Detection of *Pseudomonas syringae* pv. *morsprunorum* on Cherries in Michigan with a DNA Hybridization Probe

J. M. PATERSON and A. L. JONES, Department of Botany and Plant Pathology and The Pesticide Research Center, Michigan State University, East Lansing 48824

ABSTRACT

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A PST-DNA probe, developed for differentiating *Pseudomonas syringae* pv. *tomato* from *P. s.* pv. *syringae*, hybridized with DNA extracted from *P. s.* pv. *morsprunorum* but weakly or not at all with DNA extracted from *P. s. syringae*. Purified DNA and DNA from colony blots of 18 strains of *P. s. morsprunorum*, including strains from England, Poland, South Africa, and the United States, hybridized with the radiolabeled probe, whereas 19 of 20 strains of *P. s. syringae* isolated from deciduous tree fruit crops did not hybridize or weakly hybridized with the probe. The detection limit for bacteria from pure culture was approximately 2.2×10^4 colony-forming units (cfu) per milliliter. DNA from *P. s. morsprunorum* extracted from lesions on fruit was detected by the probe, but DNA from lesions on leaves was not detected. The number of bacteria was 100-fold lower in effluent from leaves than from fruit. With field samples, identifications made with the probe were in general agreement with those made by standard biochemical and physiological techniques. *Eco*RI fragments of *P. s. morsprunorum* DNA exhibited restriction fragment length polymorphism when Southern blots were probed with the PST-DNA probe. The PST-DNA probe should aid in the rapid detection of *P. s. morsprunorum*.

Pseudomonas syringae pv. *morsprunorum* (Wormald) Young et al is the most frequent cause of leaf spots and bacterial fruit rot (bacterial canker) of sour cherries (*Prunus cerasus* L.) and sweet cherries (*P. avium* (L.) L.) and of leaf spots on prunes in Michigan. This bacterium is also an epiphyte on blossoms and leaves of these crops (7,10) and an occasional endophyte in dormant buds (10). Although *P. s. pv. syringae* van Hall incites similar symptoms on the leaves and fruit of sweet cherry in Michigan (4), in most years, it is only an epiphyte on blossoms and leaves and an occasional endophyte in dormant buds of these fruit crops (10).

The differentiation of *P. s. morsprunorum* from *P. s. syringae* normally requires that the bacteria be isolated, purified, and characterized in a series of biochemical, physiological, and pathogenic tests. These tests require about 2 wk and may need to be repeated because of ambiguous results. Epidemiological studies on these pathogens are frequently limited because of the time and effort required to confirm the identification of large numbers of isolates.

Recently, a DNA hybridization probe (PST-DNA) was developed for differentiating *P. s.* .pv. *tomato* (Okabe) Young et al from *P. s. syringae* (3). The probe also reacted with certain other pathovars of *P. syringae*, including *P. s. morsprunorum*. The objective of this study was to evaluate the PST-DNA probe for differentiating *P. s. morsprunorum* from strains of *P. s. syringae* found on deciduous tree fruit crops and to establish its potential as a diagnostic probe for *P. s. morsprunorum*.

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MATERIALS AND METHODS

Bacterial strains. Thirty-nine strains of three pathovars of *P. syringae* were used in this study (Table 1). Strains from Michigan were isolated in 1988 and 1989 from washings of blossoms collected from stone fruit crops. Those from other geographical areas were obtained under permit from colleagues in various institutions around the world, including: R. Gitaitis, University of Georgia Coastal Plain Research Station, Tifton; C. M. E. Garrett, Institute of Horticultural Research, East Malling, Maidstone, Kent, England; D. C. Gross, Department of Plant Pathology, Washington State University, Pullman; M. J. Hattingh, Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa; P. Sobiczewski, Institute of Pomology and Floriculture, Skierniewice, Poland; and W. Zeller, Federal Biological Research Center for Agriculture and Forestry, Institute for Plant Protection in Fruit Crops, Dossenheim, Germany. The strains were maintained on King's medium B (KB) (5).

