Detection of Streptomycin-Resistant *Pseudomonas syringae* pv. *papulans* in Michigan Apple Orchards

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ABSTRACT

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Streptomycin-resistant *Pseudomonas syringae* pv. *papulans* was recovered from blister spot lesions on fruit of the cultivar Mutsu collected from 12 of 17 apple (*Malus domestica*) orchards in Michigan in 1989. Resistant, but not sensitive, bacteria grew on King's medium B amended with 100 μg of streptomycin·ml^{-1}. A 500-base pair DNA probe (SMP3) that is specific for the streptomycin-resistance gene in strain Psp36 of *P. s. papulans* hybridized with 238 of 275 streptomycin-resistant, but none of 122 streptomycin-sensitive, strains of *P. s. papulans* from Michigan orchards. Hybridization occurred with plasmids of several different sizes and not solely with the 108-kb plasmid associated with streptomycin resistance in New York State. DNA from resistant strains isolated from one orchard did not hybridize with the probe, indicating that these strains contain a resistance system unrelated to that in strain Psp36.

Blister spot is an economic problem of the apple (*Malus domestica* Borkh.) cultivar Mutsu in the apple-growing area extending from Michigan through southern Ontario, Canada, to New York. The pathogen, *Pseudomonas syringae* pv. *papulans* (Rose) Dhanvantari, overwinters primarily in buds and leaf scars (1,3) and infects fruit during a 6-wk period beginning about 2 wk after petal fall (2). The disease has been controlled in New York orchards by applications of streptomycin, but this treatment is currently ineffective (4). An explanation for the failure of streptomycin came with the isolation of streptomycin-resistant strains of *P. s. papulans* and the association of resistance with a 108-kb conjugative plasmid. The streptomycin-resistance gene from strain Psp36 of *P. s. papulans* was cloned and used to develop a gene-specific DNA probe (8).

Growers in Michigan have applied streptomycin for control of blister spot for several years, but streptomycin is no longer effective for many growers. The presence of streptomycin-resistant *P. s. papulans* in Michigan apple orchards is described below. The shared DNA homology of some, but not all, of these bacteria with the streptomycin-resistance probe obtained from *P. s. papulans* in New York State is also discussed.

MATERIALS AND METHODS

Fruit with symptoms of blister spot were collected in September 1989 from 17 apple orchards of the cultivar Mutsu. The orchards were located in western Michigan from Benton Harbor in the south to Ludington in the north (orchards A–G, I–N), in central Michigan near Lansing (orchard H), and in eastern Michigan near Detroit (orchards O–Q) (Fig. 1, Table 1). Single lesions were cut from each of 10 fruit per orchard, and the 10 lesions were crushed in 3 ml of 0.01 M potassium phosphate buffer (pH 7.2) using a 7-ml tissue grinder. Suspensions from the macerate and from a 10^{-2} dilution of the macerate were streaked (two replications) on plates containing King's medium B (KB) amended with 50 μg of cycloheximide·ml^{-1} (KBc). After 3 days, up to 24 colonies per orchard were transferred from the isolation plates to a master plate containing KB. Bacteria that were oxidase-negative, produced a fluorescent pigment in KB, and were negative for liquefaction of gelatin were considered to be *P. s. papulans*. In addition, the pathogenicity of strains from orchards D, P, and M characterized as *P. s. papulans* by these tests was determined by inoculating immature Mutsu apple in the orchard as described by Burr and Hurwitz (2). All strains caused typical blister spot symptoms, and streptomycin-resistant bacteria typical of *P. s. papulans* were reisolated from the lesions.

Each colony was transferred to two plates of KB amended with 100 μg of streptomycin·ml^{-1} (KBs) to determine resistance to the antibiotic. Bacteria that produced confluent colonies were considered to be streptomycin-resistant and those that failed to grow, streptomycin-sensitive. In addition, the level of streptomycin resistance for two strains from each orchard was tested using a slight modification of the filter paper disk method described by Burr et al (4). Bacteria were spread over the surface of KB plates. Five disks were placed directly on the surface of each plate and moistened with 20 μl of 0, 10, 100, 500, and 1,000 μg of streptomycin·ml^{-1}. Inhibition zones were measured after 48 hr.

Colony hybridizations to streptomycin-resistant probe SMP3 were performed with all *P. s. papulans* isolated from the 17 orchards. Probe SMP3 is a 500-base pair *BamHI-AvaI* fragment from the streptomycin-resistance gene of strain Psp36 isolated from Mutsu apple in New York State (8). Hybridization transfer membranes (NEF-978 Colony/Plaque Screen, Du Pont) were placed on the agar surface of KB. Bacteria were spotted onto the membrane surface with toothpicks and incubated 24 hr, after which small colonies were visible on the surface of the membranes. Strains Psp32 and Psp48 (streptomycin-sensitive) and Psp36 (streptomycin-resistant) of *P. s. papulans* from New York State were grown on all membranes as negative and positive controls, respectively.

