *P. syringae* pv. *tomato* (rows b and g), and six miscellaneous species of bacteria (rows c and h). Each dot received approximately 200 ng of DNA. The blot with rows a–c was hybridized to the PST-DNA probe, whereas the blot with rows f–h was hybridized to total ^{32}P-DNA from strain B78 of *P. s. syringae*. The strains of *P. s. syringae* included seven from tomato (column numbers 1–7) and one strain each from peach (B3A), pear (B301), lemon (B427), bean (B362), and wheat (PSC1B) (column numbers 8–12, respectively). The strains of *P. s. tomato* were the same as those in Figure 1. The other bacteria were the same as those in Figure 4. Rows d and e were blank. The exposure was for 1 hr.

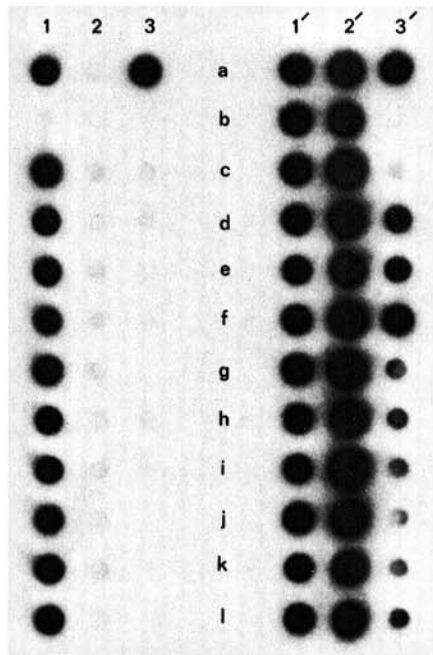

Fig. 6. Autoradiograph of replica dot blots of DNA released in situ from bacterial cells in an experiment to evaluate the reliability of this method. Columns 1 and 1′ received cells of 12 strains received as *Pseudomonas syringae* pv. *tomato*, and columns 2 and 2′ received cells of *P. s. syringae* (the same strains as in Fig. 5). Columns 3 and 3′ received cells of (row designation): strain B118 of *P. s. tomato* (a); *Escherichia coli* (b); strain 83-38 of *Xanthomonas campestris* pv. *vesicatoria* (c); strain CUCPB 1125 of *P. cichorii (d); P. fluorescens* (e); *P. viridiflava* (f); *P. aeruginosa* (g–l). The blot with columns 1–3 was hybridized to the PST-DNA probe, whereas the blot with columns 1′–3′ was hybridized to total ^{32}P-DNA from strain B78 of *P. s. syringae*. The exposure was for 2 hr.

TABLE 2. Average hybridization of the PST-DNA Probe and the B78-DNA probe to purified DNA and DNA released from cells lysed in situ from *Pseudomonas syringae* pv. *tomato* and *P. s. syringae*

Sample[a]	PST-DNA Probe		B78-DNA probe		
	CPM[b]	Ratio A[c]	CPM	Ratio B[c]	Ratio A/B
Purified DNA					
P. s. pv. *tomato*	4,647 ± 396	28.7	6,705 ± 710	0.59	48.6
P. s. pv. *syringae*	162 ± 19		11,292 ± 938		
Lysed cells					
P. s. pv. *tomato*	1,478 ± 74	25.5	1,860 ± 125	0.34	75.0
P. s. pv. *syringae*	58 ± 5		5,535 ± 434		

[a] Samples consisted of 200 ng of purified DNA or approximately 1×10^7 cells.
[b] Data are the average CPM ± standard error.
[c] Ratio of *P. s.* pv. *tomato*: *P. s.* pv. *syringae*.

using purified DNA. The differences between the two pathovars were statistically significant for both probes ($P < 0.001$).

