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Diversity in plasmid DNA content of two pathovars of Pseudomonas syringae and detection of Pseudomonas syringae pv. morsprunorum with a DNA probe.

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Major professor

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DIVERSITY IN PLASMID DNA CONTENT OF TWO PATHOVARS OF *PSEUDOMONAS SYRINGAE* AND DETECTION OF *PSEUDOMONAS SYRINGAE* PV. *MORSPRUNORUM* ON CHERRIES WITH A DNA PROBE

By

James M. Paterson

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ABSTRACT

DIVERSITY IN PLASMID DNA CONTENT OF TWO PATHOVARS OF *PSEUDOMONAS SYRINGAE* AND DETECTION OF *PSEUDOMONAS SYRINGAE* PV. *MORSPRUNORUM* ON CHERRIES WITH A DNA PROBE

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Forty five isolates of *Pseudomonas syringae* pv. *morsprunorum* (Psm) and *Pseudomonas syringae* pv. *syringae* (Pss) were examined for plasmid DNA content and restriction fragment length polymorphism of the plasmid DNA. All strains of Psm harbored three to seven plasmids. Only seven of 22 strains of Pss harbored one to two plasmids. *Eco*RI restriction enzyme digests of plasmid DNA of isolates of Pss produced fewer bands in agarose gels than did digested plasmid DNA from isolates of Psm.

Two genomic DNA fragments cloned in plasmids pJCA2 and pJCA11 were combined and referred to as probe PST-DNA. The PST-DNA probe, developed for differentiating *P. s.* pv. *tomato* from Pss, was tested as a possible diagnostic probe for Psm. DNA from bacterial cells in effluent from fruit lesions was readily detected. DNA from bacterial cells in effluent from leaf lesions was not detected by the probe. There was good agreement between results obtained with the probe and those obtained with standard biochemical and physiological tests.

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PART II

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Bacterial canker is an important economical disease affecting stone fruit crop production through out the world (2,7,8,10). The disease has been referred to by several names due to the pathogens ability to infect several stone fruit trees. Blossom blast, dead bud, gummosis, die-back, and bacteriosis are names that have been used to refer to this disease. However, bacterial canker was recommended by Crosse (4) as the common name for this disease since it leaves little doubt as to the cause of the most severe symptom.

The disease is cyclic with a winter phase associated with cankers in the bark of stems and branches and a summer phase associated with spots on leaves, fruits and other green tissues (4). Canker development results from infection through blossoms that moves systemically into fruiting spurs and branches. The pathogen overwinters in the woody tissue and in early spring the bacteria multiply and spread (4,8). On leaves, symptoms first appear as water-soaked spots that progress into necrotic lesions which may drop out resulting in a tattered appearance (9). Blossom tissues may also become infected resulting in blighting and death of the

blossoms. Fruit lesions appear as irregular, dark brown spots on the side or calyx end of the fruit. Lesions are often water-soaked at their margin in the early stages of infection (9).

Pseudomonas syringae pv. morsprunorum (Wormald) Young et al and P. s. pv. syringae van Hall are capable of inciting bacterial canker. The first report of a bacterial pathogen causing cankers and gumming on fruit trees was by van Hall (13) in Germany in 1902. In England, the cause of bacterial canker was attributed to the pathogen P. mors-prunorum by Wormald (15) in 1932. The pathogen was found to occur on plum, cherry, and other stone fruit crops. Wormald also described a second bacterium as the cause of bacterial canker of stone fruit which he named P. prunicola (15). The designation P. prunicola was changed to P. syringae by Wilson (14) in 1936. P. syringae encompasses a diverse group of pathogens related to a pathogen originally isolated from lilac.

In 1980, Young et al (16) proposed the use of the term pathovar to designate strains of P. syringae which were not adequately described based on physiological characteristics. Without the pathovar designation, pseudomonad bacteria pathogenic on different crops would be lumped into P. syringae by the International Committee on Systematic Bacteriology (ICSB). Currently, strains of P. syringae and P. mors-prunorum from fruit crops are recognized as P. s. pv. syringae and P. s. pv. morsprunorum, respectively.

Both pathovars are capable of infecting stone fruit crops (4,8,9,10), however P.s. pv. morsprunorum is the pathovar most commonly associated with outbursts of bacterial canker in Michigan. P. s. pv. morsprunorum may be differentiated from P. s. pv. syringae by differentiated by physiologic and biochemical tests (10). Both pathovars are capable of existing as epiphytes on the leaf surface during the summer (4,10). P. s. pv. morsprunorum exists in the epiphytic flora throughout the summer in England, United States (MI), and South Africa(4,11,8).

Control of bacterial canker is difficult, no one method is suitable for complete control. Uninfested budwood should be used for propagation material. Chemical control of the canker phase of the disease involves spraying with copper or Bordeaux mixtures in the fall to prevent infection through leaf scars and in the spring before blossoms occur to slow the spread of the epidemic (1). Spraying of the leaves to prevent spread of the pathogen from leaf lesions had no significant effect on the incidence of cankers on plum or cherry (4).

Previous experiments have established the presence of epiphytic populations of P. s. pv. morsprunorum and P. s. pv. syringae on sweet cherry (Prunus avium L.) and sour cherry (P. cerasus L.) in Michigan (10,12). Further to this, isolates of P. s. pv syringae but not P. s. pv. morsprunorum were found to contain plasmids which encoded resistance to copper. Attempts to transfer copper

resistance between P. s. pv. syringae and P. s. pv. morsprunorum by conjugation were unsuccessful and further support the taxonomic separation of the two pathogens. Numerous physiologic and biochemical tests have been used to identify and and differentiate P. syringae pathovars associated with stone fruits (4,10). These tests are useful but require up to 2 weeks after pure culturing isolates and may need retesting due to ambiguous results. Serologic investigations were specific for identification at the species level only (11). Bacteriophages have been used but could not distinguish between pseudomonad pathogens and saprophytes (5).

The purpose of this research was to evaluate if the presence of plasmid DNA in P. s. pv. morsprunorum and P. s. pv. syringae was suitable for differentiating isolates in Michigan stone fruit orchards. A DNA probe was also used to detect P. s. pv. morsprunorum, the most common incitant of bacterial canker in Michigan and differentiate it from P. s. pv. syringae.