Membranes were floated on 750 μl of 0.5 N NaOH for 2 min, blotted on filter paper, and refloated for 2 min. Next, the membranes were floated on 750 μl of 1.0 M Tris-HCl (pH 7.5) for 2 min, blotted, and refloated to neutralize the base. Excess debris was removed from the membranes with SSC buffer (3 M NaCl, 3 M sodium citrate [pH 7.0]) and by gently rubbing the membrane with a small brush. Probe SMP3 was excised from a low-melting temperature agarose gel and radiolabeled with ^{32}P by the randomized oligonucleotide labeling procedure (5,9). Membranes were hybridized with ^{32}P-labeled DNA probe SMP3 for 18 hr in 5× SSPE, 5× Denhardt's reagent, 0.6% sodium dodecyl sulfate (SDS), 5% polyethylene glycol (average molecular weight 7,000–9,000), and denatured, fragmented salmon sperm DNA (100 μg/ml) (9). Hybridizations were followed by three successive 20-min washes in 2× SSC, 1% SDS; 1× SSC, 0.5% SDS; and 0.5× SSC, 0.5% SDS. Hybridizations and all stringent washes were done at 65 C. Colony hybridizations were performed twice for all strains.

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Fig. 1. Location in Michigan of apple orchards of the cultivar Mutsu sampled for streptomycin-resistant strains of *Pseudomonas syringae* pv. *papulans*.

Bacteria from each orchard were screened for plasmids using a modification of the method of Kado and Liu (6) as described by Burr et al (4). Plasmid DNA was electrophoresed on agarose gel, stained, and photographed as described by Sundin et al (10). Southern blots were prepared of the plasmid DNA, and the blots were probed with ^{32}P-labeled DNA probe SMP3, washed, and autoradiographed.

RESULTS

Streptomycin-resistant strains of *P. s. papulans* were isolated from 12 of 17 orchards (Table 1). Almost all (97.8%) of the 281 strains from the 12 orchards grew on KBs, but none of the 116 strains from the five remaining orchards did. Orchards C and F contained a mixture of sensitive and resistant strains.

The resistant and sensitive strains grew identically to control strains obtained from New York. Zones of inhibition were observed with all sensitive strains, including strain Psp32, around disks treated with 2–20 μg of streptomycin but not around those treated with 0 or 0.2 μg of streptomycin. Resistant strains, including control strain Psp36, were not inhibited regardless of the concentration of streptomycin.

In colony hybridization studies, streptomycin-resistant strains from 11 orchards contained DNA that hybridized to probe SMP3 (Table 1). Although most streptomycin-resistant strains and control strain Psp36 contained DNA that hybridized with probe SMP3, DNA of 22 resistant strains from orchard K, six strains from orchard B, and nine strains from orchard F did not hybridize with the probe. None of the 122 streptomycin-sensitive strains nor the two sensitive control strains contained DNA that hybridized with probe SMP3. The results from both hybridizations were identical.

The plasmid content of resistant and sensitive *P. s. papulans* was highly diverse (Fig. 2A; data for sensitive strains not shown). Probe SMP3 hybridized with a single 108-kb plasmid in Psp36 and with plasmids of several different sizes in all resistant strains from Michigan except those from orchard K (Fig. 2B, lanes 2–13). However, the strains from orchard K did contain plasmids (Fig. 2A, lane 13). The probe did not hybridize with any DNA extracted from streptomycin-sensitive strains (*data not shown*).

Table 1. Frequency of recovery of streptomycin-resistant *Pseudomonas syringae* pv. *papulans* from apple orchards in Michigan and hybridization of their DNA to a ^{32}P-labeled streptomycin-resistance DNA probe (SMP3) from resistant strain Psp36 of *P. s. papulans* isolated in New York State

Orchard code[a]	Location by county	Cultures tested (no.)	Resistant cultures (no.)[b]	Hybridization to probe SMP3 (no.)
A	Cass	24	0	0
B	Van Buren	24	24	18
C	Van Buren	24	19	19
D	Berrien	24	24	24
E	Berrien	24	24	24
F	Berrien	24	23	14
G	Berrien	24	24	24
H	Clinton	24	0	0
I	Kent	24	0	0
J	Kent	24	0	0
K	Mason	22	22	0
L	Oceana	20	0	0
M	Oceana	19	19	19
N	Oceana	24	24	24
O	Oakland	24	24	24
P	Genesee	24	24	24
Q	Lapeer	24	24	24

[a] Orchard selection independent of prior streptomycin usage.
[b] Growth on King's medium B with 100 μg of streptomycin·ml^{-1}.