Isolation of DNA. Genomic DNA was extracted from bacteria by the miniprep method described by Wilson (11). Bacteria from saturated overnight cultures were lysed with sodium dodecyl sulfate (SDS). Proteins were digested with proteinase K. Cell debris, polysaccharides, and remaining proteins were removed by selective precipitation with hexadecyltrimethylammonium bromide (CTAB). Genomic DNA was recovered from the supernatant by isopropanol precipitation and treated with RNase A. DNA concentrations were estimated from the relative fluorescence of 4-μl aliquots compared with DNA standards that were stained with ethidium bromide. Next, 200 and 20 ng of DNA from each strain was spotted onto a nylon membrane held in a dot blot manifold.

Preparation of Southern blots. Purified DNA was digested with the restriction enzyme *Eco*RI (Boehringer Mannheim, Indianapolis, IN) and then electrophoresed in a 0.8% agarose gel in Tris-borate buffer (2). Next, the DNA was denatured and transferred to the nylon membrane using the manufacturer's directions for capillary blot procedure (NEN Research Products, E. I. du Pont de Nemours & Co., Boston, MA).

PST-DNA probe and hybridizations. The PST-DNA probe was prepared from plasmids pJCA2 and pJCA11, which contained 3.6- and 3.5-kb *Eco*RI restriction fragments of DNA, respectively, from *P. s. tomato* (3). The restriction fragments for radioisotope labeling were electroeluted onto DEAE membranes (Schleicher & Schuell, Inc., Keen, NH). The manufacturer's recommended procedures were modified by incubating the DNA with a high salt solution (20 mM Tris, pH 8.0, 0.1 mM EDTA, and 1.5 M NaCl) for 45 min at 52 C. The eluted DNA was concentrated by butanol extraction, purified by phenol extraction, precipitated with ethanol, and dissolved in TE buffer (2). The DNA was radiolabeled with ^{32}P by following the manufacturer's recommended procedures for a random priming kit (United States Biochemical Corp., Cleveland, OH). Hybridizations were performed overnight and the membranes were washed according to the manufacturer's recommended procedures. Autoradiographs of membranes were carried out with XAR X-ray film at −70 C with a Cronex Lightning Plus intensifying screen (NEN Research Products).

Sensitivity of the PST-DNA probe. Cell suspensions of *P. s. morsprunorum*

and *P. s. syringae* were adjusted turbidimetrically to OD_{620nm} = 0.15, serially diluted, and applied in aliquots of 25 µl to a nylon membrane held in a dot blot manifold. The bacteria were lysed and the DNA was bound to the membrane according to the manufacturer's directions (NEN Research Products). The concentration of bacteria applied to the membrane at each dilution was verified by plating serial dilutions of each cell suspension onto KB medium. Bacterial colonies were counted after 3 days at 22 C.

The ability of the probe to detect *P. s. morsprunorum* when mixed with *P. s. syringae* was evaluated. Strains C-17 and 101-A3 of *P. s. morsprunorum* and JP 442 and 219-05 of *P. s. syringae* were grown overnight on a shaker at 250 rpm in LB broth. Cell concentrations for each isolate were adjusted turbidimetrically to OD_{620nm} = 0.12 with LB broth. *P. s. morsprunorum* was mixed with *P. s. syringae* in ratios of 1:1, 1:2, 1:4, and 1:10 in 1.5-ml Eppendorf tubes. The bacteria were resuspended in 75 µl of sodium phosphate buffer (pH 7.2) and applied to a nylon membrane as described earlier.

