Ecology and Epidemiology

Frequency, Distribution, and Characteristics of Endophytic *Pseudomonas syringae* in Pear Trees

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ABSTRACT

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Internal stem and root tissues of pear trees were sampled for presence of *Pseudomonas syringae* at seven orchards in Oregon. Isolates positive for fluorescence (F+) and negative for cytochrome oxidase reaction (Ox−) were defined as *P. syringae*. Endophytic F+ Ox− strains occurred in 84% (57/68) of the trees. Eighty-one percent (191/235) of the F+ endophytic stains were Ox−. A total of 159 and 32 *P. syringae* strains were found in root and stem tissues, respectively. The endophytic *P. syringae* strains varied with respect to ice nucleation activity, induction of hypersensitive response on tobacco, pathogenicity on pear and cherry fruitlets, and genomic DNA fingerprinting. Inoculations of *P. syringae* into root and stem tissues of potted trees resulted in detectable but limited bacterial movement up to 3.0 cm in stems and no detectable movement above the crown from root inoculations.

Blossom and bud blast, shoot-tip dieback, and stem cankers are common disease symptoms on pear (*Pyrus communis* L.); they are caused by *Pseudomonas syringae* van Hall (2,28,30). The potential sources of inoculum for this epiphytic phytopathogenic bacterium include surfaces of symptomless and diseased trees (9,10,24,36), inside buds (27), and weeds commonly found as orchard cover (18,21,31,36).

Blossom blast begins as external surface lesions on nectaries, then progresses to the inner tissues (13). Electron microscope observations have provided evidence that *P. syringae* systemically

invades shoots and spreads in plum and apple (12,32). Systemic infection of cherry and a single pear tree have also been reported (3,4). However, a better understanding of the frequency of occurrence of endophytic *P. syringae* strains in pear trees and their distribution within the tree is needed. A genomic comparison of epiphytic and endophytic isolates could be a useful tool to help determine if endophytic strains are an extended population of the epiphytic strains. A comparison of genomic fingerprints derived from restriction endonuclease analysis of total cellular DNA has been used for bacterial strain identification (7,11) and for a comparison of strains of *P. syringae* (22).

The purpose of this study was to evaluate the frequency and distribution of endophytic *P. syringae* in pear and to compare phenotypic characteristics of endophytic and epiphytic isolates. Phenotypic characteristics evaluated included ice nucleation activity, ability to induce a hypersensitive response in tobacco, pathogenicity to pear and cherry fruitlets, and genomic DNA fingerprints. Also investigated was the internal mobility of *P. syringae* strains inoculated into root and stem tissues.

MATERIALS AND METHODS

Sampling and isolation from trees. Samples from internal tissues of 68 trees were taken from one orchard in Medford and six orchards in Hood River Valley, OR, from October to March in 1987-88 and 1988-89. Trees were randomly selected and included symptomless trees as well as trees with active or inactive cankers, which are commonly associated with disease caused by *P. syringae*. Stem segments (12–20 cm long from wood 1, 2, 3, and 4 yr old) and similar length root segments up to 4.5 cm in diameter were surface-sterilized by soaking in 1.0% sodium hypochlorite for 30 min, rinsed twice in sterile distilled water, and then flamed after immersion in 95% ethanol. The ends of each section (2-cm lengths) and the outer phloem tissue were aseptically removed to avoid epiphytic contaminants and the influences of sterilant penetration.

Tree segments were cut into 2- to 5-mm thick disks that were placed directly on the surface of *Pseudomonas* agar F (Difco Laboratories, Detroit, MI), amended with 40 μg/ml cycloheximide (PAFC) to inhibit fungal contaminants, and incubated for 3 days at 21 C. At least three sections of each age stem and six root sections were sampled for each tree.

Epiphytic *P. syringae* strains were collected from leaves and blossoms of pear trees at the seven orchards described above and from three additional orchards, one in Oregon and two in Washington. Leaves and blossoms were randomly collected at about 2 m in height and were combined in each sample. Tissue was bulked into approximately 20-g samples that were washed for 60 min on a rotary shaker at 150 rpm in 600-ml beakers containing 100 ml of sterile phosphate buffer (29). A dilution series of the wash was streaked on the surface of PAFC and incubated for 3 days at 21 C. Following incubation, plates were examined under UV light at 350 nm for presence of fluorescing (F+) bacterial colonies. All F+ isolates were purified by subculturing individual colonies three times and stored in sterile distilled water with 0.02% glycerol at 5 C.