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PART I

DETECTION OF *PSEUDOMONAS SYRINGAE* PV. *MORSPRUNORUM* ON CHERRY WITH A DNA HYBRIDIZATION PROBE

ABSTRACT

Pseudomonas syringae pv. *morsprunorum* (Psm), the most common cause of bacterial canker of sweet and sour cherry in Michigan, is often confused with *P. s.* pv. *syringae* (Pss), a second causal agent for bacterial canker. Two genomic DNA fragments cloned in plasmids pJCA2 and pJCA11 were combined and referred to as probe PST-DNA. The PST-DNA probe, developed for differentiating *P. s.* pv. *tomato* from Pss, was tested as a possible diagnostic probe for Psm. Purified DNA and DNA from colony blots of 18 strains of Psm, including strains from England, Poland, South Africa, and the United States, hybridized to the radiolabeled probe, while 19 of 20 strains of Pss isolated from deciduous tree fruit crops did not hybridize or weakly hybridized to the probe. The detection limit was approximately 1.1×10^4 colony forming units per milliliter. DNA from bacterial cells of Psm in effluent from lesions on fruit readily hybridized to the probe. DNA from bacterial cells in effluent from leaf lesions was not detected by the probe, possibly because the number of bacteria in the effluent was 100-fold lower for leaves than for fruit. In testing field samples, there was good agreement between identifications made with the probe and those made with standard biochemical and physiological

techniques. EcoRI fragments of Psm DNA exhibited considerable 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 Psm and assist in the conducting of epidemiological studies of the pathogen on cherry.

INTRODUCTION

Pseudomonas syringae pv. morsprunorum (Psm) is the most frequent cause of leaf spots and bacterial fruit rot (bacterial canker) of sour cherry and sweet cherry and of leaf spots on prunes in Michigan. This bacterium is also a common epiphyte on blossoms and leaves of these crops (4) and an occasional endophyte in dormant buds (11). Although P. s. pv. syringae (Pss) occasionally incites similar symptoms on the leaves and fruit of sweet cherry in Michigan (8), it is more commonly found as an epiphyte on blossoms and leaves of all three of these fruit crops. It is also an occasional endophyte in dormant buds (11).

The differentiation of Psm from Pss normally requires that the bacteria be isolated, purified, and characterized in a series of biochemical, physiological, and pathogenicity tests. These tests require about 2 wk to complete and they may need to be repeated due to ambiguous results in one or more test. Epidemiological studies on these pathogens are frequently limited in scope because of the time and effort required to confirm the identification of large numbers of

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

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 as described by Sundin et al (10). Those from other geographical areas were obtained under permit from colleagues in various institutions around the world. These sources include the following: 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

Table 1. Bacterial strains used in the present study and a summary of results from colony blot hybridizations with lysed bacteria and from Southern blot hybridizations with purified DNA from cultures of each strain with the radiolabeled PST-DNA probe

Species and strain	Host	Geographic origin	Year isolated	Probe reaction[b]	
				Colony blot	Southern blot (fragment size)
Strains of *Pseudomonas syringae* pv. *morsprunorum*[a]					
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

Table 1. (cont'd)

Strains of P. s. pv. syringae[a]					
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	+	3.6, 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	-	-
No. 1835	Sour cherry	Poland	1977	-	-
No. 2905	Sour cherry	Poland	1977	-	-
No.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	-	-
Strain of P. s. pv. tomato					
Pst84-94	Tomato	Georgia	1984	+	3.6

[a]Pathovar identity was determined using GATTa tests (7)
[b]+ = hybridization with the PST-DNA probe; - = no hybridization with the probe. Fragment sizes are in kilobases (kb).

maintained on medium B of King et al (KB) (5).

PST-DNA probe. The PST-DNA probe was supplied by T. P. Denny, Department of Plant Pathology, University of Georgia, Athens, and consisted of two EcoRI restriction fragments of genomic DNA from P. s. pv. tomato (Pst) (3).

Isolation of DNA. Genomic DNA was extracted from bacteria by the miniprep method described by Wilson (11). Cultures were grown overnight in 5 ml of Luria-Bertani (LB) broth (2) on a rotary shaker at 250 rpm. Bacteria from 1.5 ml of each culture were lysed with a solution of sodium dodecyl sulfate (SDS) and proteinase K (final concentration of 100 µg/ml proteinase K in 0.5% SDS). Cell debris, polysaccharides, and remaining proteins were removed by selective precipitation with a solution of CTAB/NaCl (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) followed by phenol/chloroform extractions. The DNA was recovered by isopropanol precipitation. After the isolated DNA was treated with RNAase A, it was spotted onto a nylon membrane (NEN Products, du Pont de Nemours & Co., Boston, MA) held in a dot blot manifold according to manufacturer's directions. The DNA was applied to the membrane under slight suction for 1 min and then left for 30 min without suction. Suction was applied for 1 min before the membrane was removed and allowed to dry at room temperature. DNA concentrations were estimated prior to spotting onto membranes by staining 4 µl

aliquots of each DNA solution with ethidium bromide and comparing their relative fluorescence with known DNA standards.

Preparation of Southern blots. Purified DNA was digested with the restriction enzyme EcoRI (Boehringer Mannheim, Indianapolis, IN). Following electrophoresis in a 0.8% agarose gel in Tris-borate buffer (TBE), the DNA was denatured and transferred to the nylon membrane using the manufacturer's directions for capillary blot procedure (NEN Products, du Pont de Nemours & Co. Boston, MA).

Preparation of PST-DNA probes and hybridization. Two plasmids, pJCA2 and pJCA11, containing the 3.6 and 3.5 kb EcoRI restriction fragments of P. s. pv. tomato DNA, were combined and radiolabeled as described by Denny (3). Ligated DNA was used to transform E. coli strain DH5 alpha and bacteria were plated on LB medium amended with 100 µg/ml ampicillin. Plasmid DNA was isolated from transformants by alkaline lysis (2), digested with EcoRI, separated by gel electrophoresis, and electroeluted onto DEAE membranes (Schleicher & Schuell, Inc., Keen, NH) according to manufacturer's recommended procedures. The DNA was radiolabeled with ^{32}P using a Random Priming Kit (United States Biochemical Corp., Cleveland, OH) according to manufacturer's recommended procedures. Hybridizations were performed overnight and the membranes washed according to 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 Products, du Pont de Numours & Co., Boston, MA).