Detection of bacteria in cherry tissue. Sweet cherry fruit and sour cherry leaves with bacterial canker were collected from five orchards in northern Michigan on 5 June 1990 and rinsed in sterile water. Individual lesions were excised from each of five fruit per orchard and diced with a sterile scalpel. Lesions were excised from leaves with a sterile 7-mm-diameter cork borer and the disks were cut into strips with a sterile scalpel. The bits or strips of tissue were incubated in 1 ml of 0.1 M sodium phosphate-buffered saline (pH 7.2) for 1 hr at 22 C in 1.5-ml tubes on a rotary shaker. Aliquots (20 µl) of the effluent and of a 10-fold dilution of the effluent with buffer were applied to a nylon membrane presoaked in 6× SSC (1× SSC = 0.15 M sodium chloride + 0.015 M sodium citrate) and held in a dot blot manifold. The membrane was removed from the manifold after 30 min, the cells were lysed, and the DNA was bound to the membrane according to the manufacturer's instructions. Hybridizations were performed as described earlier. Also, serial dilutions of the cell effluent were plated onto KB medium amended with 50 µg/ml of cyclohexamide (KBc) for estimation of the colony-forming units (cfu) applied to the membrane. Colony counts were recorded after 48 hr.

Colony blots of bacteria isolated from cherry tissue. Bacteria were isolated from lesions on fruit of sweet cherry and leaves of sour cherry collected from nine orchards on 7 and 12 June 1990. Actively growing colonies on KBc were transferred with a sterile toothpick to colony/plaque hybridization transfer membranes (NEN Research Products) located on the surface of KB medium. Each membrane contained colonies of strain 10l-A3 of *P. s. morsprunorum* and strain Pst84-94 of *P. s. tomato* as positive controls and strain 110-A1 of *P. s. syringae* as a negative control. The bacteria were allowed to grow for 12 hr at 20 C before the cells were lysed and the DNA was bound to the membrane according to the manufacturer's instructions. Hybridizations were performed with the PST-DNA probe as described earlier.

Identification of bacteria. Putative pseudomonads were selected randomly from dilution plates made from effluent taken from bacterial canker lesions on fruit and leaves. Up to 10 colonies were selected per orchard. The bacteria were tested for fluorescence on KB medium and for oxidase activity (6) and subjected to four determinative tests (GATTa tests) consisting of gelatin liquefaction (G), aesculin hydrolysis (A), tyrosinase activity (T), and tartrate utilization (Ta) (7). Isolates that produced variable results in the GATTa tests were tested for arbutin hydrolysis, arginine dihydrolase, fermentation of glucose, production of levan, and nitrate reduction (9). The pathogenicity of 20 representative strains characterized as *P. s. morsprunorum* and *P. s. syringae* by GATTa tests was confirmed by inoculation of immature cherry fruits; all caused typical bacterial canker lesions. In addition, the identity of all strains from geographic areas outside Michigan was verified with the oxidase and GATTa tests.

RESULTS

Sensitivity of PST-DNA probe. The PST-DNA probe detected to a limit of 2.2×10^4 cfu per dot of *P. s. morsprunorum*, but it did not hybridize with DNA extracted from strains of *P. s. syringae* applied at concentrations as high as 1.3×10^7 cfu (Fig. 1). With extracts from mixed cultures, hybridization with DNA from *P. s. morsprunorum* was not diminished until the population of *P. s. syringae* exceeded the population of *P. s. morsprunorum* by 10-fold (*data not shown*).

The specificity of the PST-DNA probe for *P. s. morsprunorum* was verified by dot blot and Southern blot hybridization assays with purified DNA (Fig. 2 and Table 1). The probe hybridized with DNA from all strains of *P. s. morsprunorum* and from the strain of *P. s. tomato* used as a control. The probe failed to hybridize with DNA from most but not all strains of *P. s. syringae*. However, hybridization with DNA from probe-positive strains of *P. s. syringae* was weak compared with the strong hybridization with DNA from strains of *P. s. morsprunorum*.