One experiment was performed to determine whether the PST-DNA probe would differentiate *P. s. tomato* from *P. s. syringae* when both samples were prepared by using cells released from disease lesions. Samples from 24 individual *P. s. tomato* lesions were prepared as described previously. Twelve samples were prepared from 4-mm-diameter leaf disks that contained four to eight lesions that developed 7 days after inoculating young tomato leaves with *P. s. syringae* strain B76. Tissue with more lesions was used because the individual lesions were so much smaller than those produced by *P. s. tomato*. Replica dot blots were hybridized to the PST-DNA probe and to the B78-DNA probe, the results were quantified, and averages were calculated when appropriate. The samples of *P. s. tomato* retained about 1.8 times more of the PST-DNA probe (627 ± 128 CPM) than of the B78-DNA probe (374 ± 27 CPM). Three of the samples of *P. s. syringae* retained approximately the same low amount of each probe and were considered to lack detectable DNA. Although all of the nine remaining samples of *P. s. syringae* hybridized preferentially to the B78-DNA probe (35–455 CPM), only the five that retained two times more of the B78-DNA probe than of the PST-DNA probe were considered to be positive for *P. s. syringae* DNA. When compared to a *P. s. tomato* standard, the five positive samples of *P. s. syringae* had ratio A/ratio B values that ranged from 2.4 to 4.1 (see Table 2 for comparison).

The final experiment demonstrated that the PST-DNA probe was not absolutely specific for *P. s. tomato*, but also hybridized to DNA from other pathovars of *P. syringae* (Figs. 7 and 8). Purified DNAs from a total of 20 pathovars, some represented by several strains, were examined on a dot blot (Fig. 7). All of the strains tested of pathovars *berberidis*, *glycinea*, *maculicola*, *persicae*, and *striafaciens* were positive. One or more strains of pathovars *lachrymans*, *mori*, *morsprunorum*, *pisi*, and *tabaci* were positive. That these isolated positives were not due to strain misidentification was demonstrated by restriction fragment length polymorphism analysis, which showed that strains within each pathovar were closely related (Denny, *unpublished data*). The results of the dot blot were confirmed by Southern blot analysis of representative strains (Fig. 8). Some of these pathovars (e.g., *berberidis* and *maculicola*) had restriction fragment length patterns very similar to some strains of *P. s. tomato*, whereas others were dissimilar. The very strong hybridization of *P. s. glycinea* on both the dot and Southern blots was due to accidental overloading with DNA.

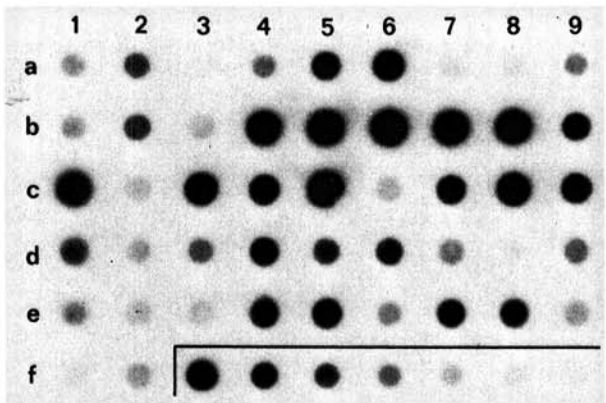

Fig. 7. Autoradiograph of a dot blot of purified DNA from *Pseudomonas syringae* pv. *tomato* and 20 other pathovars of *P. syringae* hybridized to the PST-DNA probe. The *P. s. tomato* DNA (row f3–9) was applied as a twofold dilution series from 200 to 3 ng. The other *P. syringae* pathovars were (row designation): *antirrhini* (a1–2), *atrofaciens* (a3), *atropurpurea* (a4), *berberidis* (a5-6), *coronafaciens* (a7-9), *delphinii* (b1-2), *glycinea* (b4–9), *lachrymans* (c1–2), *maculicola* (c3–5), *mori* (c6–7), *morsprunorum* (c8-9, d1), *papulans* (d2-3), *persicae* (d4), *passiflorae* (d5), *phaseolicola* (d6-9), *pisi* (e1–5), *savastanoi* (e6), *striafaciens* (e7), *tabaci* (e8–9), and *tagetis* (f1-2). The sample at dot b3 was later discovered to be from an unidentified fluorescent bacterium. The exposure was for 6.5 hr.

