Streptomycin-Resistant Epiphytic Bacteria with Homologous DNA for Streptomycin Resistance in Michigan Apple Orchards

P. SOBICZEWSKI, Institute of Pomology and Floriculture, 96-100 Skierniewice, Poland, and C.-S. CHIOU and A. L. JONES, Department of Botany and Plant Pathology and Pesticide Research Center, Michigan State University, East Lansing 48824-1312

ABSTRACT

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Streptomycin-resistant bacteria were recovered from leaves of apple (*Malus domestica*) and from leaves and stems of various weed species collected from six orchards in Michigan that had been treated with streptomycin in recent years. Populations of streptomycin-resistant bacteria ranged from 2.0×10^3 to 5.7×10^5 cfu per apple leaf and from 2.0×10^4 to 1.4×10^6 cfu per gram fresh weight of tissue from weed species. In DNA colony hybridization studies, 97% of 152 strains of streptomycin-resistant gram-negative bacteria contained DNA that hybridized with a 500-bp DNA probe associated with streptomycin resistance in *Pseudomonas syringae* pv. *papulans*. These bacteria included strains of *P. syringae* (several pathovars), *P. fluorescens, P. aeruginosa, P. putida, Erwinia amylovora, E. herbicola, Acinetobacter, Aeromonas, Flavobacterium*, and a yellow-pigmented *Pseudomonas* sp. In contrast, DNA from 28 gram-positive bacteria (mostly yellow-pigmented *Corynebacterium*), three strains of *E. herbicola*, one strain of *P. viridiflava*, and one unidentified yellow gram-negative bacterium did not hybridize with the probe. In Southern hybridizations, there was restriction fragment length polymorphism in the SMP3 streptomycin-resistance region among the gram-negative bacteria isolated from apple orchards.

Intensive usage of streptomycin for the control of some plant diseases has been followed by the appearance of streptomycin-resistant strains of plant-pathogenic bacteria (3–7,10,12,16,20,22,25). Streptomycin resistance in strains of *Pseudomonas syringae* pv. *papulans* (Rose) Dhanvantari from apple (*Malus domestica* Borkh.) orchards of the cultivar Mutsu is associated with a conjugative plasmid (3). A 500-bp DNA fragment has been cloned from the interior of the streptomycin-resistance gene in *P. s. papulans* (17). This fragment was used as a probe (SMP3) to confirm the presence of streptomycin-resistant *P. s. papulans* in apple orchards in Ohio, Michigan, and New York (10,17) and the presence of streptomycin-resistant *Erwinia amylovora* (Burrill) Winslow in an apple orchard in Michigan (4). Streptomycin resistance in *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye from pepper and tomato is associated with plasmids in strains from Argentina and Florida (16). The DNA associated with streptomycin resistance in *X. c. vesicatoria* hybridizes with the 108-kb plasmid from streptomycin-resistant *P. s. papulans*. The detection of homologous DNA associated with streptomycin resistance in three diverse genera of plant-pathogenic bacteria from widespread geographic locations is evidence that this resistance mechanism is an important determinant for streptomycin resistance.

Recently, the DNA associated with streptomycin resistance in *P. s. papulans* was found to be distributed widely among gram-negative streptomycin-resistant bacteria present in apple orchards in New York State (17). The distribution of this DNA among populations of epiphytic bacteria in other apple-growing regions has not been investigated. Because streptomycin-resistant *E. amylovora* and *P. s. papulans* from Michigan contain DNA that hybridizes with probe SMP3 (4,10), information on the presence of this DNA in other bacteria in Michigan apple orchards contributes to our understanding of the source of streptomycin resistance in plant-pathogenic bacteria.

The objectives of this study were to isolate and identify streptomycin-resistant bacteria present in Michigan apple orchards and to determine if the bacteria contained DNA homologous to that found in streptomycin-resistant strains of *P. s. papulans* and *E. amylovora* from Michigan.

