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Genetic and Plasmid Diversity within Natural Populations of *Pseudomonas syringae* with Various Exposures to Copper and Streptomycin Bactericides

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We examined the genetic and plasmid diversity within natural populations of *Pseudomonas syringae* isolated from three ornamental pear nurseries in eastern Oklahoma. The bactericide spray regimen differed at each nursery; copper and streptomycin, only copper, and no bactericides were applied at nurseries I, II, and III, respectively. Resistance to copper (Cu^r) and resistance to streptomycin (Sm^r) were determined for 1,938 isolates of *P. syringae*; isolates from nurseries I and II were generally Cu^r Sm^s; whereas most isolates from nursery III were Cu^s Sm^s. The plasmid profiles of 362 isolates were determined, and six, one, seven, and four plasmid profiles were obtained for Cu^r, Sm^r, Cu^r Sm^r, and Cu^s Sm^s isolates, respectively. All Sm^r plasmids contained sequences homologous to the *strA* and *strB* Sm^r genes from broad-host-range plasmid RSF1010 and were associated with Sm^r transposon Tn*5393*. Plasmids were placed into two groups on the basis of hybridization to the *oriV* and *par* sequences from pOSU900, a cryptic plasmid in *P. syringae* pv. syringae. A total of 100 randomly chosen *P. syringae* isolates from nurseries I and III were analyzed for genetic diversity by using the arbitrarily primed PCR (AP-PCR) technique. An analysis of chromosomal genotypes by AP-PCR revealed a high degree of genetic diversity among the isolates, and the results of this analysis indicated that the isolates could be clustered into two distinct groups. The plasmid profiles were specific to isolates belonging to particular AP-PCR groups. Within each AP-PCR group, identical plasmid profiles were produced by isolates that had different chromosomal genotypes, implying that plasmid transfer has played an important role in the dissemination of Cu^r and Sm^r within the populations studied.

The effective management of many important plant diseases caused by phytopathogenic bacteria has been compromised during the past three decades because of the evolution of resistance to the commonly used bactericides copper and streptomycin. Plasmid-encoded resistance to copper (Cu^r) and resistance to streptomycin (Sm^r) have become increasingly widespread in *Erwinia amylovora*, *Pseudomonas syringae*, and *Xanthomonas campestris* (11).

Several Cu^r determinants from *P. syringae* and *X. campestris* have been cloned and characterized (3, 21, 32, 64). The plasmid-encoded Cu^r determinant in *P. syringae* pv. tomato (the *cop* operon) has been extensively studied (13) and has been detected in other Cu^r bacteria (12, 14, 32, 35, 54). However, workers have also isolated Cu^r strains whose operons do not exhibit homology to the *cop* operon (59) or differ from the *cop* operon in the mechanism of resistance (1).

Sm^r determinants have been cloned from a number of phytopathogenic bacteria, and recent results have revealed homology among the Sm^r determinants from *P. syringae* pv. papulans, *P. syringae* pv. syringae, and *X. campestris* pv. vesicatoria (59). These bacteria were isolated from diverse geographic areas, indicating that the Sm^r determinant has been widely disseminated. Further characterization of the Sm^r determinant revealed homology to the *strA-strB* genes of broad-host-range plasmid RSF1010 (59) and showed that this determinant was encoded by Tn*5393*, a member of the Tn*3* family (8). Tn*5393* was originally characterized by using *Erwinia amylovora* strains recovered from orchards in Michigan and was also detected in phylloplane and soil bacteria inhabiting apple orchards in regions where streptomycin was applied as a bactericide (45, 56).

In clinical bacteria, the evolution of antibiotic resistance occurs by the dissemination of a resistant strain, the spread of a plasmid among bacterial species or genera, or the spread of a specific resistance determinant among different plasmids and chromosomes (53). The evolution of copper and streptomycin resistance in phytopathogenic bacteria probably occurs via these same mechanisms, although the contributions of these mechanisms to the evolution of resistance in natural populations are for the most part unknown. Conjugal transfer of indigenous plasmids encoding copper and/or streptomycin resistance has been demonstrated in vitro for *Erwinia amylovora*, *P. syringae*, and *X. campestris* (2, 5, 7, 57, 59, 62). However, dissemination of resistance plasmids in natural populations of phytopathogenic bacteria is not well understood. In *P. syringae* pv. tomato, a single Cu^r plasmid, pPT23D, was detected in all Cu^r strains examined, and genomic digests of these strains could be distinguished from genomic digests of Cu^s strains (9, 15). Since the genetic diversity within *P. syringae* pv. tomato is relatively low (19), the lack of many competing genotypes, coupled with selection pressure from copper sprays, may have enhanced the selection of a single Cu^r *P. syringae* pv. tomato genotype in California. However, the genetic diversity within *P. syringae* pv. syringae is inherently higher than the genetic diversity that has been described for *P. syringae* pv. tomato (19, 33). Therefore, the consequences of exposing a heterogeneous population of *P. syringae* pv. syringae to copper

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TABLE 1. Bacterial strains and plasmids used and their relevant characteristics

Strain or plasmid	Chromosomal phenotype	Plasmid profile	Relevant characteristics	Reference
P. syringae pv. syringae strains				
7B44		1	Cu^r Sm^s; pPSR7	This study
7G14		1A	Cu^r Sm^s; pPSR8, pPSC6	This study
2H12		2	Cu^r Sm^s; pPSR9	This study
3C1		3	Cu^r Sm^s; pPSR10	This study
8B48		4	Cu^r Sm^s; pPSR11	This study
7A36		5	Cu^r Sm^s; pPSR12, pPSC7	This study
7C12		6	Cu^r Sm^r; pPSR13, pPSR21	This study
7B12		7	Cu^r Sm^r; pPSR14	This study
8C32		8	Cu^r Sm^r; pPSR15	This study
9A26		8A	Cu^r Sm^r; pPSR16, pPSC8	This study
2E49		9	Cu^r Sm^r; pPSR17	This study
7B22		10	Cu^r Sm^r; pPSR18	This study
7F14		11	Cu^r Sm^r; pPSR19	This study
9A22		12	Cu^s Sm^r; pPSR20	This study
7G43		C1	Cu^s Sm^s; pPSC1	This study
7E42		C2	Cu^s Sm^s; pPSC2	This study
8B24		C3	Cu^s Sm^s; pPSC3	This study
7C27		C4	Cu^s Sm^s; pPSC4, pPSC5	This study
9C11.2	Cm^r			This study
B48			Isolated from peach	18
B61			Isolated from wheat	18
B64			Isolated from wheat	18
B76			Isolated from tomato	18
B78			Isolated from tomato	18
PSC1B			Isolated from corn	18
P. syringae pv. morsprunorum 3714			INA^-	J. M. Young
Plasmids				
RSF1010			*strA strB* Sm^r determinant	52
pPSR1			Cu^r Sm^r; contains Tn*5393*	59
pOSU22			*oriV* and *par* loci from pOSU900	42

and streptomycin bactericides are not known. In previous studies, resistance plasmids of various sizes were recovered from *P. syringae* pv. syringae strains inhabiting various tree hosts, implying that different plasmid genotypes were present in the populations (59, 62). However, information about the chromosomal genotypes in indigenous populations of *P. syringae* pv. syringae is also necessary in order to understand the evolution of copper and streptomycin resistance in this bacterium.