Southern blot hybridizations. When Southern blots of digested genomic DNA from one strain of *P. s. tomato* and seven strains of *P. s. morsprunorum* were

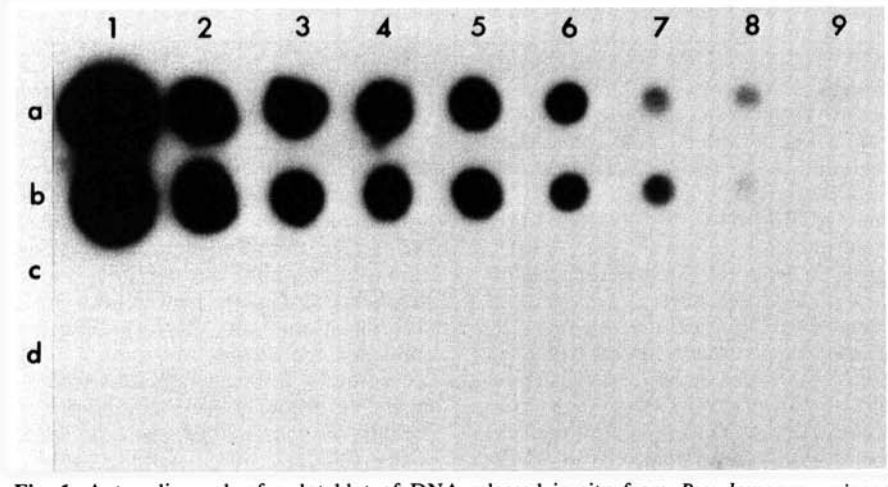

Fig. 1. Autoradiograph of a dot blot of DNA released in situ from *Pseudomonas syringae* pv. *morsprunorum* (strains C-17 and 101-A3 in rows a and b, respectively) and *P. s.* pv. *syringae* (strains JP 442 and 219-05 in rows c and d, respectively). The rows contained two-fold dilution series (1/256 endpoint) beginning with $3–4 \times 10^6$ cfu/ml.

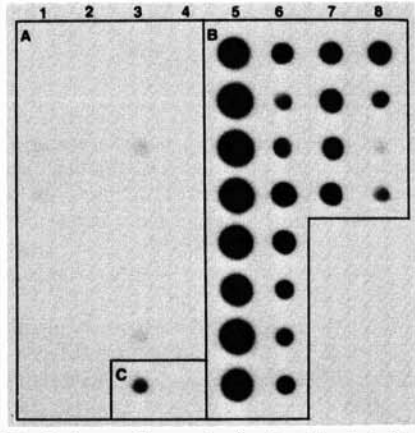

Fig. 2. Autoradiograph of a dot blot hybridization of purified DNA from **(A)** 15 strains of *Pseudomonas syringae* pv. *syringae* and **(B)** 12 strains of *P. s.* pv. *morsprunorum*. A strain of *P. s. tomato* (Pst84-94) was included as a control **(C)**. Approximately 200 ng (columns 1, 3, 5, and 7) and 20 ng (columns 2, 4, 6, and 8) of DNA were applied per strain.

probed with PST-DNA, DNA from all eight strains hybridized with the probe (Fig. 3). Among the 18 strains of *P. s. morsprunorum* tested, 14 strains shared a 3.6-kb fragment in common with strain Pst84-94 of *P. s. tomato* (Table 1). Some restriction fragment length polymorphism was observed among the seven strains of *P. s. morsprunorum* (Table 1 and Fig. 3). The probe did not hybridize or hybridized only weakly with digested DNA from the strains of *P. s. syringae* (Table 1 and Fig. 3).

Detection of *P. s. morsprunorum* in effluent from diseased tissue. Single lesions from each of 20 fruit from four sweet cherry orchards (five fruit per orchard) and five leaf lesions from one sour cherry orchard were screened with the PST-DNA probe. The probe hybridized with DNA in extracts of effluent from fruit lesions but not with DNA in effluent from leaf lesions (Table 2, orchards 306–310). Effluent from all lesions contained bacteria that were identified as *P. s. morsprunorum* based on physiological tests. Concentrations of *P. s. morsprunorum* in effluent from fruit lesions was much higher (average of 3.1×10^9 cfu/ml) than in effluent from leaves (average of 6.5×10^7 cfu/ml). Effluent from symptomless fruit and leaf tissue contained 3.9×10^2 and 1.1×10^3 cfu/ml of *Pseudomonas* spp., respectively.