DISCUSSION

Fragments of *P. s. tomato* genomic DNA that appeared to hybridize specifically to *P. s. tomato* and not to *P. s. syringae* were readily isolated from the two cosmid clones that hybridized preferentially to *P. s. tomato*. The strains of *P. s. tomato* that were examined by Southern blot analysis should be representative of this organism (6). Two fragments of *P. s. tomato* were combined in making the PST-DNA probe to decrease the possibility of a false negative when testing unknowns. Consequently, it is unlikely that a strain of *P. s. tomato* will be found that is hybridization negative for the PST-DNA probe.

The sensitivity of the PST-DNA probe was adequate, with the limits of detection being about 2 ng of purified DNA and 1×10^5 cfu of cells. Sensitivity might be improved by using a higher specific activity probe (see below). The probe also reliably detected cells of *P. s. tomato* that were recovered from individual lesions. Maceration of tissues with a glass rod or by the method of Bertoni and Mills (1) was counterproductive, because fine plant debris interfered with filtration of the sample and deposition of DNA on the membrane. It was also necessary to use fresh cells, because suspensions made from old colonies or suspensions held 24 hr at 4 C performed less well. Very old lesions might, therefore, be expected to give poor results.

Specificity of the probe appeared to be sufficient to differentiate *P. s. tomato* from *P. s. syringae*, because none of the authentic strains of *P. s. syringae*, which included seven from tomato, hybridized to the PST-DNA probe. Furthermore, an effort was made to test strains of *P. s. syringae* that are representative of this pathovar; the strains selected came from around the United States, were isolated from most of the hosts of *P. s. syringae*, and included a number of epiphytes isolated in Georgia. Because the genetic diversity of *P. s. syringae* is unknown, however, strains that hybridize to the PST-DNA probe may be found during more extensive screening. A promising preliminary finding was that the PST-DNA probe did not hybridize to any of the other tomato pathogens tested. The PST-DNA probe did hybridize to some pathovars of *P. syringae* other than *P. s. tomato*. Some of these pathovars, like *P. s. berberidis*, are closely related to *P. s. tomato*

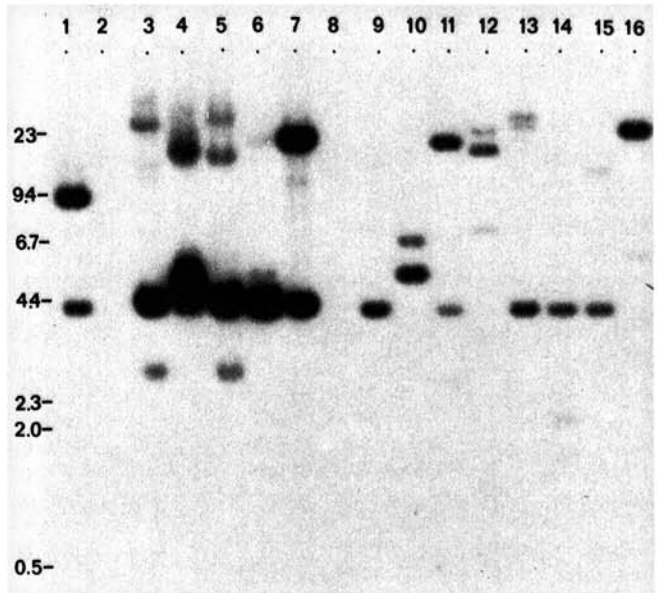

Fig. 8. Autoradiograph of a Southern blot of *Eco*RI digested total DNAs of selected *Pseudomonas syringae* pathovars hybridized to the PST-DNA probe. Lanes contained pathovars (strain designation): 1, *berberidis* (4116); 2, an unidentified fluorescent bacterium; 3–5, *glycinea* (2159, A-29-2, and R6, respectively); 6, lachrymans (PL785); 7, *maculicola* (#10); 8 and 9, *mori* (4331 and 07882-30, respectively); 10, *morsprunorum* (567); 11, *persicae* (5846); 12, *phaseolicola* (Mex1), 13, *pisi* (LH152); 14, *striafaciens* (BK375); 15, *tabaci* (2835); 16, "*maculicola*" (1038-3). The size standards are as in Figure 1.