MATERIALS AND METHODS

Isolation of bacteria. Apparently healthy apple leaves were collected at random on 13 and 25 July 1990 from five orchards (orchards A–E) in southwest Michigan and on 21 July from an orchard (orchard F) in central Michigan. Streptomycin had been used in all six orchards for several years, with the exception of orchard E during 1989 and 1990. Four samples of 25 leaves each were collected from 20–30 trees per orchard on each sampling date. Leaves and stems of broadleaf herbaceous plants and grasses (weeds) were also collected on each sampling date from under the drip line of trees in each orchard. The samples were placed in plastic bags and kept on ice in a cooler until processed later the same day. A 25-g fresh weight subsample was randomly selected, placed in a 500-ml flask with 200 ml of 0.01 M potassium phosphate buffer (pH 7.2), and then agitated for 30 min with a rotary shaker at 22 C. The washings were serially diluted and seeded on two petri plates containing King's medium B (11) amended with 100 g of streptomycin sulfate and 50 g of cycloheximide per milliliter. Plates were incubated at 25 C for 3–4 days before the total number of bacteria were counted. Representative colonies of each colony type present on the plates were selected for further studies.

Identification of streptomycin-resistant bacteria. Isolated bacteria were tested as appropriate for production of potato soft rot (2); motility (8); cytochrome oxidase activity (9); production of fluorescence on King's medium B (11); Gram stain (Hucker modification), catalase activity, oxidation/fermentation metabolism (acid production from glucose aerobically and anaerobically), nitrate reduction, arginine dihydrolase, levan production, gelatin hydrolysis, and presence of xanthomonadin pigments (14); viscosity (18); and staining of flagella and growth of bacteria at 41 C (21). Colony color of strains was checked on King's medium B. Bacteria were identified according to keys or characteristics listed in Billing (1), Bradbury (2), Fahy and Persley (8), and *Bergey's Manual of Systematic Bacteriology* (13,23).

Colony hybridization. DNA probe SMP3 was prepared from plasmid pCPP505 as described by Norelli et al (17). The fragment was radiolabeled with ^{32}P by following the randomized oligonucleotide labeling procedure (Random

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Primed DNA Labeling Kit, United States Biochemical, Cleveland, OH). Colony hybridizations with DNA probe SMP3 were performed with all the strains of streptomycin-resistant bacteria retained from the orchard survey. Each strain was transferred to Colony/Plaque Screen hybridization transfer membranes (New England Research Products, Boston, MA). The membranes were placed on the surface of King's medium B agar and incubated for 48 hr at 20 C. *Escherichia coli* (Migula) Castellani and Chalmers with plasmid pCPP505 containing a 2.1-kb insert from strain Psp36 (17), strain Psp36 of *P. s. papulans* (streptomycin resistant), and strain EL01 of *E. amylovora* (streptomycin sensitive) were included on each membrane as positive and negative controls, respectively. The bacteria were lysed and the released DNA was denatured and fixed to the membranes according to the manufacturer's instructions. Hybridizations at 65 C and autoradiographs of the membranes were carried out as described by Maniatis et al (15).

Restriction enzyme digests and Southern blots. Total genomic DNA (includes plasmid DNA) was isolated with a miniprep procedure (24) from 21 strains of streptomycin-resistant epiphytic bacteria isolated in this study and from streptomycin-resistant strains Psp36 of *P. s. papulans* and HO62-1 of *E. amylovora*. Procedures for digesting DNA with *Ava*I, agarose gel electrophoresis, and Southern analysis were as described previously (4).

RESULTS

Isolation. Streptomycin-resistant bacteria were recovered from leaves collected from apple trees in the six orchards and from crabapple trees located next to orchard A (Table 1). The number of resistant bacteria recovered per leaf ranged from 2.0×10^3 for leaves of the cultivar Jonathan in orchard E to 5.7×10^5 for leaves of the cultivar Mutsu in orchard C. Streptomycin-resistant bacteria were recovered also from leaves and stems of the weed species collected in five apple orchards (Table 1). The number of bacteria per gram of tissue ranged from 2.0×10^4 for weeds collected in orchard E to 1.4×10^6 for weeds collected in orchard C. The orchard that was not sprayed with streptomycin in 1989 and 1990 (orchard E) had the lowest number of streptomycin-resistant bacteria on apple leaves and on weeds. A total of 159 bacteria from apple leaves and 21 bacteria from weeds were saved for identification.