Hybridization with colonies isolated from diseased tissue. When primary isolates of bacteria recovered from lesions were screened with colony blot hybridization, the PST-DNA probe hybridized with DNA from 40 of 69 colonies (Table 2, orchards 311–326). Thirty six of the probe-positive colonies, but none of the probe-negative colonies, were identified as *P. s. morsprunorum*. Four colonies were false positives—strain 317-22 from orchard 317, strains 315-31 and 315-32 from orchard 315, and strain 320-11 from orchard 320. Strain 317-12 was identified based on physiological tests as *P. s. syringae*. Strains 315-31 and 315-32 were negative for arbutin hydrolysis, arginine dihydrolase, and nitrate reduction and positive for glucose fermentation and levan formation. Strain 320-11 was negative for all tests except glucose fermentation. Thus, the false-positive strains from orchards 315 and 320 were *P. syringae*, but their physiological characteristics were intermediate between those for pathovars *morsprunorum* and *syringae*.

When a Southern blot of digested genomic DNA from each strain was probed with the PST-DNA probe, the probe hybridized strongly with a 3.6-kb fragment from strains 315-31 and 320-11 (weak hybridization) and with 3.6- and 20-kb fragments from strain 315-32 (Fig. 4). The false-positive strain 317-22, identified as *P. s. syringae*, contained a single 20-kb fragment, and two additional strains of *P. s. syringae* collected

Table 1. Probe reactions of pathovars after colony-blot hybridizations with lysed bacteria and Southern-blot hybridizations with purified DNA from cultures of *Pseudomonas syringae* pv. *morsprunorum* and *P. s.* pv. *syringae* with the radiolabeled PST-DNA probe

Pathovar[y] and strain	Host	Geographic origin	Year isolated	Probe reaction[z]	
				Colony blot	Southern blot (fragment size)
morsprunorum					
101-A3	Prune	Michigan	1988	+	20.0, 9.0, 6.0, 3.6, 2.4
102-A3	Prune	Michigan	1988	+	20.0, 9.0, 6.0, 2.4
103-A8	Prune	Michigan	1988	+	24.0, 6.0, 3.6, 2.4
106-A2	Sour cherry	Michigan	1988	+	6.0
110-B2	Prune	Michigan	1988	+	4.5
111-B4	Prune	Michigan	1988	+	6.0, 3.6
115-A1	Prune	Michigan	1988	+	3.6, 2.4
211-10	Plum	Michigan	1989	+	6.0, 3.6
212-02	Sweet cherry	Michigan	1989	+	3.6
213-04	Sour cherry	Michigan	1989	+	3.6
213-05	Sour cherry	Michigan	1989	+	3.6
218-01	Prune	Michigan	1989	+	6.0, 4.5
223-03	Plum	Michigan	1989	+	6.0, 3.6, 2.4
C-17	Cherry	England	1957	+	3.6
C-185	Cherry	England	1967	+	3.6, 2.4
P-204	Sour cherry	Poland	1978	+	3.6
627	Sweet cherry	South Africa	1982	+	3.6
634	Plum	South Africa	1982	+	3.6
syringae					
105-A1	Plum	Michigan	1988	(+)	3.6
110-A1	Prune	Michigan	1988	−	−
112-A1	Sour cherry	Michigan	1988	−	−
203-02	Sour cherry	Michigan	1989	−	−
219-05	Sour cherry	Michigan	1989	(+)	2.4
222-04	Plum	Michigan	1989	−	−
223-01	Plum	Michigan	1989	−	−
W4N9	Sweet cherry	Washington	1980	−	−
W4N43	Apple	Washington	1981	−	−
W4N101	Pear	Washington	1981	−	−
W4N103	Sweet cherry	Washington	1982	−	−
W4N108	Sweet cherry	Washington	1982	−	−
1835	Sour cherry	Poland	1977	−	−
2905	Sour cherry	Poland	1977	−	−
9	Sour cherry	Poland	1976	−	−
Pss 9	Pear	Switzerland	···	−	−
Pss 10	Sour cherry	Germany	···	(+)	5.6, 4.0
S 150	Cherry	England	1978	(+)	9.4, 1.7
JP 442	Plum	England	1981	−	−
724	Plum	South Africa	1981	−	−
tomato					
Pst84-94	Tomato	Georgia	1984	+	3.6

[y] Pathovar identity was determined by standard biochemical and physiological tests (GATTa) for gelatin liquefaction (G), aesculin hydrolysis (A), tyrosinase activity (T), and tartrate utilization (Ta) (7).