Arbitrarily primed PCR (AP-PCR) analysis is a sensitive method which can be used to fingerprint genomes (66, 68). In the AP-PCR technique single oligonucleotide primers and low-stringency PCR are used to generate patterns of DNA fragments which are strain specific. The results obtained by AP-PCR are consistent with multilocus enzyme electrophoresis, DNA-DNA hybridization, and restriction fragment length polymorphism data (65, 67). The use of primer sets targeted to specific sites within the genome, such as the repetitive extrachromosomal palindromic (REP) regions of gram-negative bacteria (63), increases the efficiency of this technique for differentiating closely related bacterial strains. The relative positions of REP sequences in the genomes of gram-negative bacteria are conserved in closely related strains and distinct in unrelated species and genera (37). The REP primers have been used to determine fingerprints for the genomes of several gram-negative bacteria isolated from soil (17, 63), and the results of one study were consistent with the results generated by multilocus enzyme electrophoresis (17).

In Oklahoma, copper and streptomycin bactericides have been utilized intensively in the nursery industry for at least 10 and 5 years, respectively. We previously isolated Cu^r, Sm^r, and Cu^r Sm^r strains of *P. syringae* pv. syringae, the causal agent of a canker and tip dieback disease on *Pyrus calleryana* (ornamental pear), from three nurseries in Oklahoma (59). Our long-term goal is to understand the evolution and persistence of plasmid-encoded traits in populations of *P. syringae*. Our objective in this study was to examine the genetic and plasmid diversity within epiphytic *P. syringae* pv. syringae strains which colonized ornamental pear trees exposed to different spray regimens. To achieve this, the epiphytic *P. syringae* populations were surveyed at three nurseries, and the frequencies of resistance phenotypes were determined. We examined the plasmid diversity within a subset of isolates from each nursery and used AP-PCR to investigate the genetic diversity within populations from two of the nurseries. The distribution of selected plasmids among different *P. syringae* chromosomal genotypes was also determined. Our results suggest that the *P. syringae* pv. syringae populations on ornamental pears in Oklahoma are genetically diverse. Also, we determined that resistance to copper and resistance to streptomycin in *P. syringae* pv. syringae are conferred by distinct groups of plasmids which appear to colonize only specific *P. syringae* genotypes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 shows the plasmids used in this study and selected bacterial strains which exhibit different plasmid profiles. The medium used to isolate *P. syringae* from plant samples was medium B of King et al. (28) amended with 100 μg of cycloheximide per ml to inhibit fungal growth. Resistance to copper and resistance to strepto-

mycin were determined by using mannitol-glutamate medium (27) amended with 250 μg of cupric sulfate per ml and mannitol-glutamate medium containing 25 μg of streptomycin sulfate per ml, respectively. *Escherichia coli* was grown on Luria-Bertani medium (41). Ampicillin, chloramphenicol, streptomycin, and tetracycline were added to Luria-Bertani medium or mannitol-glutamate medium at concentrations of 40, 50, 25, and 12.5 μg/ml, respectively.

Sources of *P. syringae* isolates. Samples were obtained from three ornamental pear (*Pyrus calleryana* cv. Aristocrat) nurseries located in the eastern Oklahoma counties of Cherokee and Muskogee. The distances between the nurseries were 45 km (nurseries I and II), 47 km (nurseries II and III), and 2 km (nurseries I and III). The trees were subjected to the following spray regimens during each growing season (March to August): nursery I, 15 applications of a mixture containing cupric hydroxide (2.4 g/liter) and streptomycin sulfate (0.2 g/l); nursery II, 20 applications of cupric hydroxide (2.4 g/liter); and nursery III, no applications of copper or streptomycin. Following an initial application while the trees were dormant, the bactericides were applied once during the bloom period and then weekly throughout the growing season. Epiphytic bacteria were isolated from symptomless leaf surfaces on 13 April, 11 May, 3 June, 2 July, and 30 July 1991 and on 27 April, 26 May, and 24 June 1992. Single leaves at approximately the same position in the plant canopy were randomly selected from 25 trees, placed individually into plastic bags, and transported to our laboratory on ice. The leaves were washed individually in 20 ml of 0.01 M potassium phosphate buffer (pH 7.0) supplemented with 0.1% peptone (PK buffer) for 1 h at 250 rpm on a rotary shaker. Aliquots (50 μl) of appropriate serial dilutions were plated onto medium B of King et al. containing 100 μg of cycloheximide per ml and incubated for 48 h at 28°C. Oxidase-negative colonies (50) which were fluorescent on medium B of King et al. containing 100 μg of cycloheximide per ml and which exhibited the appropriate colony morphology were considered *P. syringae* colonies (59). Up to 10 *P. syringae* colonies per leaf were randomly chosen and examined for bactericide resistance by replica plating on mannitol-glutamate medium containing 250 μg of cupric sulfate per ml and mannitol-glutamate medium containing 25 μg of streptomycin sulfate per ml as described previously (59).

Characterization of plasmids. Plasmids were isolated from one or two randomly selected *P. syringae* isolates from each colonized leaf. The plasmid isolation method used was the method of Crosa and Falkow (16), with slight modifications (2). Preparations were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) before restriction enzyme digestion. Plasmids were separated on 0.7% agarose gels, and their sizes were estimated by comparison with known plasmid standards and by determining combined sizes of restriction fragments generated by three different enzymes. Plasmids from different isolates were placed into profile groups on the basis of size and bactericide resistance phenotype. The conjugative abilities of resistance plasmids belonging to each profile group were determined by conducting mating experiments on medium B of King et al., using a previously described method (59). *P. syringae* pv. syringae 9C11.2, a plasmid-free Cu^s Sm^s strain with chloramphenicol resistance (Cm^r), was used as the recipient.