Identification of bacteria. Of 152 strains of gram-negative bacteria, 25 were identified as fluorescent pseudomonads (Table 2): 19 strains of *P. syringae* (several pathovars or groups), 1 each of *P. fluorescens* biovar III and biovar V, 2 of *P. aeruginosa*, 1 of *P. putida*, and 1 of *P. viridiflava*. All strains of *P. syringae* were recovered from apple leaves, whereas the other fluorescent pseudomonads came from weed species. Nonfluorescent, whitish or grayish gram-negative bacteria were recovered from apple leaves from two orchards. These included 15 strains of *E. amylovora*, 3 in the genus *Acinetobacter*, 1 in the genus *Aeromonas*, and 1 in the genus *Pseudomonas*. The largest group of gram-negative bacteria was pigmented yellow. This group included 54 strains of *E. herbicola*, 50 in the genus *Pseudomonas*, and 1 in the genus *Flavobacterium*. With the exception of *Flavobacterium*, which was recovered only from weeds, these bacteria were recovered from apple leaves in each of the six orchards. Two strains of gram-negative bacteria were not identified.

Most of the 28 strains of gram-positive bacteria were yellow-pigmented *Corynebacterium* and various coryneforms (Table 3). All but one of these strains were recovered from apple leaves.

Hybridization with a DNA probe specific for streptomycin resistance. In colony hybridizations, DNA from 147 of 152 streptomycin-resistant gram-negative bacteria hybridized with probe SMP3 (Table 2). Both oxidase-positive and oxidase-negative strains from apple leaves and from weed species contained DNA that hybridized with the probe. Only DNA from one strain of *P. viridiflava*, three strains of *E. herbicola*, and one unidentified gram-negative bacterium failed to hybridize with the probe. None of the streptomycin-resistant gram-positive bacteria contained DNA that hybridized with probe SMP3 (Table 3).

Southern hybridization analysis. Probe SMP3 hybridized with single restriction fragments of 2.7, 1.5, or 0.9 kb when total genomic DNA from streptomycin-resistant gram-negative epiphytic bacteria was digested with *Ava*I and subjected to Southern analysis (Fig. 1, Table 1). The probe hybridized with a 2.7-kb fragment in digested DNA from strain HO62-1 of *E. amylovora*, from two strains of *E. herbicola*, and from one strain of a yellow *Pseudomonas*. It hybridized with a 1.5-kb fragment in digested DNA from three strains of *P. s. papulans*, three strains of yellow *Pseudomonas*, and single strains of *P. s. syringae*, *P. fluorescens* biovar III, *P. fluorescens* biovar V, *P. aeruginosa*, *Acinetobacter*, *E. herbicola*, and *Flavobacterium*. Hybridization with a 0.9-kb fragment occurred with single strains of *P. putida*, *Aeromonas* sp., and *E. herbicola*. No hybridization occurred with digested DNA from single strains of *P. viridiflava*, *E. herbicola*, and an unidentified yellow bacterium.

DISCUSSION

The detection of streptomycin resistance in epiphytic gram-negative bacteria isolated from apple orchards in Michigan supports the concept that various bacteria in a common environment may have a homologous gene for resistance. Our results were generally consistent with those from the study in New York (17), except we observed a higher percentage of bacteria with homologous DNA associated with streptomycin resistance. That may have been due to our sampling of orchards with a recent history of streptomycin usage. In New York, both streptomycin-sprayed and nonsprayed orchards were sampled, and sprayed orchards yielded a much higher percentage of bacteria with DNA that hybridized with probe SMP3 than did nonsprayed orchards (17).

Streptomycin-resistant gram-negative epiphytic bacteria with DNA that hybridized with probe SMP3 were detected in all six orchards we sampled, but streptomycin-resistant *P. s. papulans* and *E. amylovora* with DNA that hybridized with SMP3 were detected in separate orchards. Thus, streptomycin resistance is selected and builds up in nonpathogenic epiphytic bacteria before it is selected and builds up in plant-pathogenic bacteria. The detection of

Table 1. Streptomycin-resistant bacteria isolated from leaves of apple and leaves and stems of weed species in Michigan apple orchards with King's medium B + 100 μg/ml of streptomycin sulfate and 50 μg/ml of cycloheximide

Orchard			Streptomycin applications (no.)		Bacteria per apple leaf ($\times 10^4$)		Bacteria per gram fresh weight of weed species ($\times 10^4$)	
Code	County	Cultivar	1989	1990	13 July	25 July	13 July	25 July
A	Van Buren	Jonathan	7	8	1.2	21.0	4.3	140.0
		Crabapple	0	0	3.4[a]	ND[b]	ND	ND
B	Van Buren	Jonathan	8	8	1.3	12.0	86.0	75.0
C	Berrien	Mutsu	4	5	57.0	37.0	140.0	130.0
D	Berrien	Jonathan	1	2	29.0	33.0	26.0	65.0
E	Van Buren	Jonathan	0	0	0.2	0.4	2.0	18.0
F	Ingham	Jonathan	2	1	ND	1.2[c]	ND	ND

[a] Bacteria isolated from leaves of wild crabapple trees adjacent to orchard A.
[b] Not done.
[c] Isolations made on 21 July 1990.