Bacterial isolate characterization. Purified F+ isolates were cultured on nutrient agar (NA) for 24 hr at 21 C, then tested for cytochrome oxidase reaction (17), gelatin liquefaction (G) (14), aesculin hydrolysis (A) (33), tyrosinase activity (T) (5), and tartrate utilization (Ta) (34). Hypersensitivity response (HR) in tobacco (*Nicotiana tabacum* L. cv. Samsun MN) (16) and pathogenicity on pear and sweet cherry (*Prunus avium* L.) fruitlets using a stab inoculation technique (10) were evaluated for isolates grown for 48 hr on PAF at 21 C.

Ice nucleation activity of all isolates was evaluated at −5 C for 48-hr-old cultures grown on PAF at 21 C. A suspension of about 10^8 colony forming units (cfu) per milliliter was used with four replicates of ten 10-μl drops placed onto paraffin-coated aluminum foil boats floating on a 50:50, ethylene glycol:water solution in a refrigerated bath. The number of drops that froze within 2 min were recorded (19).

DNA restriction-fragment profile analysis. Twenty-four endophytic, eight epiphytic, and five reference strains of *P. syringae* were used for genomic DNA fingerprinting. This included DNA profile comparisons of isolates taken from both root and stem tissues of the same tree (five trees) and comparisons of endophytic and epiphytic isolates from the same tree (two trees). DNA profile comparisons were also made of selected isolates recovered from root and stem inoculations. The five reference strains (obtained from D. C. Gross, Washington State University, Pullman, WA) included extensively characterized phenotypes selected to provide a range of DNA profiles for comparison with other isolates and with each other.

Pure cultures were grown in King's medium B broth (15) and incubated on a rotary shaker at 150 rpm and 28–30 C for about 16 hr. Five milliliters of bacterial suspension were placed into 25-ml screw-capped Corex tubes and centrifuged at 8000 × g for 10 min at 4 C (Beckman J2-21, Beckman Instruments, Palo Alto, CA). Pelleted cells were resuspended in 10 ml of SET buffer (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid [EDTA]), centrifuged at 8000 × g for 10 min at 20 C, and resuspended in 4.5 ml of SET. Twenty-five microliters of Proteinase K (Bethesda Research Laboratories, Gaithersburg, MD [BRL]) (20 μg/ml) and 0.5 ml of 10% sodium dodecyl sulfate were added, mixed by inversion, and then incubated at 42 C for 60 min. Following incubation, 0.9 ml of 5M NaCl was added and mixed well until the precipitate dissolved. After complete dissolution, 0.65 ml 10% hexadecyltrimethylammonium bromide in 0.7M NaCl was added, mixed by inversion, and then incubated for 20 min at 65 C.

Nucleic acids were extracted in a three-step process with chloroform, phenol/chloroform (1:1 ratio), and chloroform. At each step the reagent was added and the mixture was agitated vigorously, then centrifuged at 10,000 × g for 10 min at 20 C. The aqueous phase was removed by pipette and placed into a sterile, clean test tube for the next step. After final extraction, the nucleic acids were precipitated with isopropanol and transferred to sterile Eppendorf tubes for a 95% ethanol wash. Finally, excess ethanol was decanted and the precipitated nucleic acids were dried at 21 C for 15 min and redissolved in 300–400 μl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Approximately 15–20 μl of each DNA preparation was digested with 15–25 units of EcoRI (BRL) at 37 C for 3 hr. Ten micrograms of RNase A (Sigma Chemical Co., St. Louis, MO) was added 10 min before the end of the digestion period. Digestion was terminated by adding 0.15M EDTA (pH 7.5).

The digested samples were electrophoresed, stained, and photographed as described by Maniatis et al (23). Electrophoresis was done at 4 V/cm for 0.25 hr, then at 2 V/cm for 13.75 hr in 5-mm-thick gels prepared with 0.6% agarose. HindIII-digested Lambda DNA (BRL) was loaded into each gel as a DNA molecular weight standard. Banding patterns in the DNA fingerprints were compared visually.