Molecular genetic techniques. Restriction enzyme digestion, agarose gel electrophoresis, Southern transfer, and isolation of DNA fragments from agarose gels were performed by using standard procedures (38). When the plasmid profile groups included two plasmids, the resistance plasmids that we were interested in were conjugated into plasmid-free strain 9C11.2. Hybridization analyses were performed to determine the locations of specific genetic determinants on plasmids from each profile group. The DNA fragments used as probes were (i) the 1.5-kb *Sst*I-*Eco*RV fragment containing Sm^r genes *strA* and *strB* from broad-host-range plasmid RSF1010 (52); (ii) the 3.2-kb *Sst*I fragment which contains parts of the transposase (*tnpA*) and resolvase (*tnpR*) genes and the recombination site (*res*) from Sm^r transposon Tn*5393* (8); and (iii) the 1.2-kb *Hin*dIII and 1.4-kb *Eco*RI fragments from pOSU22 containing the *oriV* and *par* loci from pOSU900 (42). DNA fragments were labeled with digoxigenin-11-dUTP by following the instructions of the manufacturer (Genius labeling and detection kit; Boehringer Mannheim, Indianapolis, Ind.). Hybridization was performed at 68°C, and this was followed by high-stringency washes as described previously (59).

Phenotypic characterization of *P. syringae* isolates. The phenotypic characteristics of 100 *P. syringae* isolates that were randomly selected from the isolates obtained from nurseries I and III were also investigated by assessing the hydrolysis of esculin, liquefaction of gelatin, utilization of sodium tartrate, and tyrosinase activity (25). The ability of each strain to elicit a hypersensitive response was evaluated by infiltrating a bacterial cell suspension (approximately 10^7 CFU/ml in PK buffer) into the intercellular spaces of tobacco (*Nicotiana tabacum* cv. Xanthi) leaves. The plants were incubated for 24 h on a greenhouse bench and then examined for the presence of collapsed tissue characteristic of the hypersensitive response (50). Ice nucleation activity was assessed by incubating 1-ml suspensions of bacterial cells (approximately 10^7 CFU/ml in PK buffer) at −5°C in a controlled temperature bath. Suspensions which froze within 15 min were considered ice nucleation active (INA^+); PK buffer and a suspension of cells of an INA^- strain, *P. syringae* pv. morsprunorum 3714, were used as negative controls.

Genetic analysis of a subset of *P. syringae* pv. syringae strains by using AP-PCR. A total of 100 *P. syringae* isolates were identified as *P. syringae* pv. syringae isolates by phenotypic tests. A complete description of the characteristics of these 100 *P. syringae* pv. syringae strains, including their resistance phenotypes and plasmid profiles, has been published elsewhere (58). Genomic fingerprints of these bacteria were determined by using AP-PCR (67, 68) and the following oligonucleotide primers: (i) the 18-bp REP primers (63) 5′-ICGICTTATCIGGCCTAC-3′ and 5′-IIIICGICGICAT CIGGC-3′ (Genosys Biotechnologies, Inc., The Woodlands, Tex.), which were used in combination; and (ii) a 20-bp oligonucleotide complementary to the IS*50* portion of Tn*5* (47), 5′-GGTTCCGTTCAGGACGCTAC-3′ (Oklahoma State University Recombinant DNA/Protein Resource Facility), which was used alone. PCR were conducted in 25-μl volumes containing the REP primers (50 pmol each) or the IS*50* primer (100 pmol), 4 μg of bovine serum albumin (Boehringer Mannheim), 1.25 mM deoxynucleoside triphosphates (Boehringer Mannheim), 10% dimethyl sulfoxide (Fluka Chemical Corp., Ronkonkoma, N.Y.), and 1.5 U of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in the salt buffer described by Kogan et al. (29). To this reaction mixture we added approximately 10^6 cells (previously grown on medium B of King et al. for 12 h) with a sterile toothpick, and the mixture was covered with 1 drop of mineral oil. Amplification reactions were also performed with purified DNAs from plasmids belonging to the different plasmid profile groups. Plasmid DNA was purified on CsCl-ethidium bromide gradients or by electroelution from 0.7% agarose gels. Amplification reactions were performed with a thermal cycler (Perkin Elmer Cetus) as follows: 1 cycle at 95°C for 6 min; 5 cycles consisting

of 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; 25 cycles consisting of 94°C for 1 min, 50°C for 1 min, and 65°C for 8 min; 1 cycle at 65°C for 16 min; and a final soaking step at 4°C. The fidelity of the technique was validated by performing all of the amplification reactions at least twice. After the reactions, 8-μl portions of the PCR products were electrophoresed for 1.5 h at 60 V on 1.5% agarose gels, and the gels were stained with ethidium bromide and photographed by using Polaroid type 55 film.

Data analysis. After the number of PCR products generated with both primer sets was determined, the data were converted to a two-dimensional binary matrix in which 1 indicated that a PCR product was present and 0 indicated that a PCR product was not present. An analysis was performed by using the biostastical analysis program NTSYS-pc (Applied Biostatistics, Inc., Setauket, N.Y.). A similarity matrix was computed by using Dice's coefficient, $2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of bands that isolates x and y have in common and n_x and n_y are the total numbers of bands in isolates x and y, respectively. A cluster analysis was performed by using the unweighted pair group method with arithmetic average (55).

RESULTS

Colonization of *Pyrus calleryana* trees by *P. syringae*. Epiphytic colonization by *P. syringae* was consistently detected at all sampling times in each nursery. The populations were generally higher in the early spring, which is consistent with observations reported previously for epiphytic colonization of tree hosts by *P. syringae* (22). The populations typically decreased in the summer months when ambient temperatures were high and rainfall was low (data not shown). The mean *P. syringae* population sizes on colonized leaves were $\log_{10}$ 4.59, 4.17, and 4.38 CFU/g (fresh weight) at nurseries I, II, and III, respectively. A total of 1,938 *P. syringae* isolates recovered during the survey were examined for their resistance phenotypes. Figure 1 shows the total percentage of leaves colonized by each resistance phenotype at each nursery. Although both copper and streptomycin were applied at nursery I, Cu^s Sm^s strains were detected on 19.1% of the colonized leaves at this site. Cu^r Sm^s and Cu^r Sm^r *P. syringae* strains were detected on approximately equal numbers of leaves at nursery I. At nursery II, a large percentage (86.6%) of the colonized leaves harbored Cu^r Sm^s *P. syringae*. Although streptomycin was not sprayed at nursery II, Cu^r Sm^r *P. syringae* was detected on 13.4% of the colonized leaves. Neither copper nor streptomycin was sprayed at nursery III; however, approximately equal numbers of colonized leaves harbored Cu^s Sm^s and Cu^r Sm^r *P. syringae*. We found that 26.2, 14.6, and 9.1% of the colonized leaves obtained from nurseries I, II, and III, respectively, contained populations that were heterogeneous with respect to resistance phenotypes and that two or three resistance phenotypes could be detected on a single leaf (Fig. 1).