Table 2. Characteristics and identification of streptomycin-resistant gram-negative bacteria (rods) recovered from apple orchards and their hybridization with streptomycin-resistance probe SMP3 from *Pseudomonas syringae* pv. *papulans*

Bacteria	Isolates (no.)	Orchard code[a]	Colony color[b]	Motility and flagellation	Oxidase	Catalase	Oxidative(O)/fermentative(F) metabolism	Nitrate reduction	Arginine dihydrolase	Levan production	Growth at 41 C	Gelatin hydrolysis	DNA homology: Colony	DNA homology: Southern
Fluorescent														
P. s. papulans	5	C	W	+	−	+	O	−	−	−	−	− or weak	+	+(2)[c]
P.s. syringae	3	C,D,E	W	+	−	+	O	−	−	+	−	+	+	+(1)
P. syringae group 1	7	A,B,C,D,E	W	+	−	+	O	−	−	−	−	−	+	
P. syringae group 2	4	D,E	W	+	−	+	O	−	−	−	−	+[d]	+	
P. viridiflava	1	Aw	W	+	−	+	O	−	−	−	−	+[e]	−	−(1)
P. fluorescens biovar III	1	Cw	W	+	+	+	O	+	+	−	−	+	+	+(1)
P. fluorescens biovar V	1	Cw	W	+	+	+	O	−	+	−	−	+	+	+(1)
P. aeruginosa	2	Aw,Cw	W	+	+	+	O	−	+	−	+	+	+	+(1)
P. putida	1	Cw	W	+	+	+	O	−	+	−	−	−	+	+(1)
Nonfluorescent														
Erwinia amylovora	15	A	W	+	−	+	O/F	−	−	+	ND[f]	ND	+	
Acinetobacter	3	A	W	+	−	+	O	−	−	−	ND	ND	+	+(1)
Aeromonas	1	D	W	+	+	+	O/F	+	−	−	ND	ND	+	+(1)
Pseudomonas	1	D	W	+	+	+	O	−	−	−	ND	ND	+	
E. herbicola group 1	51	A,B,C,D,E,F	Y	+	−	+	O/F	+	−	−	ND	ND	+	+(4)
E. herbicola group 2	3	B,D	Y	+	−	+	O/F	+	−	−	ND	ND	−	−(1)
Pseudomonas[g]	50	A,B,Bw,C,D,E,F	Y	+	−	+	O	−	−	−	ND	ND	+	+(4)
Flavobacterium	1	Bw	Y	−	+	+	O	+	−	−	ND	ND	+	+(1)
Unidentified														
Yellow	1	Cw	Y	−	+	+	O/F	−	−	−	ND	ND	−	−(1)
White	1	D	W	+	−	+	O/F	+	−	−	ND	ND	+	

[a] Code letter followed by w indicates strain was isolated from weeds in that orchard.
[b] W = whitish or grayish, Y = yellow.
[c] Number of isolates tested by Southern blot analysis is shown in parentheses.
[d] Negative for production of potato soft rot.
[e] Positive for production of potato soft rot.
[f] Not done.
[g] All isolates failed to produce xanthomonadin pigment and had negative viscosity test results.

Table 3. Morphological and cultural characteristics of streptomycin-resistant gram-positive bacteria recovered from apple leaves in six apple orchards

Organism Orchard code	Isolates (no.)	Colony color	Rod	Coryneform	Cocci	Oxidase	Nitrate reduction	Catalase	Endospores	DNA homology
Corynebacterium and other coryneforms										
B,D,E,F	9	Yellow	+	+	−	−	−	+	−	−
A,B,D,Dw,[a]E,F	11	Yellow	+	+	−	+	−	+	−	−
A,C	2	Pink	+	+	−	+	−	+	−	−
B	1	Pink	+	+	−	−	−	+	−	−
Unidentified bacteria										
F	2	Yellow	+	−	−	−	−	+	−	−
F	1	Yellow	+	−	−	+	−	+	−	−
E	1	Yellow	−	−	+	−	−	+	−	−
F	1	Pink	−	−	+	−	−	+	−	−

[a] Bacteria isolated from weeds in orchard D.