DISCUSSION

Resistance to streptomycin in *P. s. papulans* presents a problem to growers of the cultivar Mutsu because no alternative bactericides are available for the control of blister spot (4). Several of the growers in this study indicated they could no longer control blister spot with streptomycin. As production of the cultivar Mutsu is declining in Michigan because of blister spot, poor yields, and difficulties in training such vigorous trees, the

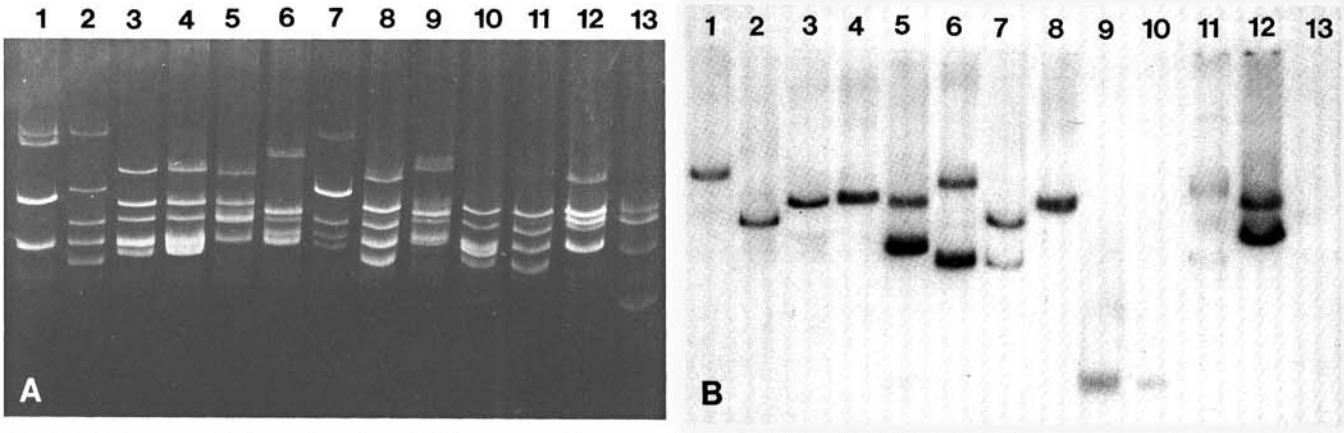

Fig. 2. (A) Agarose gel electrophoresis and **(B)** corresponding autoradiograph of plasmid DNA isolated from streptomycin-resistant strains of *Pseudomonas syringae* pv. *papulans* isolated from Mutsu apple orchards in Michigan and hybridized with ^{32}P-labeled streptomycin-resistance gene probe SMP3 from strain Psp36 of *P. s. papulans*. Lane 1 is strain Psp36 from New York State, and lanes 2–13 are strains from Michigan orchards C, E, G, M, N, O, Q, P, D, B, F, and K, respectively.

economic importance of the disease is also declining.

The recovery of streptomycin-resistant *P. s. papulans* from fruit of Mutsu apple in Michigan follows the detection of resistant strains in apple orchards in New York State (4,8). Although spray schedules were not available, the Michigan growers did confirm that streptomycin has been used in all orchards where resistant strains were detected. In contrast, no or very little streptomycin has been used in orchards where resistant strains were not detected. Also, resistant strains isolated from 11 of 12 Michigan orchards contained DNA that shares homology with the streptomycin-resistance gene found in strains of *P. s. papulans* isolated in New York orchards (8). Because of the geographic isolation of Michigan orchards from New York orchards, resistant strains probably were selected independently in the two states. The recent detection of the same streptomycin-resistance gene in *Xanthomonas campestris* pv. *vesicatoria* (7) is additional evidence for the potential of independent selection of this gene.

The failure of probe SMP3 to hybridize with some Michigan strains provides evidence for the existence of a resistance system that is unrelated to the resistance system in strain Psp36. The detection of streptomycin-resistant strains in Ohio with no homology with probe SMP3 (8) also is evidence for an unrelated resistance system. Whether the resistance in strains from orchard K is the same as the resistance in the strain from Ohio has not been determined. The occurrence of the streptomycin-resistance gene on plasmids of different sizes and of other streptomycin-resistant genes is consistent with the genetics of resistance in the diverse group of streptomycin-resistant bacteria found in apple orchards (8).

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