Sensitivity of the PST-DNA probe. To evaluate the sensitivity of the probe, cultures of Psm and Pss were grown overnight in LB broth shaken at 250 rpm. Cell concentrations were adjusted turbidimetrically to OD_{620} = 0.15, serially diluted (1/256 endpoint) with 0.1 M sodium phosphate buffer (pH 7.2), and applied in aliquots of 25 µl to a nylon membrane held in a dot blot manifold. The bacteria were lysed and the DNA bound to the membrane according to manufacturer's directions (NEN Products, du Pont de Nemours & Co. Boston, MA). To verify the concentration of bacteria applied to the membrane at each dilution, the remaining portion of each cell suspension was serially diluted and plated onto KB medium. Bacterial colonies were counted after 3 days at 22 C. To evaluate the ability of the probe to detect Psm when mixed with Pss, strains C-17 and 101-A3 of Psm and strains JP 442 and 219-05 of Pss were grown overnight at 250 rpm in LB broth. Cell concentrations for each isolate were adjusted turbidimetrically to OD_{620} = 0.12 with LB broth. Psm was mixed with Pss in ratios of 1:1, 1:2, 1:4, and 1:10 in 1.5 ml Eppendorf tubes. Each mixture was obtained by adding successive 1 ml aliquots from stock solutions and pelleting the bacteria in a microcentrifuge. The bacteria were resuspended in 75 µl sodium phosphate

buffer (pH 7.2) and applied to a nylon membrane as described above. After drying the membrane at room temperature, the bacteria were lysed and the DNA bound to the membrane.

Detection of bacteria in cherry tissue. Sweet cherry fruit and sour cherry leaves with bacterial canker lesions 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 macerated with a sterile scalpel. Lesions were excised from leaves with a sterile 7-mm-diameter cork borer and the disks were macerated with a sterile scalpel. The mascerated tissues were incubated in 1 ml of 0.1 M sodium phosphate buffered saline (pH 7.2) for 1 hr in 1.5 ml tubes on a rotary shaker. Aliquots (20 µl) of the cell effluent and of a 10-fold dilution in buffer were applied onto a nylon membrane presoaked in 6X SSC (1X SSC: 0.15 M sodium chloride - 0.015 M sodium citrate) and held in a dot blot manifold. The membrane was removed from the apparatus after 30 min and the DNA released from the cell and bound to the membrane according to manufacturer's instructions. Hybridization was performed as described above. Also, serial dilutions of the cell effluent were plated onto KB medium amended with 50 µg/ml cyclohexamide (KBc) to estimate the number of 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 diseased lesions on fruit and leaves collected from nine orchards on 7 and 12 June 1990. Actively growing colonies on isolation plates of KBc were transferred with a sterile toothpick onto Colony/Plaque Hybridization Transfer Membranes (NEN Products, du Pont de Nemours & Co., Boston, MA) located on the surface of KB medium. Each membrane contained colonies of Psm 101-A3 and Pst84-94 as positive controls and Pss 110-A1 as a negative control. The bacteria were allowed to grow for 12 hr at 20 C before cell lysis and release of DNA to the membrane surface, according to manufacturer's instructions. Hybridizations were performed with the PST-DNA probe as described above.

Identification of bacteria. Colonies exhibiting a morphology typical of 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), then subjected to four determinative tests (GATTa tests) consisting of: gelatin liquefaction (G), aesculin hydrolysis (A), tyrosinase activity (T), and tartrate utilization (Ta) as described by Latorre and Jones (7). Isolates that produced variable results in the GATTa tests were tested further. The additional tests included: arbutin hydrolysis,

arginine dihydrolase, fermentation of glucose and levan, and nitrate reduction according to procedures described by Schaad (9). The pathogenicity of 20 representative strains characterized as Psm and Pss by GATTa tests was determined by inoculation of immature cherry fruits and all caused typical bacterial canker lesions. In addition, all strains from geographic areas outside Michigan in Table 1 were subjected to the oxidase and GATTa tests to verify their identity.

RESULTS

Sensitivity of PST-DNA Probe. The sensitivity of the PST-DNA probe was determined by applying a series of two-fold dilutions of cell suspensions of Psm and of Pss to a nylon membrane in a dot blot manifold (Fig. 1). The probe detected down to 1.1×10^4 cfu per dot of Psm (limit of detection) but it did not hybridize to DNA from strains of Pss applied at concentrations as high as 1.3×10^7 cfu per dot. To examine the sensitivity of the probe in the presence of non-homologous DNA, 1×10^7 cfu of Psm were mixed with increasing concentrations of Pss before the bacteria were applied to the filter. The hybridization signal was not diminished until the concentration of Pss was increased to about 10 times the concentration of Psm (photo not shown). The probe failed to hybridize to Pss in the absence of Psm regardless of the concentration of Pss.

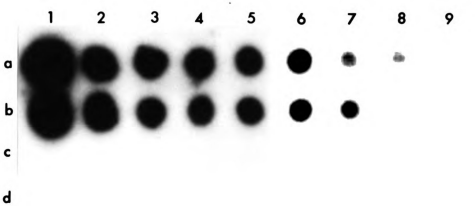

Fig. 1. Autoradiograph of a dot blot of DNA released *in situ* from bacterial cells of *Pseudomonas syringae* pv. *morsprunorum* (rows a and b) and *P. s.* pv. *syringae* (rows c and d). The rows contained two-fold dilution series (1/256 endpoint) of cells from the following strains: row a, C 17 (3.0×10^6 to 1.1×10^4 cfu); row b, 101-A 3 (3.2×10^6 to 1.2×10^4 cfu); row c, JP 442 (3.3×10^6 to 2.5×10^4 cfu); row d, 219-05 (3.8×10^6 to 1.5×10^4 cfu).

Specificity of the PST-DNA probe with purified DNA. The specificity of the PST-DNA probe for detecting P. s. pv. morsprunorum was verified by dot blot and Southern blot hybridization assays with purified DNA (Table 1). A representative dot blot containing 200 and 20 ng of DNA from each strain is shown in Figure 2. The strains of Psm and Pss selected for this experiment were from diverse geographic regions. They were all isolated from stone fruit crops except three strains of Pss were isolated from apple and pear. The probe hybridized with all strains of Psm and the strain of Pst used as a control (Table 1, Fig. 2). The probe failed to hybridize with most but not all strains of Pss. However, the signals from the probe-positive strains of Pss were weak compared to the strong signals from strains of Psm. When the concentration of DNA was reduced from 200 to 20 ng, only strain 219-5 of Pss was detected (Fig. 2).

Southern blot hybridizations. When Southern blots of digested genomic DNA from one strain of Pst and seven strains of Psm was probed with PST-DNA, DNA from all eight strains hybridized with the probe (Fig. 3). Among the 18 strains of Psm tested, 14 strains shared a 3.6 kb fragment in common with strain Pst84-94 of Pst (Table 1). Some restriction fragment length polymorphisms were observed among the seven strains of Psm (Table 1, Fig. 3). Except for strains 219-05, the probe did not hybridize or hybridized weakly with the strains of Pss (Fig. 3, Table 1).