Characterization of plasmids. Plasmids were isolated from 362 *P. syringae* strains (18.7% of the strains examined); 310 of these isolates were resistant to copper or streptomycin and contained at least one plasmid (Fig. 2). Each plasmid was assigned to 1 of 14 profile groups on the basis of its size and resistance phenotype. The sizes of individual resistance plasmids ranged from 53 kb (Fig. 2A, lane 4) to 220 kb (Fig. 2B, lane 3). Ten plasmid profile groups contained single plasmids; these plasmids were found in Cu^r Sm^s (Fig. 2A, lanes 1 through 4), Cu^r Sm^r (Fig. 2A, lanes 8 and 9; Fig. 2B, lanes 1 through 3), and Cu^s Sm^r (Fig. 2B, lane 5) isolates. The other four plasmid profile groups contained two plasmids each; these plasmids were found in Cu^r Sm^s (Fig. 2A, lanes 5 and 6) and Cu^r Sm^r

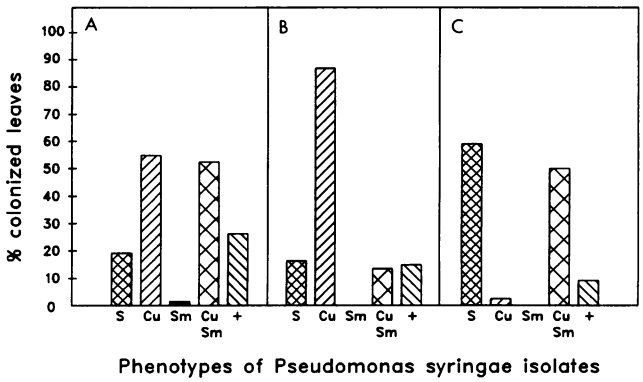

FIG. 1. Percentages of *Pyrus calleryana* cv. Aristocrat leaves colonized by *P. syringae*. (A) Nursery I. (B) Nursery II. (C) Nursery III. The values for nurseries I and II are the values for 200 leaves sampled in 1991 and 1992; the values for nursery III are the values for 75 leaves sampled in 1992. S, Cu^s Sm^s; Cu, Cu^r Sm^s; Sm, Cu^s Sm^r; CuSm, Cu^r Sm^r; +, strains with different resistance phenotypes recovered from a single leaf. The bactericide spray regimens were as follows: nursery I, 15 applications of a mixture containing cupric hydroxide (2.4 g/liter) and streptomycin sulfate (0.2 g/liter); nursery II, 20 applications of cupric hydroxide (2.4 g/liter); nursery III, no application of copper or streptomycin bactericides.

(Fig. 2A, lane 7; Fig. 2B, lane 4) isolates. Cryptic plasmids in Cu^s Sm^s isolates were assigned to four plasmid profile groups (profile groups C1, C2, C3, and C4) on the basis of size differences. Profile groups C1, C2, and C3 each contained one plasmid (size range, 30 to 65 kb), and profile group C4 contained two plasmids (49 and 190 kb) (data not shown).

Southern hybridization and conjugal transfer of selected plasmids into strain 9C11.2 were used to determine which plasmid(s) in each profile group harbored a particular resistance determinant and if the plasmids contained a common replicon. The Cu^r determinant was located on 53- to 68-kb conjugative plasmids in all Cu^r Sm^s *P. syringae* strains (Fig. 3, profile groups 1 through 6 and 1A) and on 68- to 220-kb conjugative plasmids in Cu^r Sm^r strains (Fig. 3, profile groups 7 through 11 and 8A). The *strA-strB* genes were located by hybridization to the profile group 12 plasmid (Fig. 3) and on the plasmid containing the Cu^r determinant in plasmid profiles 7 through 11 and 8A (Fig. 3). In plasmid profile group 6, the Cu^r and Sm^r determinants were not linked and were localized on 58- and 196-kb plasmids, respectively (Fig. 3). Each Sm^r plasmid which hybridized to *strA-strB* also hybridized to the 3.2-kb *Sst*I fragment containing parts of the *tnpA* and *tnpR* genes and *res* from Tn*5393*. With the exception of the Sm^r plasmid in profile group 6, the resistance plasmids in each profile group could be conjugated into recipient strain 9C11.2 at frequencies ranging from 3.42×10^{-7} to 5.73×10^{-3} exconjugant per donor cell (data not shown).

The resistance plasmids could be separated into two main groups on the basis of size and on the basis of hybridization to the pOSU900 replicon. The plasmids assigned to group 1 ranged in size from 53 to 125 kb and contained sequences which hybridized to the *oriV* and *par* loci from pOSU900, a cryptic plasmid isolated from *P. syringae* pv. syringae (42). Group 1 contained all of the Cu^r plasmids (Fig. 3, profile groups 1 through 6 and 1A), three Cu^r Sm^r plasmids (Fig. 3, profile groups 7, 8, and 8A), one Sm^r plasmid (Fig. 3, profile group 12), and three cryptic plasmids (Fig. 3, profile groups C1, C2, and C4). The plasmids in group 2 did not hybridize to the

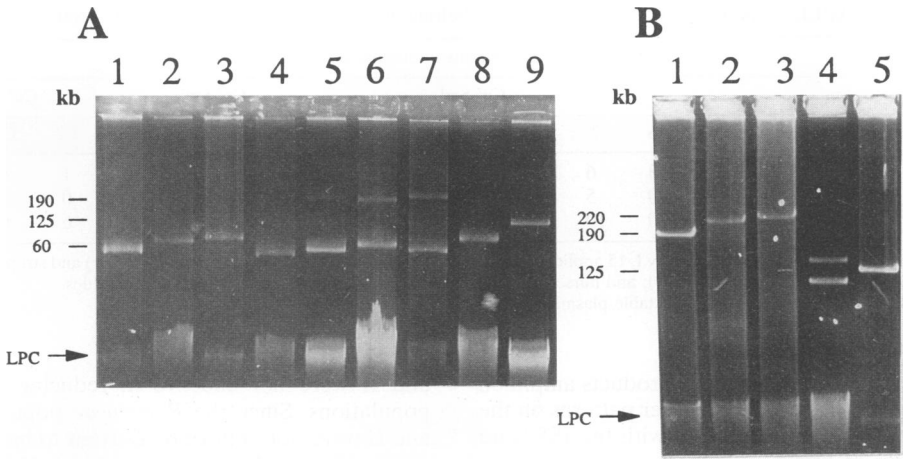

FIG. 2. Plasmid profiles of *P. syringae* isolates with resistance to copper and/or streptomycin. (A) Lane 1, strain 7B44(pPSR7) (profile group 1); lane 2, strain 2H12(pPSR9) (profile group 2); lane 3, strain 3C1(pPSR10) (profile group 3); lane 4, strain 8B48(pPSR11) (profile group 4); lane 5, strain 7G14(pPSR8, pPSC6) (profile group 1A); lane 6, strain 7A36(pPSR12, pPSC7) (profile group 5); lane 7, strain 7C12(pPSR13, pPSR21) (profile group 6); lane 8, strain 7B12(pPSR14) (profile group 7); lane 9, strain 8C32(pPSR15) (profile group 8). (B) Lane 1, strain 2E49(pPSR17) (profile group 9); lane 2, strain 7B22(pPSR18) (profile group 10); lane 3, strain 7F14(pPSR19) (profile group 11); lane 4, strain 9A26(pPSR16, pPSC8) (profile group 8A); lane 5, strain 9A22(pPSR20) (profile group 12). The positions of plasmid size standards are indicated on the left. LPC, linearized plasmid and chromosomal DNA.

pOSU900 replicon, were 190 to 220 kb, and included the large Cu^r Sm^r plasmids (Fig. 3, profile groups 9 through 11) and the Sm^r plasmid in profile group 6 (Fig. 3). The cryptic plasmids which did not hybridize to the pOSU900 replicon were not assigned to either of these plasmid groups (Fig. 3, profile groups 1A, 5, 8A, C1, and C4).

