Streptomycin-Resistant Bacteria Associated with Fire Blight Infections

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ABSTRACT

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Streptomycin-resistant strains of *Erwinia amylovora* were not detected from samples of 357 fire blight–infected apple and pear tissues collected from 38 orchards in New York State. Streptomycin-resistant strains of other species were detected in the fire blight–infected tissues from four apple (cv. Crispin) orchards. These included strains of oxidase-positive and oxidase-negative *Pseudomonas* spp. and strains of *Pantoea agglomerans*. DNA from eight of the 18 streptomycin-resistant strains hybridized with a DNA probe, SMP3, that was previously cloned from a streptomycin-resistant strain of *Pseudomonas syringae* pv. *papulans* and shown to share sequence homology with DNA from several strains of streptomycin-resistant bacterial plant pathogens and saprophytic epiphytes. All of the strains that contained sequences homologous to SMP3 carried the streptomycin resistance determinant on plasmids of varying sizes. The frequency of resistance development appears to be associated with streptomycin use patterns.

Streptomycin is widely used in the United States for control of bacterial plant diseases including fire blight, caused by *Erwinia amylovora* (Burrill) Winslow et al. Resistant strains of *E. amylovora* were first reported in 1971 in the United States (19) and have since been reported from several locations (4,11). The frequency with which streptomycin-resistant *E. amylovora* is detected in orchards appears to vary greatly between regions of the United States. In California and Washington, for example, high percentages (up to 71%) of *E. amylovora* strains isolated from orchards have been shown to be resistant to streptomycin, whereas in New York resistance was not detected (1). In Michigan, resistance in *E. amylovora* was recently detected in one orchard but not in 19 other orchards that were assayed (4,21).

Burr et al (3) reported streptomycin resistance in *Pseudomonas syringae* pv. *papulans* (Rose) Dhanvantari, the causal agent of blister spot on apples, and demonstrated that the resistance gene was present on a conjugative plasmid. Norelli et al (15) cloned the streptomycin resistance gene and developed a DNA probe, SMP3, that hybridized with DNA sequences from strains of streptomycin-resistant *P. s. papulans* and several other streptomycin-resistant phytopathogenic and saprophytic bacteria. Chiou and Iones (4) found that streptomycin-resistant *E. amylovora* strains from Michigan hybridize with SMP3 , but a resistant strain from Washington did not. Strains of streptomycin-resistant *E. amylovora* from California also do not hybridize with SMP3 (14; and J. L. Norelli, *unpublished*). At least two mechanisms of streptomycin resistance may therefore exist in *E. amylovora*: one of chromosomal origin, as found in California (19), and the other of plasmid origin, as found in Michigan (4).

Because streptomycin is an important antibiotic for fire blight management in New York State, where a high level of resistance to it exists among populations of *P. s. papulans* and other epiphytic bacteria in many apple orchards (15), we wished to determine if streptomycin-resistant *E. amylovora* was also present.

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In 1991 there was a severe outbreak of fire blight in New York, making it possible to sample strains of *E. amylovora* from many different orchards. We also isolated other streptomycin-resistant bacteria from fire blight–affected tissues to determine if they contain sequences that hybridize with SMP3, and if the resistant determinant was plasmid-borne.

MATERIALS AND METHODS

Isolation of bacteria. Shoots and young fruits with fire blight symptoms were collected from 34 apple and 4 Bartlett pear orchards in western New York and the Hudson River valley fruit growing regions of the state. Collections were made between 10 June and 10 July 1991. Apple cultivars included Crispin, Idared, Jonagold, Rome Beauty, Paulared, Cortland, Twenty Ounce, Tydeman, Rhode Island Greening, Northern Spy and Jonamac. To isolate *E. amylovora* and to determine if streptomycin-resistant strains were present, a medium semiselective for *E. amylovora* (CCT) (8) and CCT amended with streptomycin (50 μg/ml) were used. Tissue pieces from advancing margins of active fire blight infections were triturated in 0.5 ml of sterile distilled water, and the suspensions were streaked onto both media. At least five and no more than 20 tissue pieces, each collected from a different tree, were assayed per orchard. Colonies that grew on the media and were characteristic of *E. amylovora* (smooth, pulvinate, light blue opalescent with entire margins) were subcultured. All strains that resembled *E. amylovora* were tested for pathogenicity on immature pear slices by a modification of a previously described method (16). Pear fruit slices were inoculated by piercing them with a dissecting needle that had been dipped in a 48- to 72-hr-old colony of the bacteria. Pear slices inoculated with *E. amylovora* strain Ea273 or distilled water served as positive and negative controls, respectively, in all pathogenicity tests. All colonies that grew on CCT plus streptomycin were subcultured on Luria-Bertani (LB) medium (12) amended with streptomycin (50 μg/ml). A total of 39 strains of *E. amylovora* from 32 orchards that were isolated on unamended CCT were subsequently plated on LB plus streptomycin. Strains of *E. amylovora* and streptomycin-resistant bacteria were stored at −80 C in cryogenic storage medium (1.2 g of nutrient broth, 22.5 g of glycerol, and 85 ml of distilled water) amended or unamended with streptomycin.

