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Ecological and Genetic Analysis of Copper and Streptomycin Resistance in *Pseudomonas syringae* pv. Syringae†

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Strains of *Pseudomonas syringae* pv. syringae resistant to copper, streptomycin, or both compounds were recovered from symptomless and diseased tissue of four woody hosts in three nurseries in Oklahoma. In strains resistant to copper and streptomycin (Cu^r Sm^r), resistance to both compounds was cotransferred with a single plasmid which was either 68, 190, or 220 kilobase pairs (kb). All Cu^s Sm^r strains contained a 68-kb conjugative plasmid. Cu^r Sm^s strains contained one plasmid which varied in size from 60 to 73 kb. All conjugative plasmids which transferred streptomycin resistance contained sequences homologous to the *strA* and *strB* Sm^r genes from the broad-host-range plasmid RSF1010. The Sm^r determinant was subsequently cloned from a 68-kb Cu^r Sm^r plasmid designated pPSR1. A restriction map detailing the organization of the homologous Sm^r genes from pPSR1 and RSF1010 and cloned Sm^r genes from *P. syringae* pv. papulans and *Xanthomonas campestris* pv. vesicatoria revealed the conservation of all sites studied. The Cu^r genes cloned from *P. syringae* pv. tomato PT23 and *X. campestris* pv. vesicatoria XV10 did not hybridize to the Cu^r plasmids identified in the present study, indicating that copper resistance in these *P. syringae* pv. syringae strains may be conferred by a distinct genetic determinant.

In recent years, the severity of a tip dieback and canker disease of woody plants has increased in nurseries in eastern Oklahoma. The symptoms of this disease are similar to those observed on nursery trees in the Pacific Northwest which are infected with *Pseudomonas syringae* van Hall (9). Although bactericides containing copper and streptomycin have been used extensively in nurseries in Oklahoma for control of this disease, a reduction in disease incidence has not been observed.

The occurrence of copper-resistant (Cu^r) strains of the phytopathogens *P. syringae* (3, 5, 51) and *Xanthomonas campestris* pv. vesicatoria (1, 7, 15, 33) has been established. These bacteria were isolated from plant hosts on which copper bactericides had been utilized for disease control. Streptomycin was first utilized as a bactericide in agriculture in the late 1950s, and streptomycin resistance has since been reported in the phytopathogens *Erwinia amylovora* (11, 16, 31, 44), *Erwinia carotovora* (20), *Pseudomonas lachrymans* (54), *P. syringae* (8, 19, 24, 55), and *X. campestris* pv. vesicatoria (35, 48).

Genetic studies of bactericide resistance in phytopathogenic bacteria have increased in recent years (14). Copper resistance determinants have been localized to plasmid DNA in all phytopathogenic bacteria studied. In *P. syringae* pv. tomato, the copper resistance genes reside on a 35-kilobase-pair (35-kb) plasmid in strains isolated in California (13). In *X. campestris* pv. vesicatoria, the Cu^r determinant resides on large plasmids in strains isolated in Florida and Oklahoma (7, 47). Cu^r determinants have been cloned from *P. syringae* pv. tomato PT23 and *X. campestris* pv. vesicatoria XV10 (6, 21).

Sm^r determinants have been cloned from plasmids which reside in *P. syringae* pv. papulans (36) and *X. campestris* pv. vesicatoria (35). The 4.9-kb insert from the *X. campestris* pv. vesicatoria clone hybridized with plasmids indigenous to Sm^r *P. syringae* pv. papulans and other Sm^r *X. campestris* pv. vesicatoria strains (35). Additional hybridization work using a 500-bp fragment with specificity for the Sm^r determinant in *P. syringae* pv. papulans indicated that Sm^r gene homologs are found in *E. amylovora* (11), *P. syringae* pv. papulans (24), and *X. campestris* pv. vesicatoria (35). Although this Sm^r determinant appears widespread in distribution, the Sm^r genes from *P. syringae* pv. papulans and *X. campestris* pv. vesicatoria did not hybridize with DNA from all strains of Sm^r phytopathogenic bacteria tested, indicating that another resistance determinant may be present in some strains (11, 35).

Although streptomycin resistance has been intensively studied in clinical bacteria, only four types of streptomycin-inactivating enzymes are known, those being aminoglycoside phosphotransferase [APH(6) and APH(3″)] and aminoglycoside nucleotidyltransferase [ANT(6) and ANT(3″)] (18). The conservation of these four mechanisms in both gram-negative and gram-positive bacteria suggests that gene transfer has played an important role in the dissemination of Sm^r genes. For example, the nonconjugative IncQ plasmid RSF1010 (23), which encodes two tandem Sm^r genes, *strA* and *strB* (43), has been recovered from clinical isolates of *Escherichia coli*, *Proteus* spp., *Providencia* sp., *Pseudomonas aeruginosa*, *Salmonella* spp., and *Shigella flexneri* (4). The *strA* gene product is reported to be an aminoglycoside phosphotransferase [APH(3″)] (25). Two reports have indicated that Sm^r phytopathogenic bacteria also inactivate streptomycin by the APH(3″) mechanism (20, 54).

Although copper or streptomycin resistance has been identified in several phytopathogenic bacteria, we are aware of only one study reporting strains with resistance to both compounds (39). The involvement of plasmids in the resistance phenotypes of these strains was not studied, nor was the relatedness of the resistance genes contained in these strains compared with that of previously cloned resistance determinants.