Systemic movement of *P. syringae* strains in roots. Actively growing pear seedlings of the cultivar Bartlett were grown in sand:peat:perlite (4:4:1). After removal from the medium, the seedlings were gently washed in tap water. The entire root system was then immersed into a bacterial suspension for 30 hr at 20 C. Bacterial suspensions of *P. syringae* strains W4N54 (obtained from D. C. Gross, Washington State University) and Mc2xa (obtained from Hood River Valley), epiphytic and endophytic strains, respectively, were prepared at about 10^8 cfu/ml (A_{600} was 0.3 ± 0.02 on a Spectronic 20 colorimeter, Bausch and Lomb, Rochester, NY). Sterile distilled water was used as the control treatment. Following treatment, the seedlings were repotted in the original medium and placed in a greenhouse at 22 C and 65% relative humidity under a photoperiod of 14 and 10 hr of light and dark, respectively. Natural light was extended to 14 hr by fluorescent lights after dark.

Evaluations of bacterial movement were done after 20 days. Stem sections were surface-sterilized and processed with the disk-section method previously described. Root segments of the seedlings were fibrous and too small in diameter for disk-sectioning. The largest root segments were removed from each

tree and rinsed with tap water. Root segments were surface-sterilized in 0.5% sodium hypochlorite for 10 min, rinsed three times in sterile distilled water, macerated aseptically, and placed onto PAFC for 3 days at 21 C. Five stem sections, 8–12 cm in length, were sampled in sequence distally from the crown, along with 1.5 g of root tissue from each tree, 15 trees per treatment. Isolates that were recovered were compared to strains W4N54 and Mc2xa for colony morphology, pigmentation, and growth rate, and tested for fluorescence and cytochrome oxidase reaction. Selected isolates were subjected to the GATTa determinative tests. Two isolates that appeared similar to each inoculum strain were processed for genomic DNA fingerprinting as described above.

Systemic movement of *P. syringae* strains in stems. Actively growing 3-yr-old pear trees of the cultivar Easter, potted in the medium described above, were used to evaluate bacterial mobility in stems of two endophytic *P. syringae* strains, KD7 and K4 (obtained from Anjou pear trees) and two epiphytic *P. syringae* strains W4N54 and AP1 (W4N54 origin is described above, and AP1 was obtained from an Asian pear, *Pyrus pyrifolia*). A 10-μl suspension containing about 10^6 cfu/ml of each strain was applied to one leaf scar of 1- and 2-yr-old stems on each tree, 10 trees per treatment, immediately after leaf removal. A sterile dissecting needle tip was inserted through the drop of inoculum 2–3 mm into the leaf scar, and then the inoculum site was wrapped with Parafilm (American Can, Greenwich, CT). Drops of sterile distilled water were used as the control. This procedure introduced about 10^4 cfu at each site as determined by dilution plating assay. After 20 days in a greenhouse at conditions described above, the inoculated limbs were removed and surface-sterilized as previously described. Disks from 1- to 2-mm thick were cut from each side of the point of inoculation to a distance of 8 cm, plated in sequential order on PAFC, and incubated for 3 days at 22 C. Colonies that fluoresced under UV light (350 nm) were purified as previously described and classified according to cytochrome oxidase reaction and the GATTa determinative tests. Selected isolates recovered from each treatment were prepared as described above for DNA fingerprinting.

RESULTS

Frequency and distribution of endophytic *P. syringae*. F+ Ox− isolates were recovered from internal tissues of 57 of 68 (84%) Anjou trees. F+ Ox− isolates were recovered exclusively from the roots of 35 (51.5%) trees and from the stems of six (9.0%) trees. F+ Ox− isolates occurred in both root and stem tissues in 16 (23.5%) of the trees. There was no apparent relationship between isolation of F+ Ox− isolates from internal tissues of a tree and whether the tree was symptomless or had suffered blossom blast and/or had cankers from which *P. syringae* had been previously isolated.

A total of 235 isolates were recovered from the interior of 68 Anjou trees: 83% (196/235) from root tissues and 17% (39/235) from stem tissues. Of the total F+ isolates, 81% (191/235) were cytochrome oxidase negative. No further tests were given to the F+, cytochrome oxidase positive isolates. A total of 159 F+ Ox− isolates were recovered from root tissues (Table 1). F+ Ox− isolates were randomly distributed in root sections ranging in diameter from 3 to 45 mm. Roots were sampled at various distances from the crown to obtain a diversity in size of the sections. Roots larger than 45 mm in diameter were difficult to sample and surface-sterilize, and removal of larger roots from a tree was discontinued once bacteria were isolated frequently from smaller roots.