Identification of bacteria. The following tests were used to characterize all strains of the streptomycin-resistant bacteria that shared sequence homology with the streptomycin resistance gene probe SMP3. Methods as described by Schaad (18) were used except where otherwise noted. The tests included Gram stain reaction, oxidase reaction, hypersensitivity in tobacco, levan production, gelatin liquefaction, and production of catalase. The strains were also tested for Gram stain reaction, using 3% KOH (22); anaerobic growth on Tryptic soy agar (Difco), using the BBL GasPak anaerobic system (Becton Dickinson, Cockeysville, MD); fluorescence on King's medium B (10); pigment production on LB medium; phenylanine deaminase (20); nitrate reduction (5); production of orange colonies on medium of Miller and Schroth (13); mucoid growth; growth at 37 C; and growth on MacConkey agar (Difco). Arrangement of flagella was determined by staining a suspension of 24-hr-old cells (about 10^8/ml grown on LB medium) for about 30 sec with 2% potassium phosphotungstate (18) and observing them with a transmission electron microscope. The bacteria were also characterized by the Biolog GN MicroPlate system (Biolog, Inc. Hayward, CA) and the API 20E system (Analytab Products, Plainview, NY). Reactions of yellow-pigmented bacteria were compared to those of a saprophytic strain of *E. herbicola* (Eh112y, supplied by S. V. Beer, Cornell University).

Colony hybridization with SMP3. Colony hybridizations were conducted with streptomycin-resistant bacteria isolated from fire blight infections as previously described (15). Strains were grown on the surface of Colony/Plaque Screen hybridization transfer membrane (Dupont, NEN Research Products, Boston, MA), which was placed on the surface of LB agar and incubated at 28 C for 24 hr. Each filter contained a positive (*P. s. papulans* streptomycin-resistant strain PSP39) and negative (streptomycin-sensitive PSP38) control. Colonies were lysed, following the recommendations of the manufacturer. Filters were hybridized at 65 C in aqueous solution with ^{32}P-labeled SMP3 probe (6) and washed at high stringency (17). SMP3 was previously cloned from streptomycin-resistant *P. s. papulans* strain Psp36 and consists of a 500-bp *Bam*HI-*Ava*I fragment from plasmid pCPP505 (which contains a 2.1-kb insert that encodes streptomycin resistance from Psp36 in pBR322) (6). Hybridizations were repeated at least once.

Southern analysis. All strains that hybridized with SMP3 in the colony hybridization were analyzed to determine if the streptomycin resistance gene was carried on a plasmid, and if the plasmid was similar in size to the streptomycin resistance plasmid of *P. s. papulans*. Plasmid DNA was isolated by a modification of the method of Kado and Liu (9) as previously described (15). Plasmids were visualized by electrophoresis in a horizontal 0.5% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) at 5 V/cm. Gels were stained with ethidium bromide, photographed, and transferred under alkaline conditions to GeneScreen Plus hybridization transfer membrane (Dupont, NEN Research Products). Hybridizations with SMP3 were performed as described above.

RESULTS

Isolation of bacteria. *E. amylovora* was isolated on unamended CCT medium from all 357 fire blight infections collected from 38 of the apple and pear orchards. Strains produced typical colonies on CCT and incited a characteristic pathogenic response (discoloration and ooze production within 48 hr) on immature pear slices. No streptomycin-resistant strains of *E. amylovora* were detected on CCT amended with streptomycin. None of the 39 strains of *E. amylovora* that were subcultured and saved from unamended CCT grew when they were plated on LB plus streptomycin (50 μg/ml).