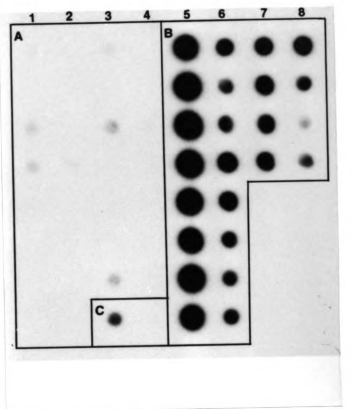

Fig. 2. Autoradiograph of a dot blot hybridization of purified bacteria DNA from 15 strains of Pseudomonas syringae pv. syringae (area A) and 12 strains of P. s. pv. morsprunorum (area B). Pst control was included (area C). Approximately 200 ng (columns 1, 3, 5, and 7) and 20 ng (columns 2, 4, 6, and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

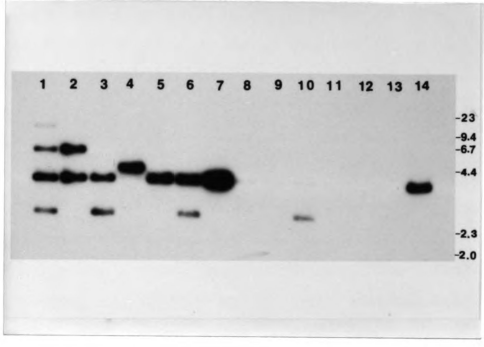

Fig. 3. Autoradiograph of a Southern blot of total DNA of Pseudomonas syringae pv. morsprunorum (lanes 1-7), P. s. pv. syringae (lanes 8-13) and P. s. pv. tomato (lane 14) digested with EcoRI and hybridized to the PST-DNA probe. Lanes contained: 1, 101-A3; 2, 211-10; 3, 115-A1; 4, 110-B2; 5, C-17; 6, C-185; 7, P-204; 8, 105-A1; 9, 110-A1; 10, 219-05; 11, JP 442; 12, No. 2905; 13, No. 1835; 14, Pst84-94. The sizes (in kb) of lambda DNA digested with HindIII are given at the right.

The probe hybridized with a 2.4 kb restriction fragment of Pss strain 219-05 and of Psm strains 101-A3, 115-A1, and C-185.

Detection of P. s. pv. 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. Effluent from all lesions contained bacteria identified as Psm (Table 2). Concentrations of Psm 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). The effluent from symptomless fruit and leaf tissue contained 3.9×10^2 and 1.1×10^3 cfu/ml of Pseudomonas spp., respectively. The probe hybridized with effluent from all 20 fruit but not with effluent from leaves (Table 2). It also hybridized with DNA from cells of Psm (6.5×10^5 cfu/ml) and Pst (2.0×10^6 cfu/ml), but not with DNA from cells of Pss (9.5×10^5 cfu/ml) nor with effluent from symptomless fruit and leaves (Fig. 4).

Hybridization with colonies isolated from diseased tissue. When primary isolates of bacteria recovered from disease lesions were screened using colony blot hybridization, the PST-DNA probe hybridized with DNA from 40 of 69 colonies (Table 2, Fig. 5). Thirty six of the probe-positive colonies but none of the probe-negative colonies contained bacteria identified as Psm (Table 2, Fig. 5). Among four

Table 2. Comparison of results from hybridizations with the PST-DNA probe of DNA extracted from bacterial canker lesions or released *in situ* from bacterial colonies isolated from lesions with the results of conventional isolation and identification of the bacteria based on physiological tests

Orchard (code no.)	Lesions or colonies (no.)	Probe reaction[a]	Bacterial population (log (cfu/fruit))	Species present[b]			Agreement (%)[c]
				Psm	Pss	Other	
DNA detected in cell effluent from bacteria canker lesions							
306	5	$5^+/0^-$	9.50	5	0	0	100
307	5	$5^+/0^-$	9.57	5	0	0	100
308	5[d]	$0^+/5^-$	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
DNA detected in bacterial colonies isolated from lesion effluent							
311	10	$10^+/0^-$	-	10	0	0	100
312	10[d]	$10^+/0^-$	-	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

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

[b]Species designation determined by GATTa tests (7) conducted on individual colonies. Psm = *Pseudomonas syringae* pv. *morsprunorum*, Pss = *P. s.* pv. *syringae*.

Table 2. (cont'd)

[c]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 standard biochemical and physiological tests (7).
[d]Bacteria were from lesions on sour cherry leaves, all other bacteria were from lesions on sweet cherry fruit.

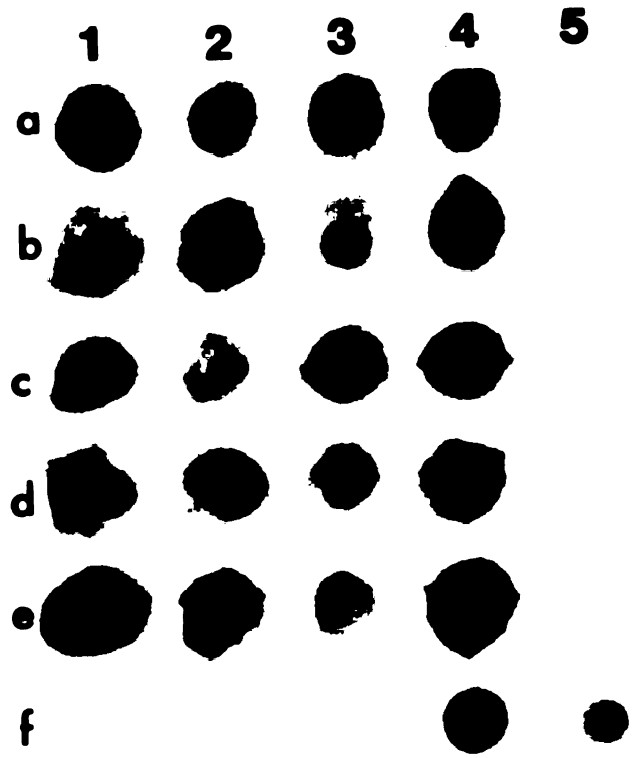

Fig. 4. Autoradiograph of dot blot of DNA released *in situ* from bacterial cells recovered from diseased fruit of sweet cherry and leaf samples of sour cherry. Twenty ul aliquots from 1 ml of lesion effluent and a 10-fold dilution were applied to the membrane and hybridized to the PST-DNA probe. Columns 1-4 contain DNA from fruit lesions collected in four orchards, column 5 contains DNA from leaf lesions. Each column contains five subsamples (row a-e) from a single location. Row f contains controls consisting of cell effluent from; 1, healthy fruit tissue; 2, healthy leaf tissue; and cell suspensions of; 3, *Pseudomonas syringae* pv. *syringae*; 4, *P. s.* pv. *morsprunorum*; 5, *P. s.* pv. *tomato*.