The distributions of the plasmid profiles among the isolates obtained from the three nurseries revealed that specific plasmids were predominant at each nursery (Table 2). At nursery I, the most common Cu^r plasmids were profile group 1, 1A, and 5 plasmids, while the most common Cu^r Sm^r plasmids were profile group 8 and 8A plasmids. The *P. syringae* isolates obtained from nursery I produced 15 different plasmid profiles and exhibited more variability in plasmid content than the isolates obtained from the other two nurseries. At nursery II, the predominant Cu^r plasmids were profile group 1 and 2 plasmids. At nursery III, the major Cu^r Sm^r plasmids were profile group 7 and 10 plasmids, which were isolated only infrequently from nursery I strains (Table 2). The cryptic plasmids also differed; profile group C3 plasmids predominated at nurseries I and II, and profile group C1 and C2 plasmids were isolated more frequently from nursery III strains (Table 2).

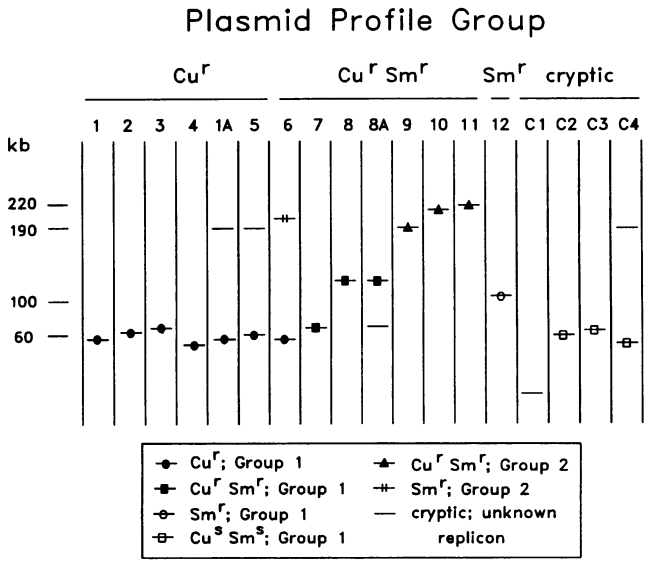

FIG. 3. Characterization of 18 plasmid profile groups found in *P. syringae* strains isolated from *Pyrus calleryana*. Plasmids assigned to group 1 contained sequences which hybridized to a probe containing the *oriV* and *par* loci from pOSU900 (42). Group 2 comprised 190- to 220-kbp resistance plasmids which did not hybridize to the pOSU22 probe.

Phenotypic characterization of a subset of *P. syringae* isolates. The phenotypic traits of 100 randomly selected *P. syringae* isolates from nurseries I and III revealed that 98% of these isolates were homogeneous with respect to all seven diagnostic tests used. These strains were oxidase negative, hydrolyzed esculin, liquefied gelatin, did not utilize sodium-tartrate, had no tyrosinase activity, were ice nucleation active at −5°C, and elicited a hypersensitive response in tobacco. Only two strains, 8H1 and 8H2, differed; these strains lacked ice nucleation activity at −5°C. On the basis of these characteristics, all 100 strains were identified as *P. syringae* pv. syringae isolates.

AP-PCR analysis of *P. syringae* pv. syringae strains. A genome analysis was performed with the 100 *P. syringae* pv. syringae strains identified as described above and 6 *P. syringae* pv. syringae strains which were examined previously for phenotypic and genetic diversity (18, 19). The total genomic DNAs released from intact cells of the 106 *P. syringae* pv. syringae strains were used as templates for PCR amplification with the IS*50* primer and the REP primers. We identified 29 and 36 DNA products, ranging in size from approximately 0.3 to 4.0 kb, in the 106 strains when we used the IS*50* and REP primers, respectively. No bands were observed when purified plasmid DNA from a member of each plasmid profile group was used as a template for the PCR, indicating that differences in plasmid content among the strains were not reflected in the

TABLE 2. Numbers of *P. syringae* strains belonging to 18 distinct plasmid profile groups

Nursery[a]	No. of strains in plasmid profile groups																			Total
	Cu^r Sm^s						Cu^r Sm^r							Cu^s Sm^r	Cu^s Sm^s					
	1	1A	2	3	4	5	6	7	8	8A	9	10	11	12	C1	C2	C3	C4	0[b]	
I	34	25	4	4	0	25	3	6	56	15	4	3	0	3	0	1	8	2	3	196
II	47	0	30	4	3	0	0	5	3	0	0	1	0	0	0	0	5	0	2	100
III	0	0	0	0	0	1	0	10	2	0	0	18	4	0	8	6	0	0	17	66

[a] The bactericide spray regimens were as follows: nursery I, 15 applications of a mixture containing cupric hydroxide (2.4 g/liter) and streptomycin sulfate (0.2 g/liter); nursery II, 20 applications of cupric hydroxide (2.4 g/liter); and nursery III, no application of copper or streptomycin bactericides.
[b] Cu^s Sm^s *P. syringae* isolates which contained no detectable plasmids.

PCR analysis results. The patterns of PCR products amplified from specific strains were compared with other patterns on the same gel. Examples of the results obtained with the IS*50* and REP primers are shown in Fig. 4.

A cluster analysis of the data matrices generated with the IS*50* primer and the REP primers revealed that the 106 *P. syringae* pv. syringae strains could be differentiated into two distinct groups (groups A and B) which were separated at levels of genetic similarity of 39.7 and 47.0%, respectively (data not shown). A comparison of the dendrograms generated with the IS*50* and REP primers revealed that they were not identical. Four strains (B48, B61, B64, and PSC1B) were placed into group A on the IS*50* PCR dendrogram and into group B on the REP PCR dendrogram. One strain (B76) was placed into group B on the IS*50* PCR dendrogram and into group A on the REP PCR dendrogram. There were also minor differences in the grouping of closely related strains on the IS*50* and REP dendrograms. The data obtained with the types of primers suggested that each primer type detected differences in the genomes of strains which were not evident when the other primer type was used. A combined matrix was then generated by using the IS*50* and REP PCR data. When this matrix was analyzed, 84 distinct patterns were observed among the 106 *P. syringae* pv. syringae strains. A total of 70 of these patterns were unique, and 14 patterns were produced by more than one isolate; the maximum number of isolates that produced identical IS*50* and REP PCR patterns was six.