A total of 18 streptomycin-resistant bacterial strains were isolated from fire blight infections collected from four Crispin orchards in western New York. None of the streptomycin-resistant bacteria had colony morphologies on CCT that were characteristic of *E. amylovora*.

Identification of streptomycin-resistant bacteria. All streptomycin-resistant strains were gram-negative and could be separated into two groups consisting of fluorescent-pigmented bacteria and yellow-pigmented bacteria. Two of the fluorescent bacteria (SRE1 and SRE18) were identified as *P. syringae* and one as an oxidase-positive, hypersensitive-negative *Pseudomonas* sp.(SRE2). The yellow-pigmented bacteria (SRE5, SRE9, SRE11, SRE13, and SRE14) were identified as *E. herbicola* (Lohnis) Dye, which has recently been renamed *Pantoea agglomerans* (Beijerinck 1888) comb. nov. (7). Results of diagnostic tests are given in Table 1.

Colony hybridization. Eight of the 18 streptomycin-resistant bacteria shared sequence homology with probe SMP3 (Fig. 1). These eight strains belonged to two genera and at least three species, including *Pseudomonas* sp., *P. syringae*, and *Pantoea agglomerans*. Six of the eight strains (SRE1, SRE2, SRE5, SRE9, SRE11, and SRE13) were from three orchards in Wayne County, and two were from one orchard in Orleans County (SRE14 and SRE 18). The farthest distance between these specific orchards was about 120 km. From each of these orchards, streptomycin-resistant strains that did not hybridize with SMP3 were also isolated.

Southern analysis. Results of the plasmid profile and Southern hybridizations are shown in Figure 2A and B. All of the strains that were isolated from fire

blight infections and that hybridized with SMP3 carried the streptomycin resistance determinant on a plasmid or plasmids. Plasmid number and size were quite variable between strains of *Pseudomonas* and *Pantoea*. The plasmids carrying the streptomycin-resistant determinant in SRE 18 (*Pseudomonas* sp.) and in SRE5, SRE9, and SRE11 (*Pantoea agglomerans*) are similar in size to the previously characterized conjugative plasmid of PSP39 (3).

DISCUSSION

Streptomycin-resistant *E. amylovora* was not detected in any samples collected from apple or pear orchards in New York State. Sample size was not a limiting factor, since isolations were made from 357 *E. amylovora*-infected tissue samples taken from 38 orchards in a year when disease pressure was high and fire blight was severe. Streptomycin-resistant *E. amylovora* was detected in one of 20 orchards in Michigan (with more than 200 tissue samples assayed), and resistance was shown to be plasmid-encoded (4). Our results and those from Michigan greatly contrast with findings in California (19) and, more recently, in Washington (11), where strains of streptomycin-resistant *E. amylovora* are ubiquitous, and 98 of 138 strains from 44 orchards were resistant to streptomycin. Reasons for this difference are not known, but they may be related to the pattern and intensity of streptomycin use as affected by climatic conditions and phenological development of susceptible blossom tissues. In general, the blossom period of apple and, particularly, pear in New York is significantly shorter than in California and Washington. This can affect the number of environmental events that occur that are conducive to infection by *E. amylovora* and thus the number of streptomycin sprays applied. As discussed below, streptomycin use in New York is infrequent. In some California orchards where streptomycin resistance was detected, the antibiotic had been applied extensively (19). In a recent survey in Washington, up to 5 sprays of streptomycin were applied per season in some orchards (11). It was concluded that resistance in California and Washington resulted from mutations of many diverse strains of *E. amylovora* that eventually became widespread and appeared even in orchards where streptomycin was not used.

Results from previous studies suggest that the genetic basis of streptomycin resistance in *E. amylovora* in California and Washington is different from that in Michigan. Whereas streptomycin resistance strains from Michigan are encoded on a plasmid that hybridizes with SMP3, DNA from the few streptomycin resistance strains from California

Table 1. Characterization of gram-negative streptomycin-resistant bacteria isolated from fire blight infections and that hybridize with DNA probe SMP3[a]