Stem wood from 1 to 4 yr old was selected for sampling because, on most trees, wood this age was identified easily and was within a satisfactory size range for sampling. The distribution of F+ Ox− isolates in 1-, 2-, 3-, and 4-yr-old stems was 8, 7, 5, and 12 isolates, respectively (Table 1). F+ Ox− isolates were recovered from more than one age of stem tissue in only four trees. The remaining stem isolates were recovered from only one age of stem tissue per tree.

Bacterial isolate characterization. Of the total endophytic F+ Ox− isolates, 17.3% (33/191) were positive for gelatin liquefaction and aesculin in hydrolysis and negative for tyrosinase activity and tartrate utilization (GATTa+) (Table 1). Of the total epiphytes and reference strains collected for comparison, 72.5% (29/40) and 80% (8/10), respectively, were GATTa+. Also listed in Table 1 are the numbers of isolates that were classified GATTa-1, which indicates they were positive for gelatin liquefaction and aesculin hydrolysis, variable for tyrosinase activity, and negative for tartrate utilization. Tyrosinase activity was the most subjective of the four determinative tests and generally was repeated more than twice before the evaluation could be completed for many isolates. Tyrosinase activity for this group (GATTa-1) was listed as variable. If the negative reaction, which occurred in many single tests, was given to isolates in this category, then 41.9% (80/191), 100% (40/40), and 100% (10/10) of the endophytic, epiphytic, and reference isolates, respectively, would be classified as GATTa+ (Table 1). A third group of endophytes (111/191, 58%) was positive and negative in various combinations in the four tests and was classified as GATTa±.

Only 2.1% (4/191) of the total endophytic isolates were positive for ice nucleation activity (INA+) (Table 1). These four INA+ isolates were recovered from 4-yr-old stem tissues of three trees at different orchards. In contrast, 42.5% (17/40) and 30% (3/10) of the epiphytic and reference isolates, respectively, were INA+.

Of the total endophytic F+ Ox− isolates, 7.9% (15/191) elicited a hypersensitive response (HR) in tobacco, whereas 60% (24/40) and 100% (10/10) of epiphytic and reference isolates,

TABLE 1. Characterization of fluorescent, oxidase-negative bacteria isolated from internal root and stem tissues and surfaces of leaves and blossoms of Anjou pear

Source of isolates	Number of isolates	Number of isolates with positive reactions						
		Grouping according to Latorre & Jones (1979)[w]			Ice nucleation activity	Hypersensitive reaction	Pathogenicity[y]	
		GATTA+	GATTa-1[x]	GATTA±			Cherry	Pear
Endophytes								
Roots	159	24	44	91	0	2	2	1
Stems								
1 yr	8	2	1	5	0	2	0	0
2 yr	7	1	2	4	0	2	1	1
3 yr	5	0	0	5	0	2	0	0
4 yr	12	6	0	6	4	7	7	6
Epiphytes	40	29	11	0	17	24	23	21
Reference strains[z]	10	8	2	0	3	10	9	9

[w] GATTa+ isolates were positive for gelatin liquefaction and aesculin hydrolysis, negative for tyrosinase activity and tartrate utilization; GATTa± isolates were positive and negative in various combinations for the four tests.
[x] GATTa-1 isolates were positive for gelatin liquefaction and aesculin hydrolysis, variable for tyrosinase activity and negative for tartrate utilization.
[y] Pathogenicity tests were conducted on three replicates each of pear and cherry fruitlets.
[z] *Pseudomonas syringae* pv. *syringae* strains B-5, Ps-3, 5D246, 5D4105, B-301D, W4N54, W4N1613, B-326, HS191, Pss2-3RNH.

respectively, elicited a HR (Table 1). Eleven of the 39 endophytic and epiphytic isolates positive for HR were GATTa+, as were five of the 10 endophytic isolates positive for pathogenicity to fruitlets. These five came from 4-yr-old stem tissue. All isolates positive for fruitlet pathogenicity (except four) were pathogenic to both pear and cherry fruitlets. Two endophytic and two epiphytic isolates, each isolated from pear, were pathogenic to cherry but not to pear fruitlets (Table 1). There were no apparent relationships among the phenotypic determinative GATTa tests with INA, HR, and pathogenicity.