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TABLE 1. Bacterial strains and plasmids and their relevant characteristics

Strain or plasmid	Host[a]	Chromosomal phenotype	Relevant characteristics	Reference or source
Strains				
Pseudomonas syringae pv. syringae				
A2	P		Cu^r Sm^r, pPSR1 (68 kb)	This study
AA2	P		Cu^r Sm^s, pPSR6 (70 kb)	This study
E2	C		Cu^r Sm^r, pPSR2 (190 kb)	This study
FF3	P		Cu^s Sm^r, pPSR5 (68 kb)	This study
FF5	P		Cu^s Sm^s	This study
FF5.1		Rif^r		This study
FF5.2		Cm^r		This study
G1	W		Cu^r Sm^r, pPSR3 (220 kb)	This study
H12	W		Cu^r Sm^r, pPSR3 (220 kb)	This study
pv. papulans (Psp36)			Sm^r, pCPP501	36
Xanthomonas campestris pv. vesicatoria BV5-4a			Sm^r	35
Plasmids				
RSF1010			*strA strB* Sm^r determinant	43
pSM1			3.7-kb insert containing Sm^r determinant from pPSR1 cloned into pBluescript SK	This study
pCPP505			2.5-kb insert containing Sm^r determinant from pCPP501 cloned into pBR322	36
pBV10			4.9-kb insert containing Sm^r determinant from BV5-4a cloned into pLAFR3	35
pCOP2			4.5-kb insert containing Cu^r determinant from PT23 cloned into pRK415	6
pCuR2			6.4-kb insert containing Cu^r determinant from XV10 cloned into pRK415	21

[a] P, ornamental pear; C, cottonwood; W, willow.

The long-term use of copper and streptomycin in nurseries in Oklahoma and the lack of disease control led us to believe that strains of *P. syringae* pv. syringae with resistance to both compounds were present in these nurseries. In this study, we surveyed three nurseries in Oklahoma to determine whether Cu^r and Sm^r strains of *P. syringae* pv. syringae were present and whether the resistance genes were encoded on self-transmissible plasmids. The relatedness of the Cu^r and Sm^r determinants contained in strains of *P. syringae* pv. syringae was compared with that of previously characterized resistance genes from other phytopathogenic bacteria. In this study, we detected sequences homologous to *strA* and *strB* from RSF1010 on conjugative plasmids which reside in strains of the phytopathogenic bacteria *P. syringae* pv. syringae, *P. syringae* pv. papulans, and *X. campestris* pv. vesicatoria.

(A preliminary report of this work has been published previously [49].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids and their relevant characteristics are listed in Table 1. The medium used for isolation of *P. syringae* from plant samples was medium B of King et al. (27) (KB) amended with 100 μg of cycloheximide per ml (KBc). Resistance to copper and streptomycin was determined with mannitol-glutamate medium (26) containing 0.25 g of yeast extract per liter (MGY) and amended with 250 μg of cupric sulfate per ml (MGYcu) or 25 μg of streptomycin sulfate per ml (MGYsm). Cupric sulfate and streptomycin sulfate were added to autoclaved, cooled medium from filter-sterilized stock solutions. The spontaneous mutants FF5.1 and FF5.2 were selected on MGY with 100 and 50 μg of rifampin (MGYrif) and chloramphenicol (MGYcm) per ml, respectively. *E. coli* was cultured on LB medium (34), and the antibiotics ampicillin and streptomycin were added to LB medium at concentrations of 40 and 25 μg/ml, respectively.

Source and identification of bacteria. Bacteria were isolated from cottonwood (*Populus* spp.), dogwood (*Cornus florida* L.), ornamental pear (*Pyrus calleryana* Dcne.), and willow (*Salix* spp.) in July 1988 and July 1990 from three nurseries in the eastern Oklahoma counties of Cherokee and Muskogee. Copper and streptomycin bactericides had been applied to trees in these nurseries for at least 3 consecutive years. Epiphytic bacteria were isolated by washing leaf surfaces in 0.01 M potassium phosphate buffer, pH 7.0 (K buffer), and plating dilutions on KBc. Bacteria were also recovered from cankers, dormant buds, and stem lesions by previously described methods (42). Individual plant samples were selected randomly from different trees. Isolates which were fluorescent on KBc and oxidase negative (42) were further characterized for gelatin liquefaction, esculin hydrolysis, tyrosinase activity, and utilization of tartrate (GATTa determinative tests; 29). Isolates which liquefied gelatin, hydrolyzed esculin, lacked tyrosinase activity, and did not utilize tartrate were identified as *P. syringae* pv. syringae.

Phenotypic characterization of copper and streptomycin resistance in *P. syringae* pv. syringae. Resistance to copper and streptomycin was tested by inoculating strains to MGYcu and MGYsm. Prior to testing, strains were grown for 48 h on KBc. Strains which exhibited growth on MGYcu or MGYsm equivalent to that on MGY after 72 h of growth at 24°C were considered resistant. MICs of copper and streptomycin were determined for resistant strains by inoc-

ulating them to MGY agar amended with 375, 500, or 750 μg of copper sulfate per ml or 50, 75, or 100 μg of streptomycin per ml.