	Reaction of bacterial group or strain		
Test	**SRE5, 9, 11, 13, 14**	**Eh122y**	**SRE1, 2, 18**
Pigment on KB[b]	Yellow	Yellow	Fluorescent
Growth on MS[c]	Orange	Orange	Blue
Flagella	Peritrichous	Peritrichous	Polar
Anaerobic growth	+	+	–
Oxidase	–	–	(–)[d]
Growth at 37 C	+	+	–
HR[e] on tobacco	–	–	(+)[d]
Mucoid on 5% sucrose	–	ND[f]	+
Gelatin liquefaction	+	ND	+
Catalase	+	+	+
Nitrate reduction	+	+	ND
Levan production	ND	ND	(–)[d]
Phenylalanine deaminase	+	+	–
MacConkey agar	+	+	–
BioLog GN[g]	*Pantoea agglomerans*	*P. agglomerans*	*Pseudomonas syringae*
API 20E[h]	*Enterobacter agglomerans*	ND	ND

[a] Cloned streptomycin resistance gene from *Pseudomonas syringae* pv. *papulans*.
[b] King's medium B.
[c] Miller-Schroth medium.
[d] Strain SRE2 was oxidase +, hypersensitive –, and levan +.
[e] Hypersensitive reaction.
[f] ND = not determined.
[g] Identification according to Biolog GN MicroPlate System.
[h] Identification according to API 20E System.

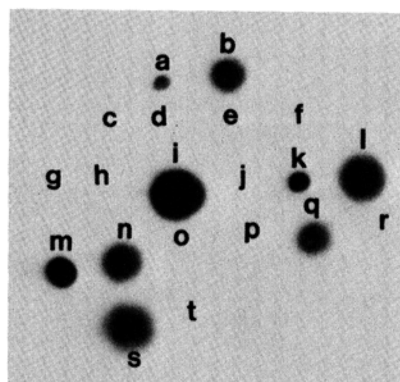

Fig. 1. Colony hybridization of streptomycin-resistant bacteria isolated from fire blight infections from four different Crispin apple orchards using probe SMP3. Letters correspond to strains: a = SRE1, b = SRE5, c = SRE12, d = SRE6, e = SRE16, f = SRE17, g = SRE7, h = SRE15, i = SRE14, j = PSP38, k = PSP39, l = SRE2, m = SRE18, n = SRE13, o = SRE10, p = SRE3, q = SRE11, r = SRE4, s = SRE9, t = SRE8. PSP38 and PSP39 are streptomycin-sensitive and streptomycin-resistant *Pseudomonas syringae* pv. *papulans* strains, respectively.

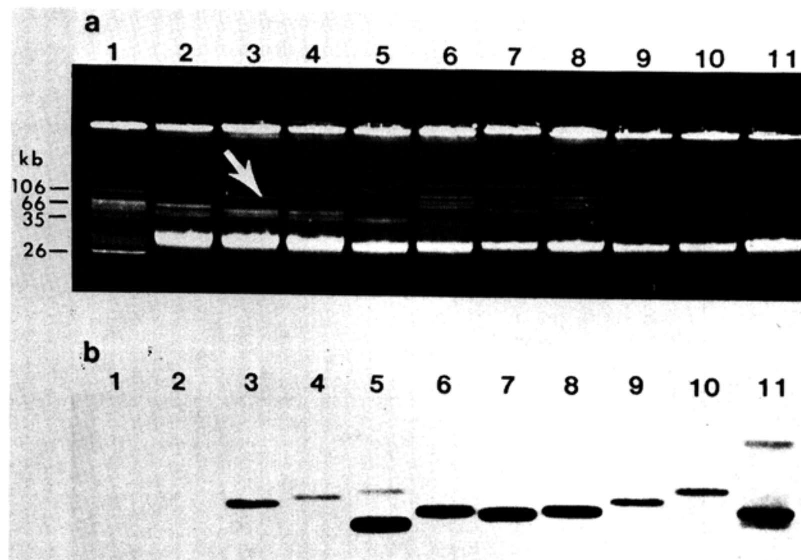

Fig. 2. (A) Plasmid profiles and **(B)** Southern analysis of streptomycin-resistant strains that hybridized with SMP3. Lane I represents *Erwinia stewartii* strain SW2, which is used as a plasmid size marker (received from D. Coplin, Ohio State University). Lanes 2 and 3 represent strains PSP38 and PSP39, which are streptomycin-sensitive and streptomycin-resistant *Pseudomonas syringae* pv. *papulans*, respectively. The arrow points to a plasmid of about 106 kb from PSP 39 that carries streptomycin-resistant determinant. Lane 4 = SRE 1, 5 = SRE2, 6 = SRE5, 7 = SRE 9, 8 = SRE11, 9 = SRE13, 10 = SRE14, and 11 = SRE 18.