The dendrogram generated from the combined matrices also differentiated the strains into two groups at a level of similarity of 45.8% (Fig. 5). Fourteen clusters contained multiple strains that produced identical IS*50* and REP PCR patterns (Fig. 5); we observed differences in plasmid profiles among strains belonging to four of these clusters (Fig. 5, clusters 1, 3, 15, and 38). When the plasmid data were included in the analysis, 87 unique *P. syringae* pv. syringae genotypes were identified among the 100 strains isolated from ornamental pear trees. The two PCR groups (groups A and B) contained strains that produced distinct plasmid profiles; group A contained Cu^r Sm^s strains that produced plasmid profiles 1, 1A, and 5 dispersed among several clusters and one Cu^r Sm^r strain that produced plasmid profile 6 (Fig. 5). Group B contained Cu^r Sm^r strains that produced plasmid profiles 7 through 11 and 8A dispersed among several clusters and Cu^s Sm^r strains that produced plasmid profile 12 (Fig. 5). In addition, 21 Cu^s Sm^s strains, 11 of which did not contain plasmids, were found in several clusters throughout group B.

DISCUSSION

The sizes of the *P. syringae* populations on individual ornamental pear leaves were similar at all three nurseries, indicating that the intensive bactericide spray programs at nurseries I and II were not effective in reducing epiphytic *P. syringae* populations. Since the *P. syringae* populations at nurseries I and II were not uniformly resistant to bactericides, our results suggest that the bactericides were not present at toxic concentrations on all of the leaves during the time between treatments. However, the predominance of resistant strains at nurseries I and II, indicated that copper resistance and streptomycin resistance are important characteristics in the bacterial populations at these nurseries and are probably responsible for the long-term maintenance of *P. syringae* populations on the trees there. At nursery I, most of the *P. syringae* isolates were Cu^r Sm^s, despite the fact that both copper and streptomycin bactericides were applied weekly in this nursery during the growing season. Because copper has been used as a bactericide in Oklahoma on nursery trees for a longer period of time than streptomycin, it is possible that copper resistance evolved in *P. syringae* prior to streptomycin resistance. Subsequent usage of streptomycin probably selected for acquisition of resistance to this antibiotic and for a population containing a larger percentage of Sm^r strains. At nursery II, 9.3% of the *P. syringae* isolates were Cu^r Sm^r even though streptomycin was not applied, and 43.6% of the *P. syringae* isolates at nursery III were Cu^r Sm^s or Cu^r Sm^r, although neither copper nor streptomycin was applied. These results suggest that *P. syringae* strains with resistance to copper or streptomycin colonized the phylloplane in the absence of bactericides, thus corroborating previous observations (1, 10, 60, 62).

The plasmid contents of Cu^r Sm^s, Cu^s Sm^r, and Cu^r Sm^r *P. syringae* isolates were diverse, and 12 resistance plasmids were placed into 14 plasmid profile groups on the basis of size and resistance phenotype. The skewed distribution of plasmid profile groups among *P. syringae* isolates suggests that different plasmids were selected at each nursery. However, it is unlikely that *P. syringae* isolates would be restricted to only one of the three sites because the relative proximity of the nurseries would favor dissemination between nurseries from plant surfaces by rain and wind. The dominance of certain plasmids within a nursery could result from physical or biological factors, such as the influx of plasmid-containing *P. syringae* cells from other hosts or variations in the phylloplane environment which could select other plasmid-encoded traits that enhance fitness.

In this study, streptomycin resistance in *P. syringae* was conferred by the *strA-strB* homologs present on Sm^r transposon Tn*5393*. This transposon was located on various *Eco*RI fragments in group 1 and group 2 plasmids, an observation which suggests that Tn*5393* was mobile in the *P. syringae* populations studied and was transposed to several different plasmids (58). Other investigators have described the detection of copper and streptomycin determinants on plasmids which differed in size (14, 26, 45, 59, 62), thus suggesting that mobilization of resistance determinants among plasmids does occur.