Characterization of plasmids. Plasmids from *P. syringae* pv. syringae were isolated by the procedure of Crosa and Falkow (17) with slight modifications (5). Plasmids were separated on 0.7% agarose gels, and their sizes were estimated with previously characterized plasmid size standards. The size of pPSR3 was calculated from the sum of fragments generated by five restriction enzymes. The plasmids were initially grouped according to size and phenotype of the host strain. Representative strains from each plasmid group were chosen for conjugation experiments. To determine whether plasmids were conjugative, matings were conducted with *P. syringae* pv. syringae AA2 and FF3 as donors and FF5.1 as the recipient. The matings were conducted by mixing together 25 μl of donor and recipient cells (10^8 CFU/ml), inoculating the 50-μl suspension to KB agar, and incubating the cells for 12 h at 24°C. The cells were then suspended in K buffer, and appropriate dilutions were plated on MGYrif to enumerate recipients and MGYrif-cu or MGYrif-sm to enumerate transconjugants. Matings involving *P. syringae* pv. syringae A2, E2, and G1 with FF5.1 were conducted in the same manner, and transconjugants were initially selected on MGYrif-cu and MGYrif-sm. Twenty-five colonies from each medium were then transferred to the other medium to determine whether both resistance markers were transferred.

Molecular genetic techniques. Agarose gel electrophoresis, DNA restriction digests, and Southern transfers to nylon membranes (Amersham Corp., Arlington Heights, Ill.) were done by standard procedures (32). Specific DNA fragments for cloning or labeling were separated on agarose gels and isolated by electroelution (32). DNA fragments used as probes were labeled with digoxigenin (Genius kit; Boehringer Mannheim, Indianapolis, Ind.) as described by the manufacturer. Prehybridizations were conducted for a minimum of 1 h at 68 or 37°C for high- or moderate-stringency conditions, respectively. Hybridizations at high stringency were conducted at 68°C and were followed by two posthybridization washes of 5 min at 24°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0)–0.1% sodium dodecyl sulfate (SDS) and two washes of 15 min at 68°C in 0.1× SSC–0.1% SDS. Hybridizations at moderate stringency were conducted at 37°C and were followed by two posthybridization washes of 5 min at 24°C in 2× SSC–0.1% SDS and two washes of 15 min at 37°C in 0.1× SSC–0.1% SDS.

Cloning and characterization of the streptomycin resistance genes from pPSR1. The 1.5-kb *Sac*I-*Eco*RV fragment, which contains the Sm^r genes *strA* and *strB* from the broad-host-range plasmid RSF1010 (43), was used to identify homologous sequences on plasmids from Cu^s Sm^r and Cu^r Sm^r strains of *P. syringae* pv. syringae. The *Sac*I site is located 138 bp downstream of the translational initiation site for *strA*, and the *Eco*RV site is located 3 bp downstream from the stop codon for *strB*. Plasmids from Sm^r strains of the phytopathogens *P. syringae* pv. papulans Psp36 and *X. campestris* pv. vesicatoria BV5-4a were also included in the analysis. pPSR1, a 68-kb Cu^r Sm^r plasmid from *P. syringae* pv. syringae A2, hybridized with the *Sac*I-*Eco*RV fragment and was chosen for further study. *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I, which do not cut *strA* or *strB* internally (43), were used to identify restriction fragments in pPSR1 containing the *strA* and *strB* homologs.

A 3.7-kb *Pst*I fragment in pPSR1 hybridized to the *Sac*I-*Eco*RV fragment and was chosen for cloning experiments because it was the smallest hybridizing fragment and would presumably contain less extraneous DNA. This fragment was ligated into the *Pst*I site of pBluescript SK (Stratagene, La Jolla, Calif.), transformed into *E. coli* DH5α, and plated onto LB medium amended with streptomycin. pSM1, a recombinant plasmid resulting from this experiment, contained the 3.7-kb *Pst*I fragment from pPSR1 and conferred streptomycin resistance to *E. coli* DH5α.

The coding sequence of *strA* and *strB* in RSF1010 encompasses positions 63 to 863 and 866 to 1699, respectively, with the numbering convention from the published sequence (43). Restriction sites internal to *strA* and *strB* in RSF1010 and additional restriction sites outside the coding sequence were obtained from the published sequence (43). To determine whether the restriction sites in *strA* and *strB* and adjacent sequences were conserved in the Sm^r genes from the phytopathogenic bacteria, pSM1, pBV10, and pCPP505 were digested with the following enzymes and enzyme combinations: *Ssp*I-*Eco*RV, *Nru*I, *Nru*I-*Eco*RV, *Sac*II-*Eco*RV, *Ava*II, *Sac*I-*Ava*II, *Sac*I-*Eco*RV, *Ava*I-*Eco*RV, *Rsa*I, *Nco*I-*Eco*RV, *Ava*II-*Eco*RV, *Dde*I-*Eco*RV, *Dde*I-*Sac*I, *Bst*YI-*Sac*I, *Not*I-*Sac*I, *Eco*RI-*Eco*RV, and *Hin*cII-*Eco*RV. The digestion products were electrophoresed in agarose gels adjacent to RSF1010 digested with the same enzyme combinations, blotted, and probed with the 1.5-kb *Sac*I-*Eco*RV fragment from RSF1010.

Hybridization experiments with DNA probes containing copper resistance determinants from phytopathogenic bacteria. DNA-DNA hybridization experiments were conducted to evaluate the relationship of the Cu^r determinants in strains of *P. syringae* pv. syringae with Cu^r genes from other phytopathogens. DNA fragments used as probes were (i) the 4.5-kb *Pst*I fragment in pCOP2 containing the Cu^r determinant from *P. syringae* pv. tomato PT23 (6) and (ii) a 4.6-kb *Eco*RI-*Hin*dIII fragment in pCuR2 containing the Cu^r determinant from *X. campestris* pv. vesicatoria (21). Southern blot hybridizations were first conducted under the high-stringency conditions described above, and following negative results, under the conditions described for moderate stringency.