(Denny, *unpublished data*). A close genetic relationship does not appear to be a good predictor, however, because *P. s. glycinea* strongly hybridized to the probe in spite of being only 60% homologous (20). Fortunately, these other pathovars are unlikely to be isolated in large numbers from lesions on tomato leaves in the South (10,12).

Known quantities of *P. s. syringae* DNA, whether purified or from cells lysed in situ, retained so little of the PST-DNA probe (less than 4% on average) that only the PST-DNA probe needs to be used to differentiate *P. s. tomato* from *P. s. syringae*. The detection of misidentified strains of *P. s. syringae* and *P. s. tomato* was a fortuitous demonstration of this method. The situation was not so simple when the samples were from disease lesions, because the number of cells recovered varied and hence the amount of DNA on the blot was unknown. In this case, one must be able to determine whether an unknown sample hybridizes weakly to the PST-DNA probe because it has an insufficient amount of *P. s. tomato* DNA, or whether the sample has sufficient DNA that is not homologous to the PST-DNA probe. A type of internal standard that compensates for the amount of DNA on a blot is provided by using a second, nonspecific probe that hybridizes to both *P. s. tomato* and *P. s. syringae* and comparing the results to those for a known sample of *P. s. tomato*. This was one of the reasons for using the *P. s. syringae* DNA probe, and was why the ratio A/ratio B values were calculated in Table 2. An unknown that contains *P. s. tomato* should have a ratio A/ratio B value = 1.0 (in this study the ratio was 0.73). This approach appeared to be valid, because ratio A/ratio B values of 45 and 75 were found for *P. s. syringae* when using purified DNA and DNA from cells lysed in situ, respectively. Much lower ratio A/ratio B values were recorded for lesion samples of *P. s. syringae*, apparently due to unexpectedly high retention of the PST-DNA probe, which in turn depressed the value of ratio A. Further testing, preferably of field samples, will be needed to determine the minimum ratio A/ratio B value that is acceptable for identifying an unknown.

There are several ways that the PST-DNA probe itself could be improved. One possibility would be to reclone the two fragments into a vector with adjacent RNA polymerase binding sites. Then, without prior isolation of the cloned fragments, very high specific activity RNA probes could be produced from just the cloned *P. s. tomato* DNA. Continued use of a radioactive probe has the advantage that, for a small number of samples, quantitative data are available in minutes by counting the dots in a scintillation counter. A second possibility, which will probably be necessary if this method is to be used outside of research laboratories, is to develop a stable, nonradioactive version of the PST-DNA probe. There are a variety of techniques for nonradioactive labeling of DNA (e.g., biotinylation, modification with antigenic sulfone groups, attachment of single-stranded binding protein, covalent attachment of an enzyme), all of which ultimately use an enzyme reaction to deposit a colored pigment where the probe has hybridized. The nonradioactive procedures should be as fast as the radioactive procedures, but because their results are not easily quantified, and extra steps may be required to prevent nonspecific binding of the probe when using crude DNA (i.e., when cells are lysed in situ), they may prove impractical for screening lesion samples.

This research explored the possibility of using hybridization probes for the detection and differentiation of bacteria. Establishing a standardized, reliable assay will require further testing of field samples that are simultaneously characterized by standard techniques. It will be especially important to determine whether the recognition of other pathovars of *P. syringae* by the PST-DNA probe is a problem. More specific, host-range related probes would greatly enhance the utility of this method. Before such probes are likely to become available, however, we must greatly increase our knowledge of the genetic relationships of phytopathogenic bacteria.

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