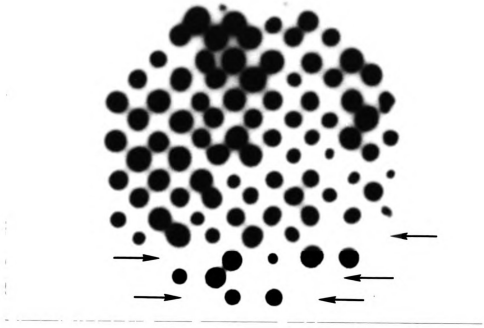

Fig. 5 Representative autoradiograph of a colony blot of DNA released *in situ* from bacterial cells isolated from lesion effluent. Ninety seven colonies were applied to the membrane and probed with the PST-DNA probe. All colonies hybridizing with the probe were identified as *Pseudomonas syringae* pv. *morsprunorum* by GATTa tests (7). The arrows indicate regions containing DNA from nine colonies that failed to hybridize with the probe and were identified later as *P. s.* pv. *syringae* or other species by the GATTa tests.

colonies that were false positives, two colonies from orchard 315 and one colony from 320 contained bacteria that differed from Psm or Pss in one or more of the GATTa tests. The bacteria in the colony obtained from orchard 317 was identified as Pss.

Additional biochemical tests on the two strains from orchard 315 were negative for arbutin hydrolysis, arginine dihydrolase, and nitrate reduction and positive for glucose fermentation and levan formation. The strain from orchard 320 were negative for all tests except glucose fermentation. These tests indicate false positive strains from orchard 315 and 320 were P. syringae but there physiologic characteristics were intermediate between those for Psm and Pss. 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 in strain 315-31 and 320-11 and to a 3.6 and 20 kb 20 kb fragment in strain 315-32 (Fig. 6). The false positive strain 317-22, identified as Pss contained a single 20 kb fragment which hybridized with the probe.

DISCUSSION

We confirmed that the PST-DNA probe hybridizes with DNA from Psm as reported by Denny (3). In addition, it hybridized with all strains of Psm obtained from lesions or isolated as epiphytes from cherries, plums, and prunes in Michigan. It also hybridized with strains of Psm obtained

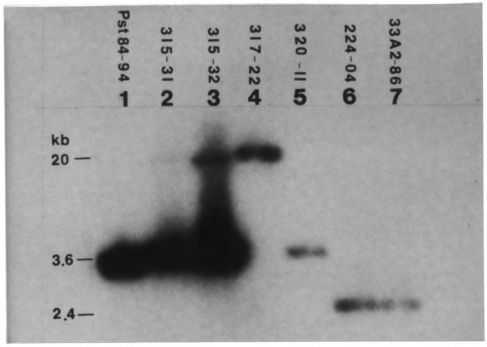

Fig. 6 Autoradiograph of a Southern blot of total genomic DNA of Pseudomonas syringae pv. tomato (lane 1), intermediates of P. syringae (lanes 2-5), and P. s. pv. syringae (lanes 6-7) digested with EcoRI and hybridized to the PST-DNA probe. Lanes contained: 1, Pst84-94; 2, 315-31; 3, 315-32; 4, 317-22;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

from stone fruit crops in Poland, England, and South Africa. The PST-DNA probe proved to be an effective tool for identifying Psm in the presence of Pss. The probe enabled us to inform extension agents and farm advisors within 48 hr after collecting samples that Psm was the primary pathovar involved in an epidemic of fruit spotting on sweet cherries in 1990.

Although the probe was not specific for Psm (3), its recognition of other pathovars of P. syringae or of saprophytic pseudomonads was not a serious problem in this study. When characterizing pseudomonads from stone fruit crops by standard diagnostic methods, it is common to detect strains with biochemical and physiological characters intermediate between those for Psm and Pss (7,8). Four of these intermediate-type strains hybridized to the PST-DNA probe. Further evaluation of these strains with a Southern blot revealed hybridization patterns similar to strains identified as Psm. Also, one colony that hybridized with the probe contained bacteria identified as Pss. However, the possibility that this colony contained a mixture of Pss and Psm cannot be ruled out. As the PST-DNA probe is not unique to Psm, it is important when using this probe to check occasionally for the possible detection of other pathovars.

The probe failed to detect Psm taken from leaf lesions despite the fact that numbers of Psm applied to nylon membranes were greater than the concentration of cells from pure cultures normally detected by the probe. It is

possible that leaf debris interfered with deposition of the DNA on the membrane (3). This problem did not occur when Psm were taken from fruit lesions because of the high population of bacteria in fruit lesions early in the growing season. As noted recently by 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, the possibility of overgrowth of older lesions with miscellaneous bacterial opportunistic colonizers later in the season and during periods of wet weather interfere with detection. As the probe works well for detecting colonies of Psm, it should prove highly effective for screening bacteria that are increased on membranes before probing. This would provide a method for selectively studying the epidemiology of Psm in the presence of Pss.

Fragment sizes of Psm hybridizing with the PST-DNA probe included 24 and 2.4 kb fragments in addition to the 20, 9, 6, and 3.5 kb fragments of Pst reported by Denny (3). This observation is consistent with the hypothesis that the greater amount of polymorphism in Psm is due to the phylogenetic distance between Psm and Pst.

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PART II

DIVERSITY IN PLASMID DNA CONTENT OF TWO PATHOVARS OF *PSEUDOMONAS SYRINGAE* FROM STONE FRUIT IN MICHIGAN

ABSTRACT

A total of 45 isolates of *Pseudomonas syringae* pv. *morsprunorum* (Psm) and *P. s.* pv. *syringae* (Pss) were examined for plasmid DNA content and restriction fragment length polymorphism of the plasmid DNA. Most of the strains were from Michigan, but representative strains from other states and countries were included for comparison. All strains of Psm regardless of geographic origin harbored three to seven plasmids and the size of the plasmids differed widely from strain to strain. Only two of 22 strains of Pss harbored one to two plasmids. Each of these strains contained a different size plasmid. In Southern blots, DMA from all isolates of Psm hybridized with a DNA probe from *P. s.* pv. *tomato* but DNA from only one strain of Pss, S-150 from England, hybridized with this probe. Numerous restriction fragments (12-17 fragments) were produced in *Eco*RI restriction enzyme digests of DNA from Psm compared to few fragments (5-6 fragments) in digests of DNA from Pss. All strains showed different restriction enzyme digest patterns.