RESULTS

Source and identification of bacteria. In 1988, 11 isolates, each from a single colony, were recovered from two stems of willow showing tip dieback symptoms. In 1990, six, four, and eight isolates were recovered from stems of cottonwood, dogwood, and willow, respectively, which also exhibited tip dieback symptoms. A total of 10 isolates were recovered in 1990 from the surfaces of two leaves of cottonwood, 3 isolates were recovered from two symptomless petioles of ornamental pear, and an additional 66 isolates were recovered from the interior of 13 symptomless buds of ornamental pear. All of these isolates were oxidase negative, liquefied gelatin, hydrolyzed esculin, lacked tyrosinase activity, and did not utilize tartrate as the sole carbon source. These biochemical traits are characteristic of *P. syringae* pv. syringae (29).

Phenotypic characterization of copper and streptomycin resistance. Growth of bacteria on MGYcu (250 μg/ml) or MGYsm (25 μg/ml) was sufficient to identify strains with resistance to copper or streptomycin because Cu^s Sm^s strains of *P. syringae* pv. syringae did not grow on these media. A summary of the resistance phenotypes from the 108 *P. syringae* pv. syringae strains is presented in Table 2.

TABLE 2. Number of *P. syringae* pv. syringae strains with resistance to copper and/or streptomycin

Host[a]	Resistance phenotype (no. of strains)			
	$Cu^s\ Sm^s$	$Cu^r\ Sm^s$	$Cu^s\ Sm^r$	$Cu^r\ Sm^r$
Cottonwood	0	0	0	16
Dogwood	4	0	0	0
Ornamental pear	7	46	11	5
Willow	8	0	0	11

[a] Host plants originated from three nurseries in eastern Oklahoma.

The MIC of cupric sulfate varied among Cu^r strains from 375 to 500 μg/ml. The MIC of streptomycin was 75 μg/ml for all Sm^r strains studied.

Characterization of plasmids. A single plasmid of 68 kb (pPSR1, Fig. 1, lane 2) was detected in all $Cu^r\ Sm^r$ strains of *P. syringae* pv. syringae isolated from ornamental pear. A single plasmid of 190 kb (pPSR2; Fig. 1, lane 4) was detected in all $Cu^r\ Sm^r$ strains of *P. syringae* pv. syringae isolated from cottonwood. A 220-kb plasmid (pPSR3; Fig. 1, lane 5) was detected in all 11 $Cu^r\ Sm^r$ strains isolated from willow. Ten of these strains also contained a smaller plasmid of 60 to 90 kb. All $Cu^r\ Sm^s$ and $Cu^s\ Sm^r$ strains isolated from ornamental pear contained one plasmid which varied in size from 60 to 73 kb (Fig. 1, lanes 1 and 3). No plasmids were detected in $Cu^s\ Sm^s$ strains of *P. syringae* pv. syringae isolated from dogwood, ornamental pear, or willow.

Plasmids representing all sizes and all resistance phenotypes were tested for conjugative ability in plate matings with the $Cu^s\ Sm^s\ Rif^r$ recipient, FF5.1. The frequency of spontaneous mutations to streptomycin and copper resistance in FF5.1 was 2.0×10^{-9} and less than 1.8×10^{-10}, respectively. Strains containing pPSR1, pPSR2, and pPSR3 transferred Cu^r and Sm^r to FF5.1 at frequencies of 9.3×10^{-8}, 1.4×10^{-2}, and 1.8×10^{-6} per recipient cell, respectively. Following selection on MGYrif-cu or MGYrif-sm, all Cu^r or Sm^r colonies grew after transfer to the other medium (MGYrif-sm or MGYrif-cu, respectively). Putative transconjugants of FF5.1 contained the appropriate plasmid when examined by agarose gel electrophoresis (data not shown). Since pPSR1 was transferred to FF5.1 at a low frequency, a $Cu^r\ Sm^r\ Rif^r$ transconjugant of FF5.1 containing pPSR1 was mated with FF5.2 ($Cm^r\ Rif^s\ Cu^s\ Sm^s$). Transfer of pPSR1 to FF5.2 and acquisition of $Cu^r\ Sm^r$ was detected at a frequency of 9.6×10^{-6}, which is higher than the frequency of spontaneous mutations to Rif^r in FF5.2 and to Cm^r in FF5.1. pPSR5, a 68-kb plasmid from the $Cu^s\ Sm^r$ strain FF3, transferred Sm^r to FF5.1 at a frequency of 5.8×10^{-6} per recipient cell. Transfer of Cu^r was not detected when the $Cu^r\ Sm^s$ strain AA2 and five additional $Cu^r\ Sm^s$ strains were used as donors.

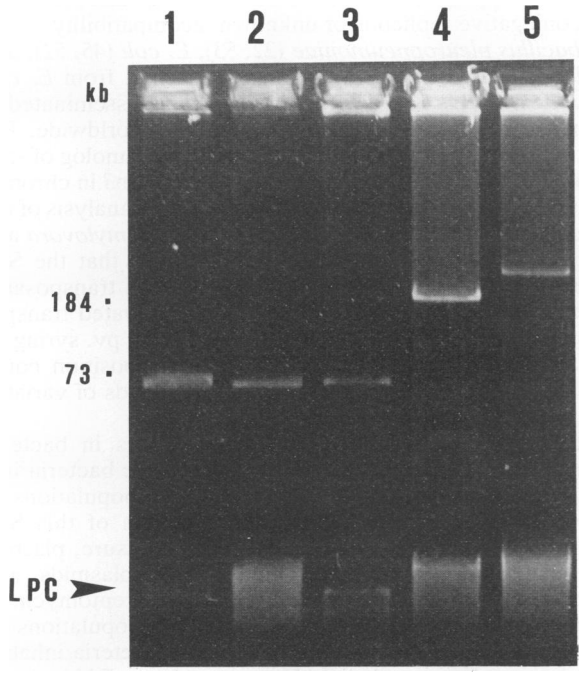

FIG. 1. Plasmid profiles of *P. syringae* pv. syringae strains isolated from woody hosts. Lanes: 1, FF3(pPSR5); 2, A2(pPSR1); 3, AA2(pPSR6); 4, E2(pPSR2); 5, H12(pPSR3). Plasmid size standards are listed at left. LPC is linearized plasmid and chromosomal DNA.