(Norelli, *unpublished*) and Washington (4) that have been tested does not hybridize with SMP3. This is also supported by Minsavage et al (14), who cloned a streptomycin-resistant determinant from *Xanthomonas campestris* pv. *vesicatoria* that hybridized with plasmid DNA from streptomycin-resistant *P. s. papulans* strains PSP34 and PSP36 but not with a streptomycin-resistant strain of *E. amylovora* from California. It is interesting that DNA homologous to SMP3 appears to be common and widespread in streptomycin-resistant plant pathogens and epiphytes in the eastern United States. In addition to *P. s. papulans* and *P. s. syringae* in New York and Ohio (4) and *E. amylovora* in Michigan, *X. c. vesicatoria* (14) and a diverse group of plant epiphytes from New York and Michigan also hybridize with SMP3 (15,21). However, several strains of streptomycin-resistant bacteria isolated in this and previous studies did not hybridize with SMP3. In these cases, the genetic basis of resistance may be similar to that of *E. amylovora* strains in the western United States.

Our recovery of streptomycin-resistant bacteria only from Crispin orchards may be related to streptomycin use patterns. In the majority of pear orchards in New York, streptomycin is applied when conditions are conducive to blossom infection by *E. amylovora*. Typically, no more that three applications are applied per season. In some years, no streptomycin is applied. Application of streptomycin for fire blight control is even less intensive in apple orchards, and in many years streptomycin is not applied at all. In contrast, one to three applications of streptomycin may be applied annually in most Crispin apple orchards to control blister spot, a disease that is usually of economic importance only on this cultivar (2). Since spray applications for blister spot do not usually coincide with spray applications for fire blight control, a total of four or more applications of streptomycin may be applied per season when growers spray for both diseases. Whereas applications for fire blight are initiated during bloom, blister spot sprays begin about 10 days after blossom petals fall. Blister spot in Crispin orchards is an important disease, annually warranting two to three spray applications of streptomycin per season. The apparent increased levels of streptomycin-resistant bacteria in Crispin orchards may therefore have been stimulated by continuous streptomycin use. This hypothesis is supported by our previous findings showing that the greatest numbers of streptomycin-resistant bacteria were generally detected in orchards where streptomycin use was greatest (15). A similar relationship was noted in Michigan, where strains of *E. amylovora* that are resistant to streptomycin were detected in an orchard with heavy use of the antibiotic (4). There are very few chemical control options for fire blight, and therefore it will be essential to instruct growers to minimize the use of streptomycin as a strategy to prevent further development of resistance.

The group of streptomycin-resistant bacteria we isolated that hybridized with SMP3 all carry the resistance gene on a plasmid. Because we used CCT medium to isolate bacteria from infected tissues, we probably did not recover all of the possible taxonomic groups of streptomycin-resistant bacteria that might grow on a less selective medium. Nevertheless, at least three different species of streptomycin-resistant bacteria belonging to the genera *Pantoea* and *Pseudomonas* were isolated that contained plasmid-encoded sequences homologous with SMP3. SMP3 homologous sequences are widespread and have been detected on different-sized plasmids from nonpathogenic epiphytic and soilborne bacteria (15) and certain plant pathogenic bacteria (4,14,15) This raises the question of possible conjugal transfer of the gene to other bacteria. We demonstrated a high frequency of conjugal transfer of the gene within *P. s. papulans* (15) and between *P. s. papulans* and *Pseudomonas* spp. A very low frequency of conjugal transfer between *P. s. papulans* and *E. amylovora* has been detected (Norelli, *unpublished*). Since *Pantoea agglomerans* is taxonomically related to *E. amylovora*, it is possible that conjugal transfer of the streptomycin gene between these two species may occur at a higher frequency. Although we did not detect streptomycin-resistant *E. amylovora*, we cannot rule out the possibility that conjugal transfer is occurring in the fire blight–affected tissues at a frequency too low to detect. It is possible that nutritional or physical conditions within infected tissues is not conducive to conjugal transfer. Further experiments are underway to determine if the streptomycin-resistant bacteria reported here can promote the spread of streptomycin resistance in *E. amylovora*.

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