INTRODUCTION

Pseudomonas syringae pv. morsprunorum (Psm) is the predominant causal organism of bacterial canker on stone fruit crops in Michigan (10). The disease affects primarily sweet cherry but sour cherry and prune may also be infected. P. s. pv. syringae (Pss) also is capable of inciting the disease on sweet cherry and is a common epiphyte on sweet and sour cherry and prune. Both pathogens produce similar symptoms. Symptoms of the disease, particularly on sour cherry appear on the leaves as water-soaked lesions which become necrotic and result in premature leaf drop. The immature fruits of cherry also may be infected resulting in premature ripening and abscission of the fruit. Canker formation occurs with the spread of the pathogen from infected spurs down into the main branch. Outbreaks of the disease, particularly under the conditions of a wet cool spring promote the rapid spread of the pathogen. In order to identify the causal organism, a series of biochemical and physiologic tests are conducted (10) which requires a minimum of 2 weeks to finalize the results.

Plasmid DNA has been successfully used by researchers in the medical field for studying the epidemiology of human bacterial pathogens (8). Outbreaks of diseases were traced to the source of occurrence and linked to the human pathogen using plasmid DNA profiles. Restriction enzyme digests of strains associated with the disease produce characteristic profiles of fragments which are separated and visualized by

agarose gel electrophoresis. Two plasmids which share the same restriction fragments are considered to be identical, and those which share a large number of same size fragments are considered to be closely related. Plasmid profile analysis enabled researchers to identify strains of bacteria responsible for disease outbreaks which were separated in occurrence over time and geographic location.

Plasmid DNA profiles have been used to characterize pathovars of Xanthomonas campestris (2,11), and have proven useful in identifying strains. Clustering of strains of X. campestris based on plasmid DNA restriction enzyme digest profiles has correlated well within the X. campestris group of pathovars. Plasmid DNA has been identified in several pathovars of P. syringae (9,12,15).

To further investigate plasmid DNA diversity, strains of Pss and Psm collected from stone fruit orchards in Michigan and in other regions of the world were evaluated to determine the variation in plasmid DNA content between the two pathovars. We also evaluated if the differences in plasmid DNA content could be used in differentiating Psm from Pss.

MATERIALS AND METHODS

Bacterial strains. The strains of P. syringae examined in this study are listed in Table 1. Strains from Michigan were isolated in 1988 and 1989 from healthy blossoms as described by Sundin et al (13). Strains from other

Table 1. Variation in plasmid content among isolates of Pseudomonas syringae pv. morsprunorum and P. s. pv. syringae collected from deciduous tree fruit crops

Strain designation	Host	Origin[a]	Plasmids (no.)	Size of plasmids (kb)
Strains of P. s. pv. morsprunorum[b]				
212-02	Sweet cherry	MI	3	120, -[c],37
C-17	Cherry	Eng.		120, 78, 47
P-243	Sour cherry	Pol.		120, 100, 78
634	Plum	S.A.		98, 74, 47
102-A3	Prune	MI	4	82, 46, 35, 5
102-A7	Prune	MI		120, 46, 35, 5
106-A2	Sour cherry	MI		100, 50, 46, 35
115-A4	Prune	MI		120, 66, 50, 35
C-185	Cherry	Eng.		120, 98, 54, 46
P-204	Sour cherry	Pol.		98, 74, 64, 2.4
101-A7	Prune	MI	5	120, 90, 66, 50,7
102-A5	Prune	MI		84, 78, 66, 50, 35
110-B2	Prune	MI		100, 66, 50, 29, 5
111-B4	Prune	MI		100, 60, 46, 40, 10
115-A1	Prune	MI		90, 60, 50, 29, 5
210-02	Plum	MI		90, 64, 46, 35, 10
211-01	Plum	MI		90, 64, 46, 35, 10
218-01	Prune	MI		100, 60, 50, -, 10
218-03	Prune	MI		90, 60, 50, 35, 10
101-A1	Prune	MI	6	120, 106, 60, 52, 46, 35
103-A2	Prune	MI		74, 58, 52, 50, 42, 35
103-A6	Prune	MI		84, 68, 50, 46, 28, 5
103-A8	Prune	MI		110, 78, 68, 46, 35, 5
627	Sweet cherry	S.A.		88, 78, 64, 56, 35, 18
103-B1	Prune	MI	7	106, 74, 60, 58, 52, 46, 35
211-10	Plum	MI		90, 60, 50, -, 29, 10, 5
Strains of P. s. pv. syringae[b]				
105-A1	Prune	MI	0	
112-A1	Sour cherry	MI		
114-A1	Prune	MI		
201-02	Sour cherry	MI		
203-02	Sour cherry	MI		
220-02	Sweet cherry	MI		

Table 1. (cont'd)

223-01	Plum	MI	0	
224-01	Sour cherry	MI		
W4N9	Sweet cherry	WA		
W4N108	Sweet cherry	WA		
JP 442	Plum	Eng.		
PSS 9	Pear	Swi.		
PSS 10	Sour cherry	Ger.		
No. 1835	Sour cherry	Pol.		
No. 2905	Sour cherry	Pol.		
110-A1	Prune	MI	1	28
203-06	Sour cherry	MI		37
203-13	Sour cherry	MI		40
219-05	Sour cherry	MI		42
222-04	Plum	MI		60
223-04	Plum	MI		25
S-150	Cherry	Eng.	2	68, 50

[a]Countries and their abbreviations are as follows: England (Eng.), Germany (Ger.), Poland (Pol.), South Africa (S. A.),and Switzerland (Swi.). States in the United States are abbreviated as Michigan (MI) and Washington (WA).

[b]Pathovar identification based on results from the GATTa tests (10). Isolates of P. syringae pv. morsprunorum were positive for gelatin liquefaction and aesculin hydrolysis and negative for tyrosinase activity and tartrate utilization. Isolates of P. s. pv. syringae were negative for gelatin liquefaction and aesculin hydrolysis but positive for tyrosinase activity and tartrate utilization.

[c] "-" indicates presence of plasmid band with no size estimate.

geographical regions were supplied by colleagues in the respective countries listed in Table 1.

Plasmid DNA isolation and electrophoresis. Plasmid DNA was isolated from bacterial isolates using a modified alkaline lysis technique (1,14). Bacterial cells from 3 ml cultures shaken overnight in Luria-Bertani (LB) broth were suspended in 50 ul SCT buffer (20% sucrose, 20 mM EDTA, 50 mM Tris-HCl, pH 8.0) and lysed with 200 ul of a 0.2 N NaOH, 1% sodium dodecyl sulfate (SDS) solution. The solution was incubated on ice for 5 min and neutralized with 3 M sodium acetate, pH 4.5. After 15 min incubation on ice, the plasmid DNA was separated from chromosomal DNA, RNA, and protein by centrifuging for 10 min at 4 C. The supernatant containing the plasmid DNA was transferred to a fresh tube, precipitated with ethanol and resuspended in 75 ul containing 50 mg/ml RNAase A. After incubating at 37 C for 20 min, 22 ul of a 10.5 M ammonium acetate solution was added and the solution was extracted once with phenol/chloroform and chloroform, ethanol precipitated, and resuspended in 50 ul TE (10 mM Tris - HCl pH 8.0, 1 mM EDTA).