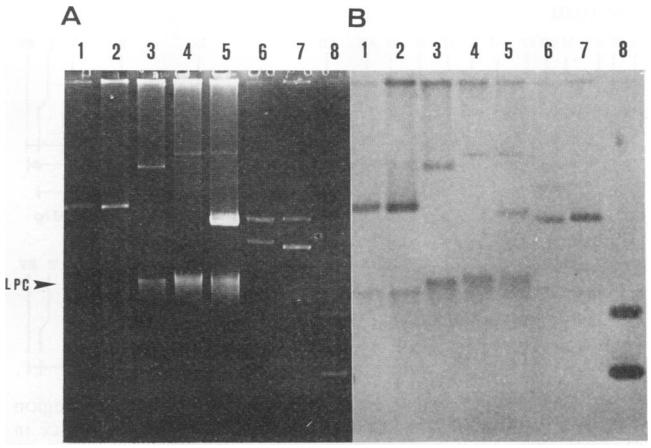

FIG. 2. Agarose gel electrophoresis (A) and hybridization (B) of plasmid DNA from Sm^r phytopathogenic bacteria with the 1.5-kb *Sac*I-*Eco*RV fragment containing *strA* and *strB* from plasmid RSF1010. Lanes 1 to 5 contain the following strains of *P. syringae* pv. syringae: 1, FF3(pPSR5); 2, A2(pPSR1); 3, E2(pPSR2); 4, H12(pPSR3); 5, G1(pPSR3); 6, *P. syringae* pv. papulans Psp36; 7, *X. campestris* pv. vesicatoria BV5-4a; and 8, *E. coli*(RSF1010). LPC is linearized plasmid and chromosomal DNA.

Cloning and characterization of the streptomycin resistance genes from *P. syringae* pv. syringae. The *Sac*I-*Eco*RV fragment from RSF1010 hybridized to self-transmissible $Cu^r\ Sm^r$ plasmids of 68, 190, and 220 kb in four *P. syringae* pv. syringae strains (Fig. 2, lanes 2 to 5) and to a 68-kb self-transmissible Sm^r plasmid in one strain (Fig. 2, lanes 1). This fragment also hybridized to the smaller plasmid present in 8 of 10 $Cu^r\ Sm^r$ strains isolated from willow. Although these strains presumably contained copies of the Sm^r determinant on plasmids which varied in size, only pPSR3 (220 kb) was self-transmissible. Additionally, the MIC of streptomycin was identical for *P. syringae* pv. syringae strains isolated from willow regardless of whether they contained pPSR3 alone or pPSR3 plus a smaller plasmid. The probe did not hybridize to plasmid DNA contained in $Cu^r\ Sm^s$ strains of *P. syringae* pv. syringae (data not shown). However, it did hybridize to plasmids present in the Sm^r strains *P. syringae* pv. papulans Psp36 (Fig. 2, lanes 6) and *X. campestris* pv. vesicatoria BV5-4a (Fig. 2, lanes 7).

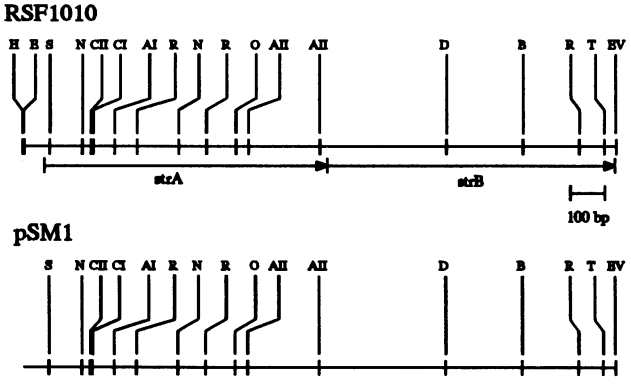

FIG. 3. Conservation of restriction sites over a 1.7-kb region encoding *strA* and *strB* in RSF1010 and streptomycin resistance in pSM1. The following position numbers (in parentheses) are from the published RSF1010 sequence (43): E, *Eco*RI (8676); H, *Hin*cII (8682); S, *Ssp*I (75); N, *Nru*I (169, 441); CII, *Sac*II (194); CI, *Sac*I (201); AI, *Ava*I (258); R, *Rsa*I (324, 520, 1594); O, *Nco*I (605); AII, *Ava*II (638, 845); D, *Dde*I (1209); B, *Bst*YI (1429); T, *Not*I (1664); EV, *Eco*RV (1702). Arrows indicate the direction of transcription.