Comparative DNA fingerprints of isolates taken from the same tree were highly variable. For example, one isolate from each age of stem tissue in the same tree had similar DNA fingerprints (Fig. 1; lanes 2 and 6–8). Two isolates from root sections of this same tree also had DNA fingerprints similar to each other and the four stem isolates (DNA fingerprints of these isolates from roots not shown). In contrast, one isolate recovered from a stem (Fig. 1; lane 9) was dissimilar to two isolates recovered from root sections (Fig. 1; lanes 10 and 11) of the same tree. Two isolates taken from root tissue of another tree had dissimilar DNA fingerprints (not shown). Also, stem isolates taken from three adjacent trees in a row had dissimilar DNA profiles (Fig. 1; lanes 3–5). Two isolates with similar DNA fingerprints from tree F17 were from 4-yr-old stem tissues, and they were two of only four endophytic INA+ isolates (Table 1). The other two endophytic INA+ isolates had different DNA fingerprints. Reference strain W4N1613 was an INA− mutant of INA reference strain B301D, and a visual comparison of their DNA fingerprints (not shown) resulted in no discernable difference in banding patterns.

Systemic movement of *P. syringae* strains in roots. F+ Ox− isolates were recovered from root segments of all trees root-inoculated with W4N54 and Mc2xa. F+ Ox− isolates were recovered from root tissues of three of the 15 trees inoculated with sterile distilled water. In all cases, F+ Ox− isolates recovered from trees inoculated with *P. syringae* strains W4N54 or Mc2xa were similar to the corresponding inoculum strains in colony morphology, pigmentation, and growth rate. Selected isolates were all GATTa+. The DNA fingerprints of the selected isolates were also similar to the corresponding inoculum strains. Stem sections of all root-inoculated trees yielded no F+ Ox− isolates, but F+ Ox+ isolates were recovered from two trees inoculated with strain Mc2xa.

Systemic movement of *P. syringae* strains in stems. Systemic movement of strains of *P. syringae* inoculated into 1- and 2-yr-old stem tissues varied with strain and age of tissues (Table 2). F+ Ox− isolates were recovered from up to 30 mm from the inoculation sites and from leaves directly adjacent to inoculation sites. There was greater ($P = 0.05$) movement in 2-yr-old tissues compared to 1-yr-old tissues for the epiphytic strains but not for the endophytic strains. There was also less movement in both ages of stem tissue for endophytic strain K4 compared to all other strains. Strain AP1 was detected at a significantly ($P = 0.05$) farther distance in 2-yr-old tissues than all other strains. There were no F+ Ox− isolates recovered from trees inoculated with sterile distilled water.

Selected isolates from inoculated trees were similar to the isolates used as inoculum (with respect to colony morphology, pigmentation, growth rate) and tested GATTa+. Genomic DNA fingerprints of selected isolates were similar to the corresponding inoculum strains.

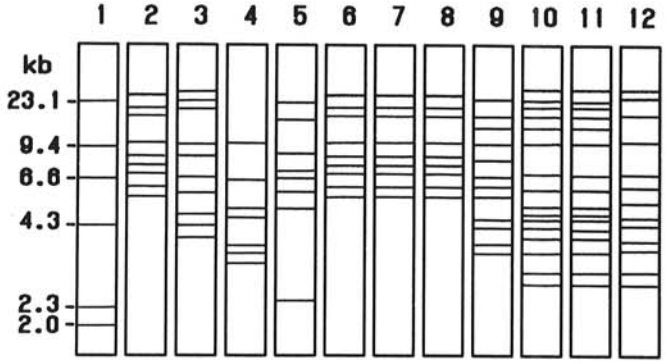

Fig. 1. Restriction-fragment banding patterns of cellular DNA from 11 different isolates of *Pseudomonas syringae* digested with EcoRI (original photograph available from second author). Lane 1 shows DNA profile standard (Lambda DNA Hind III digest) marked in kilo-based pairs in the margin; lanes 2 and 6–8 are DNA fingerprints of strains recovered from four ages of stem tissue in the same tree; lanes 3–5 are DNA profiles of isolates recovered from stems of three adjacent trees in the same row; lanes 9–11 are DNA profiles of isolates recovered from stem and two root sections, respectively, of the same tree; lane 12 is DNA profile of isolate AP1. Visual examination of bands between kb markers 2.3 and 9.4 are best for comparison.