Plasmid DNA was visualized after electrophoresis at 110 volts for 2-4 hr on 0.5% agarose gels at room temperature in Tris-borate buffer (TBE) (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). The gels were stained with ethidium bromide (0.5 ug/ml) and photographed with a red Wratten

filter under 303-nm wavelength UV light with type 55 Polaroid film. Plasmids in strain SW2 of Erwinia stewartii were used for estimating size of the detected plasmids (3).

Restriction enzyme digestion. Plasmid DNA was digested for 2 hr with the restriction enzyme EcoRI (Boehringer Mannheim, Indianapolis, IN). The restriction fragments were separated by electrophoresis on 0.8% agarose gels at 80 volts in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, and 1 mM Na_2EDTA adjusted to pH 7.2 with acetic acid). The fragments were stained with ethidium bromide and photographed as described above. Size estimates for the restriction fragments were based on comparisons with lambda DNA digested with HindIII. Size estimates for plasmids in isolates of Pss with only one plasmid were determined by totaling the sizes for EcoRI restriction enzyme digested fragments.

Preparation of Southern blots. Following electrophoresis, the digested plasmid DNA was denatured and transferred to the nylon membrane using manufacturer's direction for capillary blot procedure (NEN Products, du Pont de Numours & Co. Boston, MA).

PST-DNA probe. The PST-DNA probe is as described in Section I of this thesis.

Preparation of PST-DNA probes and hybridization. All manipulations involving probe preparations and

hybridizations were as described in Section I of this thesis.

RESULTS

The plasmid DNA content of Psm from Michigan and from England, Poland, and South Africa was highly diverse (Table 1, Fig. 1). All 26 strains that were examined by electrophoresis contained plasmids that ranged in size from about 120 to 5 kb. The number of plasmids varied from three to seven. Only two strains (210-02 and 211-01) had identical plasmid profiles and no plasmid size was common to all strains.

The plasmid DNA content for strains for strains of Pss differed considerably from the plasmid DNA content for strains of Psm. Only seven of 22 strains of Pss examined by electrophoresis contained detectable plasmids (Table 1, Fig. 1). The number of plasmids in these strains varied from one plasmid for each of six strains from Michigan to two plasmids for one strain from England. The size of the plasmids varied from 25 to 68 kb and none of the plasmids were identical in size.

When plasmid and chromosomal DNAs from seven strains of Psm from stone fruit crops in the United States (Michigan), England Poland, Switzerland, and South Africa were probed with the PST-DNA probe, all strains contained one to two plasmids of 100 to 50 kb in size that hybridized with the probe (Fig. 2, lanes 9-15). When plasmid and chromosomal

Fig. 1. Agarose (0.5%) gel electrophoresis of plasmid DNA from 14 strains of Pseudomonas syringae pv. morsprunorum. Lanes shown above contain strains: 1, 101-A1; 2, 101-A7; 3, 102-A3; 4, 102-A5; 5, 102-A7; 6, 103-A2; 7, 103-A6; 8, 103-A8; 9, 103-B1; 10, 106-A2, 11, 110-B2; 12, 111-B4; 13, 115-A1; 14, 115-B4.

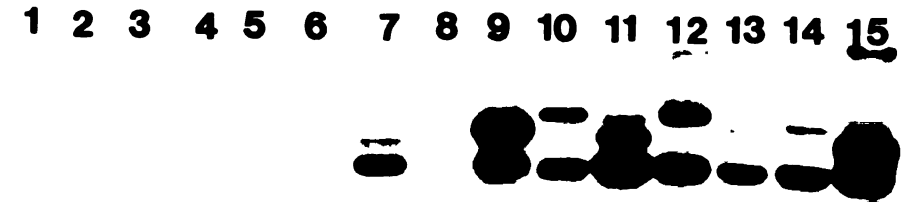

Fig. 2. Autoradiograph of plasmid DNA from eight strains of Pseudomonas syringae pv. syringae (lanes 1 - 8) and seven strains of P. s. pv. morsprunorum (lanes 9-15) probed with a 3.5 and 3.6 kb EcoRI fragment from P. s. pv. tomato. Lanes shown above contain strains: 1, No. 1835; 2, No. 2905; 3, JP 442; 4, PSS 9; 5, W4N9; 6, W4N108; 7, S-150; 8, 110-A1; 9, C-17; 10, C-185; 11, P-204; 12, P-243; 13, 625; 14, 634; 15, 103-A6.

DNAs from eight strains of Pss from fruit crops in the United States (Michigan and Washington), England, Poland, and South Africa were probed with the PST-DNA probe, only DNA from one strain from England (S-150) hybridized with the probe (Fig. 2, lanes 1-8). Chromosomal DNA and a 50 kb plasmid from strain S-150 hybridized with the probe (Fig. 2, lane 7).

Numerous restriction fragments were observed when EcoRI-digests of DNA from representative strains of Psm and Pss were separated by electrophoresis on agarose gels. Large numbers of restriction fragments (12-17 fragments, frequently more) were observed in digests of DNA from Psm (Table 2, Fig. 3). Five to six restriction fragments were observed in digests of Pss (Table 2, Fig. 4). None of the restriction patterns for the various strains were identical.

DISCUSSION

The results suggest that the pathovars differ markedly in their natural plasmid content. A number of plasmids occur in Psm while no or very few plasmids occur in Pss. There is also, a high degree of diversity in the size and number of plasmids observed within strains of both pathovars. Such variation in plasmids has been observed within other pathovars of P. syringae by Gonzales et al (9) and Piwowarski (12). The plasmid DNA profiles appear to be stable since identical profiles were observed with strains continually cultured and analyzed over a 2-yr period. This

Table 2. Approximate size of restriction fragments from EcoRI-digests of plasmid DNA from three strains of Pseudomonas syringae pv. morsprunorum and five strains of P. s. pv. syringae