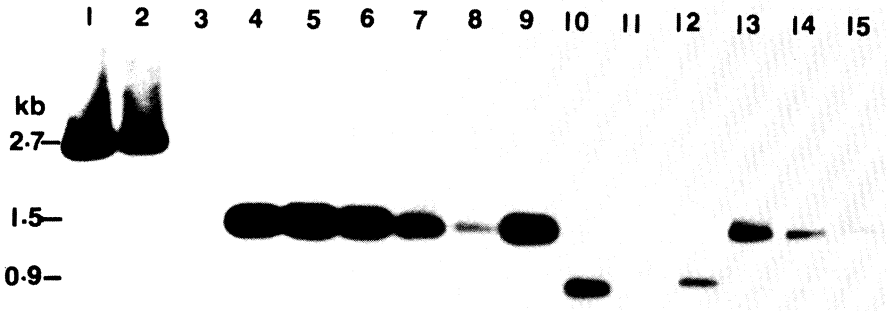

Fig. 1. Restriction fragment length polymorphism in the streptomycin-resistance region among streptomycin-resistant gram-negative bacteria from apple orchards. *Ava*I-digested total genomic DNA was hybridized with ^{32}P-labeled probe SMP3 from strain Psp36 of *Pseudomonas syringae* pv. *papulans*. Lanes 1–15 are DNA from, respectively, strain HO62-1 of *Erwinia amylovora*, strain 6a of *E. herbicola*, strain 180 of *E. herbicola*, strain 34 of *E. herbicola*, strain 40 of a yellow *Pseudomonas*, strain 45 of *P. s. syringae*, strain 57 of *P. fluorescens* biovar III, strain 154 of *P. fluorescens* biovar V, strain 60 of *P. aeruginosa*, strain 61 of *P. putida*, strain 150 of *P. viridiflava*, strain 145 of *Aeromonas*, strain 189a of a yellow *Pseudomonas*, strain 1 of *Acinetobacter*, and strain Psp36 of *P. s. papulans*.

streptomycin-resistant *E. amylovora* in orchard A and of streptomycin-resistant *P. s. papulans* in orchard C confirms previous studies on resistant plant-pathogenic bacteria in Michigan apple orchards (4,10).

A yellow nonfluorescent *Pseudomonas* and *E. herbicola* were the most common streptomycin-resistant epiphytic bacteria detected in the six orchards. Yellow nonfluorescent pseudomonads were also common among the nonfluorescent gram-negative bacteria isolated in New York, but *E. herbicola* was not (17). As *E. herbicola* is often found in association with *E. amylovora* (19), the presence of fire blight in all orchards except orchard C may explain why we detected high populations of *E. herbicola*.

Although hybridization of DNA with

probe SMP3 was common among gram-negative streptomycin-resistant bacteria, the size of the *Ava*I restriction fragment that hybridized with the probe varied among the bacteria we examined. The 2.7-kb *Ava*I fragment from streptomycin-resistant *E. amylovora* was also found in digests of DNA from some epiphytic bacteria, the 1.5-kb *Ava*I fragment associated with streptomycin-resistant *P. s. papulans* was found in digests of DNA from other epiphytic bacteria, and a third restriction *Ava*I fragment (0.9-kb) was found in other epiphytic bacteria. The two largest restriction fragments were also observed by Norelli et al (17), but the third fragment they observed was larger than the third fragment we observed. As more bacteria are examined, even more polymorphism may be detected in the SMP3 region.

Failure of the DNA probe to hybridize with DNA from a small number of streptomycin-resistant gram-negative bacteria or from 28 gram-positive bacteria is evidence that streptomycin resistance in these bacteria may not be related to that in strains of bacteria possessing DNA that hybridizes to probe SMP3. Streptomycin-resistance genes in certain strains of *P. s. papulans* found in Ohio and in Michigan lack homology with SMP3 (10,17); these genes may be related to those in the epiphytic bacteria that did not hybridize with the probe.

The presence of homologous DNA associated with streptomycin resistance in both nonpathogenic and pathogenic bacteria that inhabit apple suggests that streptomycin resistance in *E. amylovora* and *P. s. papulans* may be acquired from nonpathogenic bacteria. Not only is DNA homologous with the SMP3 region found in many bacteria, but the DNA associated with resistance is often contained on a plasmid of the same size in these bacteria (17). Whether resistance is associated with a transposable element or only with selected plasmids is being investigated.

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