DISCUSSION

Based on the results of these studies, there is a high frequency and wide distribution of a heterogeneous population of endophytic F+ Ox− bacteria in pear trees. Heterogeneity in the epiphytic pseudomonad population on host plants has been well documented (1,6,10,21). Heterogeneous populations of endophytic pseudomonads also occur in sweet cherry (3,4) and peach (6).

The use of genomic DNA fingerprinting in this study provided genotypic comparisons of isolates taken from trees at different sites, of isolates taken from different tissues, and of isolates with known genomic similarity, such as the INA+ wildtype and its INA− mutant. The genomic fingerprint was also used to confirm recovery of inoculated strains rather than the traditional tracking of an antibiotic-mutant strain. Because resolution within a gel decreased toward the cathode, we could not be certain that similar DNA fingerprints meant that strains were identical. In order to avoid the limitations of genomic DNA fingerprinting, *P. syringae* strains selected for inoculum had clearly different DNA fingerprints readily observable within the area of a gel with highest resolution. Therefore, using these criteria, we agree with Malvick and Moore (21) that DNA profile analysis can be a useful tool to compare the identity of individual isolates that cannot otherwise be distinguished morphologically or by comparison of traditional phenotypic characteristics. We also extend its usefulness as a tool to track inoculated wildtype strains.

The inability to detect movement of root-inoculated *P. syringae* strains above the crown and the relatively short (3 cm) translocation of stem-inoculated *P. syringae* strains suggests limited vascular mobility of internal strains in pear tissues. *P. syringae* spreads systemically up to 6 cm from the point of inoculation in plum shoots (32). The presence of strains with similar DNA profiles at different locations within a tree appears contrary to the observed results of the experiments concerning systemic movement and supports the hypothesis that mobility within a tree may be greater than was detected in these trials. It is possible that the limited movement of *P. syringae* in our study was related

TABLE 2. Movement of inoculum of two epiphytic and two endophytic strains of *Pseudomonas syringae* in 1- and 2-yr-old stem tissue of pear trees

Source	Inoculum strain[y]	Movement from wound site (mm)[z]	
		1-yr-old stem	2-yr-old stem
Epiphyte	W4N 54	8.3 bA	12.7 bB
	AP 1	11.9 bA	17.1 cB
Endophyte	KD 7	10.5 bA	9.3 bA
	K 4	3.0 aA	5.2 aA
	Control	0.0 aA	0.0 aA

[y] Inoculum for each strain in the infection court was approximately 10^4 cfu.

[z] Means of two replicates of 10 trees each. Means followed by the same letter (lower-case letters for rows and upper-case letters for columns) do not differ ($P = 0.05$) according to Duncan's multiple range test.

to time between inoculation and evaluation (20 days), which was not long enough to permit more extensive bacterial movement in plants. Also, the recovery techniques used in these trials may be limited in their sensitivity to detect extremely small numbers of bacterial cells that may have moved.

Colonization of internal tissue and systemic movement by *P. syringae* inoculated onto pear leaves and blossoms has been described based on scanning electron microscopy (25,26). Although endophytic *P. syringae* strains were recovered with relatively high freguency in our study, most of them were from root tissues. A low number of these strains were positive for HR or pathogenicity on fruitlets and none were INA+. These three characteristics are considered to be important predictors of pathogenic potential (10,20). *P. syringae* isolates were recovered with less frequency from stem tissues, but a greater proportion of these strains had one or more of the pathogenic characteristics. Despite the low proportion of endophytic strains positive for these characteristics, the presence of any endophytic strains with one or more of these three pathogenic capabilities suggests that the internal tissues of pear potentially provide a source of inoculum that can contribute to disease in the host, or serve as a source of inoculum for dispersal. A previous study with plum suggests that epiphytic populations of *P. syringae* are constantly replenished from systemically invaded, symptomless trees (32). The endophytic phase could also be important as a source of inoculum to be spread by grafting of infected scions. We agree with the hypothesis proposed by Sundin et al (35) that endophytic bacterial movement could occur during metabolite translocation in spring and fall, but further evidence is required to validate this hypothesis.

The ubiquitous nature of the epiphytic *P. syringae* and its ability to colonize internal tissues are thought to be responsible for the erratic success of chemical control of pear blast (2,8,12). The apparent ability of *P. syringae* to exist in an endophytic phase permits the bacterium to avoid contact with any nonsystemic bactericidal compound. We are currently evaluating bactericide sensitivity of endophytic and epiphytic isolates collected in this study.

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