Strain	(No. of plasmids)	Fragments (total no.)	Calculated molecular masses kilobase pairs (kb)
Strains of P. syringae pv. morsprunorum			
C-185	4	12	23.0, 6.6, 6.2, 4.4, 4.2, 2.4, 2.3, 1.8, 1.6, -, -, -
101-A7	5	14	23.0, 7.6, 7.3, 6.8, 6.6, 5.7, 5.0, 4.2, 2.4, 2.8, 2.0, 1.8, 1.4, 1.0
106-A2	4	17	25.0, 23.4, 14.0, 9.4, 7.5, 6.6, 6.2, 5.6, 5.0, 4.4, 4.2, 4.0, 3.4, 2.3, 2.1, 1.6, 1.0
Strains of P. s. pv. syringae			
110-A1	1	5	9.4, 6.0, 5.0, 4.8, 2.9
203-13	1	6	16.0, 9.4, 4.8, 4.0, 2.9, 2.6
219-05	1	6	14.0, 10.0, 9.4, 3.4, 3.0, 2.4
222-04	1	5	25.0, 16.0, 9.4, 6.2, 3.4
223-04	1	5	7.8, 6.0, 4.8, 4.0, 2.9

"-" indicates presence of restriction fragment but no size estimate available

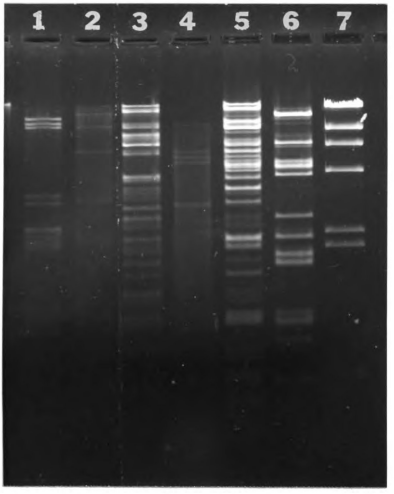

Fig. 3. Plasmid DNA from four strains of Pseudomonas syringae pv. syringae (lanes 1-2, and 4) and three strains of P. s. pv. morsprunorum (lanes 3, 5, and 6) digested with restriction enzyme EcoRI and electrophoresed on a 0.8% agarose gel. Lanes shown above contain strains: 1, 219-05; 2, 222-04; 3, 101-A7; 4, 110-A1; 5, 106-A2; 6, C-185; 7, lambda HindIII size marker.

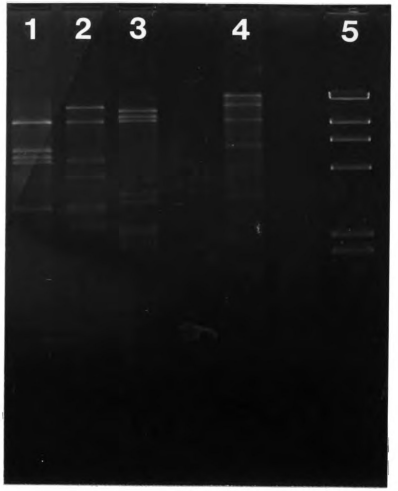

Fig. 4. Plasmid DNA from strains of Pseudomonas syringae pv. syringae digested with restriction enzyme EcoRI and electrophoresed on a 0.8% agarose gel. Lanes shown above contain strains: 1, 110-A1; 2, 203-13; 3, 219-05; 4, 222-04; 5, lambda HindIII size marker.

apparent stability of plasmid DNA may be useful in epidemiological investigations as was suggested by Lazo and Gabriel with strains of Xanthomonas campestris (11). No phenotypic function is associated with the plasmids of Psm evaluated in this study. Copper resistance has been associated with a conjugative plasmid in strains of Pss from Michigan (13). Both Psm and Pss occur as epiphytes on leaf surfaces through out the growing season (5), however conjugation studies between Psm and Pss by Sundin et al (13) indicated that the plasmids may not be able to transfer from Pss to Psm. No work was undertaken to determine if copper resistance occurred in plasmids isolated for this study, however a similar size plasmid (60 kb) was detected in a strain from a plum orchard.

Few other species of plant pathogens, particularly those of P. syringae contain as many plasmids as Psm. Coplin (4) states plasmid profiles of E. stewartii contain distinct similarities that can be used as a means of identifying this species. The situation is similar to plasmid DNA profiles in Psm. Coplin implies that there must be some reason for species like E. stewartii in maintaining so many genes on plasmids rather than chromosomal DNA. Possibly, the plasmid content of Psm represents a coadapted group of genes similar to the situation suggested by Coplin with E. stewartii (4). It was further suggested that the stability of plasmid DNA may be due to the periodic alteration of ecological niches that E. stewartii undergoes. This involves the

overwintering of the pathogen in corn flea beetles between infections of corn. A similar situation exists with Psm in that it survives for generations as epiphytes between outbreaks of bacterial canker on stone fruits. The traits which influence the ability to survive both as an epiphyte and pathogen must be stable for many generations in the absence of phenotypic selection (4). Plasmid DNA found in Psm may contain information that makes its existence possible during years of less than optimal conditions for bacterial canker.

Plasmid DNA from isolates of Pss was readily obtained and digested with restriction enzymes. Strains of Pss are similar to other pathovars of P. syringae with respect to their low variability of plasmid DNA content (7,9,12,13,15). Isolates of Pss displayed less plasmid diversity based on similar restriction fragment sizes than isolates of Psm. The plasmid DNA profiles of Pss were found to be the same within a location with either all isolates containing none or a single size plasmid. There appears to be a partial conservation of EcoRI restriction fragments in isolates of Pss similar to what was found in isolates of P. s. pv. coronafaciens reported by von Bodman & Shaw (15). Digestion of the single plasmid DNA of Pss resulted in five to six fragments and many of the fragments fragments were similar in size. Those fragments similar in size after restriction enzyme digestion indicate genetic relatedness.

Based on plasmid DNA digest profiles, strains of Psm are

clearly differentiated from Pss. There exists the possibility of various forms of the same plasmid DNA band occurring in the same profile due to nicking or linearizing of the larger plasmid bands during the isolation procedure. However, there still remains the distinct difference between strains of Pss with either none, one, or two plasmid DNA bands compared to strains of Psm with a minimum of three and as many as seven bands appearing. Plasmid DNA from strains of Psm were more complex and restriction enzyme digestion produced numerous fragments which complicated the characterization of Psm. No single EcoRI restriction fragment of isolates of Psm was common to all strains. This work reveals the diversity of Psm plasmid DNA in all isolates tested and contrasts the findings of a less variable plasmid DNA profile amongst other pathovars of P. syringae (9,12,13,15).

Although hybridization between plasmids of strains from Psm and Pss to the PST-DNA probe was detected by Southern blot analysis, the two pathovars were readily differentiated by plasmid DNA profiles.

Future work should involve hybridization studies of plasmid DNA to determine the relationship of isolates not revealed in visual examination. Hybridization studies of diverse plasmid DNA in strains of P. s. pv. glycinea by Curiale and Mills (6) indicates that plasmids which differ both in size and digest pattern nevertheless have the same sequence homology. Homology between diverse plasmids may

constitute convenient genetic markers useful in identifying unknown strains.

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