The *Sac*I-*Eco*RV fragment from RSF1010 hybridized to restriction fragments from pPSR1 which were 16.0 kb (*Bam*HI), 10.0 kb (*Hin*dIII), 9.6 kb (*Eco*RI), and 3.7 kb (*Pst*I). The 3.7-kb *Pst*I fragment was selected for cloning and was ligated into pBluescript SK, creating the plasmid pSM1. Transformants of *E. coli* DH5α containing pSM1 expressed streptomycin resistance. Restriction mapping and hybridization analyses indicated that the relative positions of selected restriction sites internal to *strA* and *strB* in RSF1010 were conserved in the *P. syringae* pv. syringae clone pSM1 (Fig. 3). The *Eco*RV site (position 1702) in RSF1010 was also present in pSM1. The restriction sites shown in Fig. 3 for pSM1 were also present in the same relative positions in pBV10 and pCPP505 (data not shown). The *Eco*RI and *Hin*cII sites which are located 71 and 65 bp, respectively, upstream from the translational start site in *strA* (Fig. 3, RSF1010 map) were not present in pSM1, pBV10, or pCPP505. The restriction mapping data, combined with the hybridization data at high stringency, demonstrated that the Sm^r determinant in *P. syringae* pv. syringae, *P. syringae* pv. papulans, and *X. campestris* pv. vesicatoria was similar if not identical to *strA* and *strB* from RSF1010.

Hybridization experiments with copper resistance gene probes. Restriction fragments containing the Cu^r determinants from *P. syringae* pv. tomato PT23 and *X. campestris* pv. vesicatoria XV10 were used as probes in Southern blot hybridization experiments with plasmids from 18 Cu^r Sm^r, Cu^r Sm^s, or Cu^s Sm^r strains of *P. syringae* pv. syringae. The restriction fragments containing the Cu^r determinants did not hybridize to any of the *P. syringae* pv. syringae plasmids under conditions of high or moderate stringency (data not shown).

DISCUSSION

To our knowledge, this is the first report demonstrating the linkage of Cu^r and Sm^r determinants on conjugative plasmids in a phytopathogenic bacterium. Presumably, in response to the selection pressure of copper and streptomycin bactericidal sprays, the *P. syringae* pv. syringae population has developed resistance to one or both of these compounds. Surprisingly, the majority of strains isolated from ornamental pear were resistant to copper or streptomycin alone. This could indicate a more recent evolution of the plasmids which encode both Cu^r and Sm^r determinants.

We identified plasmids of three distinct sizes which transferred copper and streptomycin resistance to Cu^s Sm^s recipients in laboratory matings. In our survey, a 68-kb plasmid designated pPSR1 was detected in each of five Cu^r Sm^r strains isolated from ornamental pear; a 190-kb plasmid, pPSR2, was detected in each of 16 Cu^r Sm^r strains isolated from cottonwood; and a 220-kb plasmid, pPSR3, was detected in 11 Cu^r Sm^r strains isolated from willow. Plasmids detected in Cu^r Sm^s or Cu^s Sm^r strains from ornamental pear ranged in molecular size from 60 to 73 kb. Resistance plasmids of different sizes have been isolated previously from local populations of *P. syringae* (24). The diversity in plasmid size may indicate the ability of resistance determinants to transfer among indigenous plasmids in a bacterial population.

In the present study, the Sm^r determinants from *P. syringae* pv. papulans, *P. syringae* pv. syringae, and *X. campestris* pv. vesicatoria were shown to be homologous to the *strA-strB* genes of the broad-host-range enterobacterial plasmid RSF1010 (43). Recently, Chiou and Jones (12) sequenced the Sm^r determinant in *E. amylovora* and *P. syringae* pv. papulans and demonstrated that they are identical to the *strA-strB* genes in RSF1010. Sequence analysis has also indicated that a portion of the *strA* homolog in pSM1 is identical to *strA* in RSF1010 (50). Because RSF1010 encodes an APH(3''), it is possible that Sm^r strains of *E. carotovora* and *P. lachrymans* from Japan, which produce an APH(3'') (20, 54), also contain *strA* and *strB* homologs. Among Sm^r bacteria isolated from humans and animals, sequences homologous to *strA* and *strB* from RSF1010 have been identified on related IncQ plasmids in *Bordetella bronchiseptica* (30), *Branhamella* spp. (37), *E. coli* (41), and *Neisseria* spp. (40). *strA* and *strB* homologs also occur on nonconjugative replicons of unknown incompatibility in *Actinobacillus pleuropneumoniae* (22, 53), *E. coli* (45, 52), and *S. flexneri* (10) and on a conjugative plasmid from *E. coli* (38). Clearly, this Sm^r determinant has been disseminated to plasmids inhabiting widely varied bacteria worldwide. Recently, Lacroix and Walker (28) identified a homolog of *strA* associated with sequences of the transposon Tn*3* in chromosomal DNA of *Eikenella corrodens*. Sequence analysis of the region flanking the *strA-strB* genes in both *E. amylovora* and *P. syringae* pv. papulans has also suggested that the Sm^r genes in these bacteria are contained on a transposable element (12). Although we have not demonstrated transposition of the Sm^r genes encoded by *P. syringae* pv. syringae, the acquisition of this determinant by transposition could explain the presence of these genes on plasmids of variable size.

The discovery of *strA* and *strB* homologs in bacteria pathogenic to humans and in phytopathogenic bacteria is a powerful example of the ability of bacterial populations to respond to selection pressure. Dissemination of this Sm^r determinant may be affected by selection pressure, plasmid transfer, transfer of the determinant among plasmids, and gene expression. In environments where streptomycin is used as a bactericide, Sm^r is selected in populations of phytopathogenic bacteria and in nontarget bacteria inhabiting plant surfaces and soil. For example, a DNA probe specific for the Sm^r determinant from *P. syringae* pv. papulans was used to study the distribution of Sm^r homologs in target and nontarget bacteria. Homologs were detected in miscellaneous gram-negative, Sm^r bacteria isolated from

leaves and soil of apple orchards in New York (36) and from apple leaves in Michigan (46). Streptomycin had been previously applied in some but not all of the orchards sampled. Exposure of plant surfaces and soil to streptomycin may selectively enrich organisms carrying this determinant, eventually resulting in its transfer to phytopathogenic bacteria.