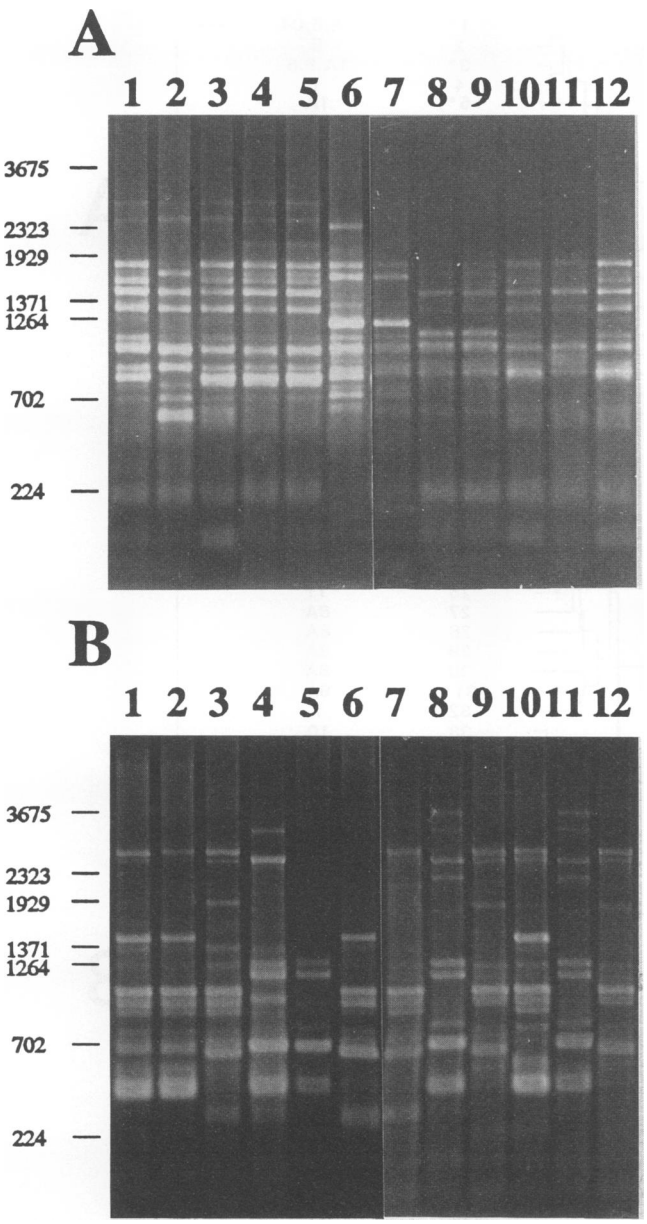

FIG. 4. Genomic fingerprinting of *P. syringae* pv. syringae strains by using the AP-PCR technique with the REP primers (A) and an IS*50* primer (B). (A) Lane 1, strain 8F21 (PCR group B); lane 2, strain 8F35 (PCR group B); lane 3, strain 8F43 (PCR group B); lane 4, strain 8G5 (PCR group B); lane 5, strain 8G10 (PCR group B); lane 6, strain 8G17 (PCR group A); lane 7, strain 8C43 (PCR group A); lane 8, strain 8D2 (PCR group B); lane 9, strain 8D6 (PCR group B); lane 10, strain 8D17 (PCR group B); lane 11, strain 8C5 (PCR group B); lane 12, strain 8D34 (PCR group B). (B) Lane 1, strain 9B36 (PCR group B); lane 2, strain 9D8 (PCR group B); lane 3, strain 8F21 (PCR group B); lane 4, strain 7F29 (PCR group A); lane 5, strain 9A3 (PCR group A); lane 6, strain 9C39 (PCR group B); lane 7, strain 8C32 (PCR group B); lane 8, strain 8C43 (PCR group A); lane 9, strain 8D6 (PCR group B); lane 10, strain 8D17 (PCR group B); lane 11, strain 8D29 (PCR group A); lane 12, strain 8E17 (PCR group B). The positions of size standards (in base pairs) are indicated on the left.

Plasmids assigned to group 1 hybridized to the probe constructed from the *oriV* and *par* sequences from pOSU900, a cryptic plasmid in *P. syringae* pv. syringae J900 (42). The pOSU900 replicon is also present on plasmids obtained from several other *P. syringae* pathovars, including *P. syringae* pv. angulata, *P. syringae* pv. apii, *P. syringae* pv. atropurpurea, *P. syringae* pv. coronafaciens, *P. syringae* pv. glycinea, *P. syringae* pv. lachrymans, *P. syringae* pv. maculicola, *P. syringae* pv. mori, *P. syringae* pv. morsprunorum, *P. syringae* pv. papulans, *P. syringae* pv. phaseolicola, *P. syringae* pv. pisi, *P. syringae* pv. savastanoi, *P. syringae* pv. tabaci, and *P. syringae* pv. tomato (42, 43, 58, 61). Therefore, the origin of replication and stability determinants from plasmid pOSU900 may be widely distributed in *P. syringae*, and plasmids containing this replicon may encode additional determinants that are important for the association of *P. syringae* with plant hosts.

Genomic fingerprinting of the 100 *P. syringae* pv. syringae strains characterized in this study revealed a high level of diversity, and many unique AP-PCR patterns were detected. The results of a cluster analysis of the combined data set differentiated the strains into two distinct groups. The strains in the two groups were closely related; 88.6 and 94.4% of the strains in PCR groups A and B were distinguished at a similarity level of >80%, respectively. This separation into two distinct groups may indicate that these strains belong to distinct subpopulations of *P. syringae*, both of which are capable of colonizing ornamental pear trees.

The results of an AP-PCR analysis of six *P. syringae* pv. syringae strains isolated from diverse hosts, including peach, tomato, and wheat plants, indicated that the strains were neither closely related to each other nor closely related to the strains isolated in this study. This finding corroborates results of other studies which indicated that some strains of *P. syringae* pv. syringae exhibit host specificity (6, 48, 49) and strains isolated from one host may be unrelated to strains isolated from other hosts (19, 33). In a previous study, Denny (18) used multilocus enzyme electrophoresis and restriction fragment length polymorphism analyses to differentiate strains isolated from dicots (B48, B76, B78) from strains isolated from monocots (B61, B64, PSC1B). However, the AP-PCR analysis performed in this study did not differentiate dicot and monocot strains.

The results of studies of Ti plasmid-chromosome associations in *Agrobacterium tumefaciens* have indicated that specific plasmid and chromosomal genotypes are highly correlated, implying that plasmid transfer between strains with different chromosomal backgrounds is rare in nature (4, 46). However, other workers have found Ti plasmids in different *A. tumefaciens* chromosomal backgrounds, suggesting that Ti plasmids can be transferred in some situations (40, 44). In *Rhizobium leguminosarum*, identical Sym plasmids were detected in different chromosomal backgrounds, and strains having the same chromosomal genotype contained different plasmids, suggesting that plasmid transfer occurs in natural populations of this species (30, 31, 51, 69). In *P. syringae* pv. syringae, strains assigned to PCR group A contained different plasmids (profile groups 1, 1A, 5, 6, and C4); however, the plasmid profiles were closely related, suggesting that plasmid rearrangements occurred in the same genetic background (61). In addition, the presence of profile group 1, 1A, and 5 plasmids and the presence of profile group 7 through 10, 8A, C1, and C2 plasmids in strains having different chromosomal genotypes in PCR groups A and B, respectively, suggest that plasmid transfer has occurred in these populations. However, the restriction of particular plasmids to either PCR group A or PCR group B implies that there are factors which limit the establishment of specific plasmids in certain chromosomal