[z] + = Hybridization, (+) = weak hybridization, and − = no hybridization with the PST-DNA probe. Fragment sizes are in kilobase (kb).

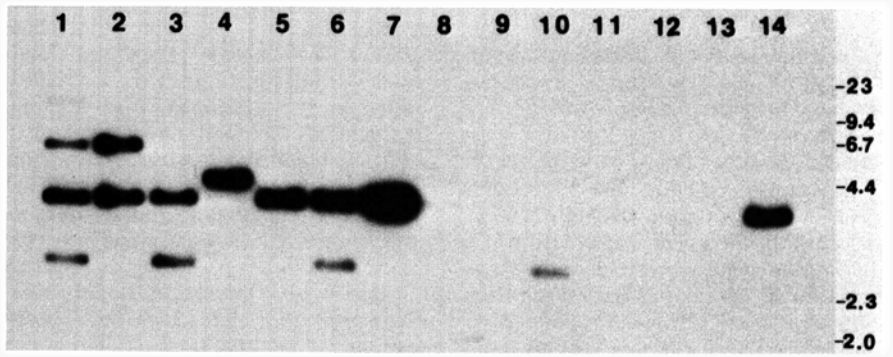

Fig. 3. Autoradiograph of a Southern blot of total DNA of *Pseudomonas syringae* pv. *morsprunorum* (lanes 1–7), *P. s.* pv. *syringae* (lanes 8–13), and *P. s.* pv. *tomato* (lane 14) digested with *Eco*RI and hybridized to the PST-DNA probe. Strains contained in the lanes include: 1, 101-A3; 2, 211-10; 3, 115-A1; 4, 110-B2; 5, C-17; 6, C-185; 7, P-204; 8, 105-A1; 9, 110-A1; 10, 219-05; 11, JP 442; 12, 2905; 13, 1835; and 14, Pst84-94. The sizes (in kb) of λDNA digested with *Hind*III are given at the left.

Table 2. Detection of *Pseudomonas syringae* pv. *morsprunorum* (Psm) or *P. s.* pv. *syringae* (Pss) by the hybridization of a PST-DNA probe with DNA extracted from bacterial canker lesions or released in situ from bacterial colonies isolated from lesions

Orchard[v]	Lesions or colonies (no.)	Probe reaction[w]	Bacterial population (log cfu/lesion)	Species present[x]			Agreement[y] (%)
				Psm	Pss	Other	
306	5	5+/0−	9.50	5	0	0	100
307	5	5+/0−	9.57	5	0	0	100
308	5[z]	0+/5−	7.81	5	0	0	0
309	5	5+/0−	9.47	5	0	0	100
310	5	5+/0−	9.45	5	0	0	100
311	10	10+/0−	···	10	0	0	100
312	10[z]	10+/0−	···	10	0	0	100
313	10	10+/0−	···	10	0	0	100
314	10	6+/4−	···	6	2	2	100
315	6	2+/4−	···	0	4	2	77
316	1	0+/1−	···	0	1	0	100
317	10	1+/9−	···	0	10	0	90
320	6	1+/5−	···	0	2	4	83
326	6	0+/6−	···	0	6	0	100

[v] For orchards 306–310, DNA was detected in effluent from bacterial canker lesions; for orchards 311–326, DNA was detected in bacterial colonies isolated from effluent.

[w] Hybridization (+) or no hybridization (−) of the PST-DNA probe with DNA of bacteria in effluent from lesions or from colonies isolated from lesions.

[x] Species designation determined by standard biochemical and physiological tests (GATTa) conducted on individual colonies for gelatin liquefaction (G), aesculin hydrolysis (A), tyrosinase activity (T), and tartrate utilization (Ta) (7).