Natural populations of *P. syringae* pv. syringae in Oklahoma appear to be in a state of flux regarding copper and streptomycin resistance. Plasmids encoding Cu^r or Sm^r phenotypes are still detected in addition to Cu^s Sm^s strains which do not contain plasmids. However, the continued practice of repeated applications of copper and streptomycin in Oklahoma nurseries may eventually result in a population which is uniformly resistant to both bactericidal compounds.

The choice of bactericides is currently limited in agriculture. Accompanying this problem for agricultural producers is the prospect of resistance to bactericides in pathogen populations following intensive use of currently available bactericidal agents. The use of medically important antibiotics in agriculture was discouraged by the Bacteriology Committee of the American Phytopathological Society in 1978, because the selection of resistance genes in plant-inhabiting bacteria could possibly have an impact on human health (2). Our results indicate that antibiotic resistance genes which have been selected in bacterial pathogens of humans have also been selected in distinct populations of phytopathogenic bacteria. Although alternative bactericides are needed in agriculture, the use of other registered antibiotics such as tetracycline may select for additional gene transfer events and result in the further development of antibiotic resistance determinants in phytopathogenic bacteria.

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REFERENCES

1. **Adaskaveg, J. E., and R. B. Hine.** 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. vesicatoria, causal agent of bacterial spot of pepper. Plant Dis. **69**:993–996.
2. **American Phytopathological Society Bacteriology Committee.** 1978. Antibiotic use for control of bacterial diseases of plants. Phytopathol. News **12**:54–55.
3. **Andersen, G. L., O. Menkissoglou, and S. E. Lindow.** 1991. Occurrence and properties of copper-tolerant strains of *Pseudomonas syringae* isolated from fruit trees in California. Phytopathology **81**:648–656.
4. **Barth, P. T., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120**:618–630.
5. **Bender, C. L., and D. A. Cooksey.** 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. **165**:534–541.
6. **Bender, C. L., and D. A. Cooksey.** 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. **169**:470–474.
7. **Bender, C. L., D. K. Malvick, K. E. Conway, S. George, and P. Pratt.** 1990. Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Appl. Environ. Microbiol. **56**:170–175.
8. **Burr, T. J., J. L. Norelli, B. Katz, W. F. Wilcox, and S. A. Hoying.** 1988. Streptomycin-resistance of *Pseudomonas syringae* pv. *papulans* in apple orchards and its association with a conjugative plasmid. Phytopathology **78**:410–413.
9. **Canfield, M. L., S. Baca, and L. W. Moore.** 1986. Isolation of *Pseudomonas syringae* from 40 cultivars of diseased woody plants with tip dieback in Pacific Northwest nurseries. Plant Dis. **70**:647–650.
10. **Chineault, A. C., V. A. Blakesley, E. Roessler, D. G. Willis, C. A. Smith, R. G. Cook, and R. G. Fenwick.** 1986. Characterization of transferable plasmids from *Shigella flexneri* 2a that confer resistance to trimethoprim, streptomycin, and sulfonamides. Plasmid **15**:119–131.
11. **Chiou, C.-S., and A. L. Jones.** 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. Phytopathology **81**:710–714.
12. **Chiou, C.-S., and A. L. Jones.** 1993. Nucleotide sequence analysis of a transposon (Tn*5393*) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. J. Bacteriol. **175**:732–740.
13. **Cooksey, D. A.** 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. Appl. Environ. Microbiol. **53**:454–456.
14. **Cooksey, D. A.** 1990. Genetics of bactericide resistance in plant pathogenic bacteria. Annu. Rev. Microbiol. **28**:201–219.
15. **Cooksey, D. A., H. R. Azad, J.-S. Cha, and C.-K. Lim.** Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. Appl. Environ. Microbiol. **56**:431–435.
16. **Coyier, D. L., and R. P. Covey.** 1975. Tolerance of *Erwinia amylovora* to streptomycin sulfate in Oregon and Washington. Plant Dis. Rep. **59**:849–852.
17. **Crosa, J. H., and S. Falkow.** 1981. Plasmids, p. 266–282. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
18. **Davies, J. E., and D. Smith.** 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32**:469–518.
19. **DeBoer, S. H.** 1980. Leaf spot of cherry laurel caused by *Pseudomonas syringae*. Can. J. Plant Pathol. **2**:235–238.
20. **Fukusawa, K., H. Sakurai, S. Shimizu, H. Naganawa, S. Kondo, H. Kawabe, and S. Mitsuhashi.** 1980. 3''-phosphoryldihydrostreptomycin produced by the inactivating enzyme of *Erwinia carotovora*. J. Antibiot. **33**:122–123.
21. **Garde, S., and C. L. Bender.** 1991. DNA probes for detection of copper resistance genes in *Xanthomonas campestris* pv. vesicatoria. Appl. Environ. Microbiol. **57**:2435–2439.
22. **Gilbride, K. A., S. Rosendal, and J. L. Brunton.** 1989. Plasmid mediated antimicrobial resistance in Ontario isolates of *Actinobacillus (Haemophilus) pleuropneumoniae*. Can. J. Vet. Res. **53**:38–42.
23. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619–630.
24. **Jones, A. L., J. L. Norelli, and G. Ehret.** 1991. Detection of streptomycin-resistant *Pseudomonas syringae* pv. *papulans* in Michigan apple orchards. Plant Dis. **75**:529–531.
25. **Kawabe, H., T. Tanaka, and S. Misuhashi.** 1978. Streptomycin resistance and spectinomycin resistance mediated by plasmids. Antimicrob. Agents Chemother. **13**:1031–1035.
26. **Keane, P. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. **23**:585–595.
27. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
28. **Lacroix, J.-M., and C. B. Walker.** 1992. Identification of a streptomycin resistance gene and a partial Tn*3* transposon coding for a β-lactamase in a periodontal strain of *Eikenella corrodens*. Antimicrob. Agents Chemother. **36**:740–743.
29. **Latorre, B. A., and A. L. Jones.** 1979. *Pseudomonas morsprunorum*, the cause of bacterial canker of sour cherry in Michigan, and its epiphytic association with *P. syringae*. Phytopathology **69**:335–339.