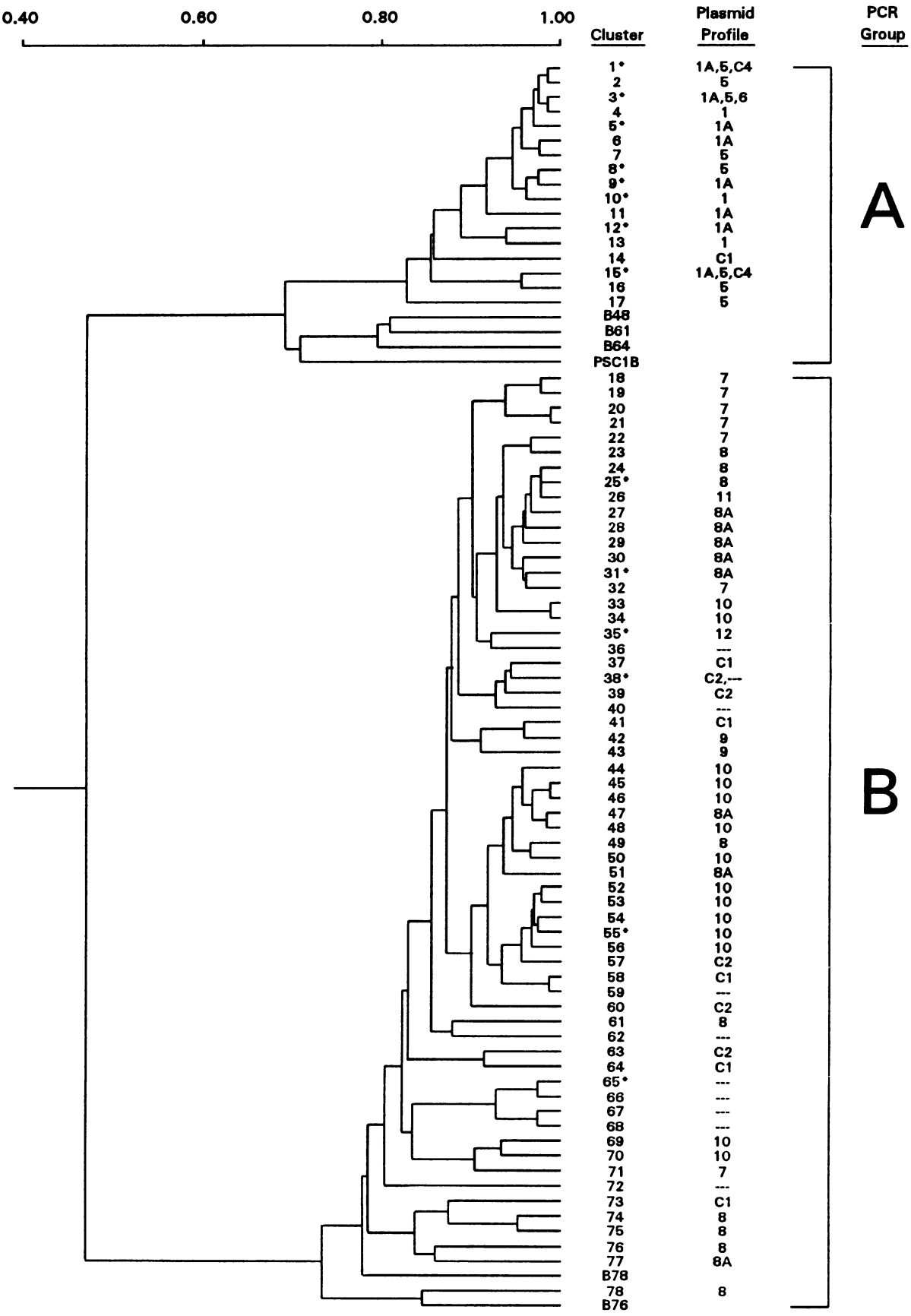
Relative Genetic Similarity
0.40
0.60
0.80
1.00
Cluster
Plasmid Profile
PCR Group
1* 1A,5,C4
2 5
3* 1A,5,6
4 1
5* 1A
6 1A
7 5
8* 5
9* 1A
10* 1
11 1A
12* 1A
13 1
14 C1
15* 1A,5,C4
16 5
17 5
B48
B61
B64
PSC1B
18 7
19 7
20 7
21 7
22 7
23 8
24 8
25* 8
26 11
27 8A
28 8A
29 8A
30 8A
31* 8A
32 7
33 10
34 10
35* 12
36 ---
37 C1
38* C2,---
39 C2
40 ---
41 C1
42 9
43 9
44 10
45 10
46 10
47 8A
48 10
49 8
50 10
51 8A
52 10
53 10
54 10
55* 10
56 10
57 C2
58 C1
59 ---
60 C2
61 8
62 ---
63 C2
64 C1
65* ---
66 ---
67 ---
68 ---
69 10
70 10
71 7
72 ---
73 C1
74 8
75 8
76 8
77 8A
B78
78 8
B76
A
B

FIG. 5. Dendrogram of *P. syringae* pv. syringae strains derived from PCR fingerprints obtained by using the IS*50* and REP primers. The dendrogram was generated from the combined IS*50* and REP primer data sets. The clusters are numbered consecutively; the clusters containing reference *P. syringae* pv. syringae strains (B48, B61, B64, B76, B78, PSC1B) were not assigned numbers. An asterisk indicates that a cluster contained more than one strain. The plasmid profiles are the profiles of plasmids contained in strains belonging to the clusters. The dashed lines indicate that plasmids were not detected in strains belonging to these clusters.

backgrounds. Young and Wexler hypothesized that the species *R. leguminosarum* is effectively compartmentalized, with plasmid transfer occurring within compartments but not between them (69). The species *P. syringae* may be compartmentalized as well; for example, Cu^r plasmids from *P. syringae* pv. syringae could not be transferred to *P. syringae* pv. morsprunorum strains in vitro and were not detected in natural populations of *P. syringae* pv. morsprunorum (62). Our observation that some plasmids are confined to particular chromosomal genotypes is interesting since the pOSU900 replicon was detected on plasmids in strains assigned to both PCR groups. The factors that contribute to the apparent compartmentalization of these plasmids are not clear since conjugal transfer of plasmids between strains belonging to PCR groups A and B has been demonstrated in vitro.

The results of our AP-PCR, plasmid profile, and resistance phenotype analyses revealed the high level of genetic diversity in the *P. syringae* strains that inhabit each nursery. There is previously published evidence that the subpopulations of *P. syringae* are dynamic, with temporal population shifts occurring in response to alterations in physical and/or biological environmental parameters (20, 23, 24, 34, 36). In this study, we found that selective pressure from bactericide usage has apparently resulted in the colonization of many *P. syringae* genotypes by individual resistance plasmids, a phenomenon which may contribute to the ultimate persistence of resistance plasmids in the populations studied. The evolution of resistance to the bactericides copper and streptomycin within *P. syringae* pv. syringae has apparently involved relatively large numbers of plasmid genotypes and chromosomal genotypes, both of which could be differentiated into two distinct groups. This may reflect the response of a population which was initially heterogeneous, a characteristic which would tend to inhibit the rapid dissemination of a single plasmid or clone. The identification of different plasmid profile groups and the results of the genome analysis of strains obtained from nurseries I and III indicated that the *P. syringae* populations at the nurseries were distinct. The populations at the nurseries could have been influenced by a number of factors, including the initial microbial community inhabiting the trees before bactericides were applied, immigration and colonization of *P. syringae* genotypes from other plant hosts, environmental factors, and the bactericide spray regimens. The results of an AP-PCR analysis of the chromosomal genotypes of the populations from nurseries I and III suggested that plasmid transfer probably occurred, although it is not known whether the frequency of plasmid transfer was affected by bactericide selection pressure. Previous observations have indicated that bactericide resistance plasmids are stably maintained in *P. syringae* and do not have an impact on epiphytic fitness (60). Thus, effective management of *P. syringae* pv. syringae is compromised by the ability of populations to evolve resistance to bactericides. Management alternatives, such as host resistance or biological control, may likewise be confounded by the inherent heterogeneity of the pathogen populations.

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