[y] Percentage of the number of lesions or colonies with DNA hybridizing with the probe divided by the number of samples found to contain Psm based on the GATTa tests.

[z] Bacteria were from lesions on leaves of sour cherry; all other bacteria were from lesions on fruit of sweet cherry.

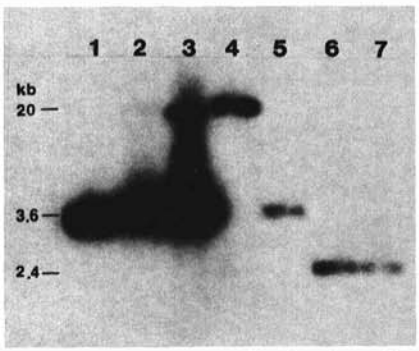

Fig. 4. Autoradiograph of a Southern blot of total genomic DNA of *Pseudomonas syringae* pv. *tomato* (lane 1), intermediates of *P. syringae* (lanes 2–5), and *P. s.* pv. *syringae* (lanes 6–7) digested with *Eco*RI and hybridized to the PST-DNA probe. Strains contained in the lanes include: 1, Pst84-94; 2, 315-31; 3, 315-32; 4, 317-22; 5, 320-11; 6, 222-04; and 7, 33A2-86.

from symptomless blossoms contained a 2.4 kb-fragment that hybridized with the probe.

DISCUSSION

We confirmed that the PST-DNA probe hybridizes with DNA from *P. s. morsprunorum* as reported by Denny (3). In addition, it hybridized with DNA from all strains of *P. s. morsprunorum* obtained from lesions or isolated as epiphytes from cherries, plums, and prunes in Michigan and with DNA of *P. s. morsprunorum* originally isolated from stone fruit crops in Poland, England, and South Africa. The PST-DNA probe proved to be an effective tool for detecting *P. s. morsprunorum* in the presence of *P. s. syringae*. The probe enabled us to inform extension agents and farm advisers within 48 hr after collecting samples that *P. s. morsprunorum* was the primary pathovar involved in an epidemic of fruit spotting on sweet cherries in 1990.

Although the probe was not specific for *P. s. morsprunorum* (3), its reaction with DNA from other pathovars of *P. syringae* or from saprophytic pseudomonads was not a serious deficiency. Pseudomonads with biochemical and physiological characters intermediate between those for *P. s. morsprunorum* and *P. s. syringae* are commonly found in stone fruit crops (7,8). Three of these intermediate-type strains contained DNA that hybridized with the PST-DNA probe. In a Southern blot analysis, these strains produced hybridization patterns similar to strains identified as *P. s. morsprunorum*. Also, one colony that hybridized with the probe was identified as *P. s. syringae*. However, this colony may have contained a mixture of *P. s. syringae* and *P. s. morsprunorum*. Because the PST-DNA probe is not specific for pathovar *morsprunorum*, one should check occasionally for the presence of other pathovars.

The probe failed to detect *P. s. morsprunorum* eluted from leaf lesions despite the fact that populations applied to nylon membranes were greater than those from pure cultures normally detected by the probe. Leaf debris may have interfered with deposition of the DNA on the membrane (3). This problem did not occur when *P. s. morsprunorum* was taken from fruit lesions because populations were about 100-fold larger, which is characteristic for bacteria in fruit lesions early in the growing season (A. L. Jones, *unpublished*). As noted by Denny (3) and Cuppels et al (1), the effectiveness of DNA probes can be improved through an awareness of the effect of lesion age on the population dynamics of cells within lesions. Also, with older lesions, miscellaneous bacterial opportunistic colonizers may overgrow pathogen populations later in the season or during periods of wet weather, and some of these bacteria may interfere with detection. The probe works well for detecting colonies of *P. s. morsprunorum*, particularly if bacteria are grown on membranes before the test. This method would allow one to study the epidemiology of *P. s. morsprunorum* in the presence of *P. s. syringae*.

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