30. **Lax, A. J., and C. A. Walker.** 1986. Plasmids related to RSF1010 from *Bordetella bronchiseptica*. Plasmid **15:**210–216.
31. **Loper, J. E., M. D. Henkels, R. G. Roberts, G. G. Grove, M. J. Willet, and T. J. Smith.** 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington state. Plant Dis. **75:**287–290.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Marco, G. M., and R. E. Stall.** 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Dis. **67:**779–781.
34. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Minsavage, G. V., B. I. Canteros, and R. E. Stall.** 1990. Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **80:**719–723.
36. **Norelli, J. L., T. J. Burr, A. M. LoCicero, M. T. Gilbert, and B. H. Katz.** 1991. Homologous streptomycin resistance gene present among diverse gram-negative bacteria in New York state apple orchards. Appl. Environ. Microbiol. **57:**486–491.
37. **Pintado, C., C. Salvador, R. Rotger, and C. Nombela.** 1985. Multiresistance plasmid from commensal *Neisseria* strains. Antimicrob. Agents Chemother. **27:**120–124.
38. **Rådström, P., and G. Swedberg.** 1988. RSF1010 and a conjugative plasmid contain *sulII*, one of two known genes for plasmid-borne sulfonamide resistance dihydropteroate synthase. Antimicrob. Agents Chemother. **32:**1684–1692.
39. **Ritchie, D. F., and V. Dittapongpitch.** 1991. Copper- and streptomycin-resistant strains and host differentiated races of *Xanthomonas campestris* pv. *vesicatoria* in North Carolina. Plant Dis. **75:**733–736.
40. **Rotger, R., F. Rubio, and C. Nombela.** 1986. A multi-resistance plasmid isolated from commensal *Neisseria* species is closely related to the enterobacterial plasmid RSF1010. J. Gen. Microbiol. **132:**2491–2496.
41. **Saano, A. K., and V. V. Zinchenko.** 1987. A new IncQ plasmid R89S: properties and genetic organization. Plasmid **17:**191–201.
42. **Schaad, N. W. (ed.).** 1988. Laboratory guide for identification of plant pathogenic bacteria, 2nd ed. American Phytopathological Society, St. Paul, Minn.
43. **Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75:**271–288.
44. **Schroth, M. N., S. V. Thomson, and W. J. Moller.** 1978. Streptomycin resistance in *Erwinia amylovora*. Phytopathology **69:**565–568.
45. **Shareck, F., M. Zollinger, and A. Sasarman.** 1983. Structure and molecular properties of the R.Sm.Su plasmid pSAS1206. Plasmid **9:**215–217.
46. **Sobiczewski, P., C.-S. Chiou, and A. L. Jones.** 1991. Streptomycin-resistant epiphytic bacteria with homologous DNA for streptomycin resistance in Michigan apple orchards. Plant Dis. **75:**1110–1113.
47. **Stall, R. E., D. C. Loschke, and J. B. Jones.** 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **76:**240–243.
48. **Stall, R. E., and P. L. Thayer.** 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. Plant Dis. Rep. **46:**389–392.
49. **Sundin, G. W., and C. L. Bender.** 1991. Characterization of copper- and streptomycin-resistant strains of *Pseudomonas syringae*. Phytopathology **81:**1145.
50. **Sundin, G. W., and C. L. Bender.** Unpublished observations.
51. **Sundin, G. W., A. L. Jones, and D. W. Fulbright.** 1989. Copper resistance in *Pseudomonas syringae* pv. syringae and its associated transfer *in vitro* and *in planta* with a plasmid. Phytopathology **79:**861–865.
52. **van Treeck, U., F. Schmidt, and B. Wiedemann.** 1981. Molecular nature of a streptomycin and sulfonamide resistance plasmid (pBP1) prevalent in clinical *Escherichia coli* strains and integration of an ampicillin resistance transposon (Tn*A*). Antimicrob. Agents Chemother. **19:**371–380.
53. **Willson, P. J., H. G. Deneer, A. Potter, and W. Albritton.** 1989. Characterization of a streptomycin-sulfonamide resistance plasmid from *Actinobacillus pleuropneumoniae*. Antimicrob. Agents Chemother. **33:**235–238.
54. **Yano, H., H. Fujii, H. Mukoo, M. Shimura, T. Watanabe, and Y. Sekizawa.** 1978. On the enzymic inactivation of dihydrostreptomycin by *Pseudomonas lachrymans*, cucumber angular leaf spot bacterium: isolation and structural resolution of the inactivated product. Ann. Phytopathol. Soc. Jpn. **44:**413–419.
55. **Young, J. M.** 1977. Resistance in streptomycin in *Pseudomonas syringae* from apricot. N.Z. J. Agric. Res. **20:**249–251.