Copper, Oxytetracycline, and Streptomycin Resistance of *Pseudomonas syringae* pv. *syringae* Strains from Pear Orchards in Oregon and Washington

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ABSTRACT

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A total of 323 strains of *Pseudomonas syringae* pv. *syringae* were collected from six pear orchards in the Mid-Columbia region of Oregon and Washington from 1989 through 1992. Of all strains of *P. s.* pv. *syringae,* 8, 25, 75, and 99% did not grow on modified casitone–yeast extract–glycerol medium amended with 0.25, 0.5, 1.0, and 2.0 mM $CuSO_4$, respectively. Strains of *P. s.* pv. *syringae* resistant to 50, 100, 250, and 500 µg of oxytetracycline per ml were recovered from six, three, two, and one of the six orchards, respectively. Nevertheless, over 70 and 90% of the strains in all six orchards were sensitive to 50 and 250 µg of oxytetracycline per ml, respectively. Strains of *P. s.* pv. *syringae* resistant to 50 and 500 µg of streptomycin per ml were found in six and four of six orchards, respectively. Twenty-five strains were resistant to both copper (1 mM) and streptomycin (100 µg/ml), and three of those were resistant to copper, streptomycin, and oxytetracycline (250 µg/ml). To our knowledge, this is the first report demonstrating resistance of strains of *P. s.* pv. *syringae* to copper, streptomycin, and oxytetracycline. Resistance correlated positively to the antibiotic spray programs in the orchards.

Pseudomonas syringae pv. *syringae* van Hall causes blossom blast and canker of pear (*Pyrus communis* L.) and has resulted in sporadic but serious losses in the Pacific Northwest (19,25). Population dynamics, distribution, and characterization of strains of *P. s.* pv. *syringae* in the Pacific Northwest have been studied (6). Also, several factors affecting severity of both Pseudomonas blossom blast (25) and canker (19) have been determined. The incidence of blossom blast is closely related to frost, moisture, bloom developmental stage, and inoculum density (12,25). Canker incidence is affected by low temperature injury, cultivar, and use of plastic trunk wraps (19).

Current recommendations for control of *P. s.* pv. *syringae* in pear orchards in Oregon include application of a fixed copper bactericide (3.84 kg a.i./acre) at bud break and streptomycin (100 µg a.i./ml) at early bloom (17). Although many growers carefully follow this program, *P. s.* pv. *syringae* continues to cause unacceptable levels of disease in some years.

Pseudomonas s. pv. *syringae* often is found as an endophyte in pear shoots and roots (24) where the bacteria are protected in the pear tissue from topical antibiotic applications. Many of the endophytic strains do not infect pear fruitlets, but some cause disease of blossoms. In addition, *P. syringae* strains found in trichomes and stomata and colonizing the stomatal complex from adjacent tissue (10) may be a source of epiphytic populations (11). Inadequate control of *P. s.* pv. *syringae* could be attributed to the occurrence of strains resistant to antibiotics. In California, strains of *P. s.* pv. *syringae* resistant to copper have been reported in almond and citrus orchards (1). Strains of *P. s.* pv. *syringae* resistant to both copper and streptomycin have been isolated from ornamental pear trees grown in nurseries in Oklahoma (21).

The objective of this study was to determine the sensitivity to copper, oxytetracycline, and streptomycin of strains of *P. s.* pv. *syringae* in pear orchards in the Mid-Columbia region of Oregon and Washington. The survey was conducted over a 4-year period in six orchards representing diverse use of antibiotics.

MATERIALS AND METHODS

Collection of strains from pear orchards. Samples of pear blossoms were obtained from six mature pear orchards (four commercial, one abandoned, and one at the Mid-Columbia Agricultural Research and Extension Center) in the Mid-Columbia region of Oregon and Washington. The orchards were selected to represent the diversity in antibiotic use patterns.

Orchard A had been abandoned for over 20 years and thus received no antibiotics. No copper was sprayed in orchards B and C, while orchards D, E, and F received one application of copper per year prior to and during the study. Streptomycin was applied about once every 2 years in orchards B, E, and F, and once per year in orchard D. Orchard C received streptomycin four times in 1988, and oxytetracycline two, two, seven, five, and three times in 1988 through 1992, respectively. By 1991, streptomycin was no longer used in this orchard. Oxytetracycline was not used in orchard F and was applied about once every 4 years in orchards B and E and once every 2 years in orchard D. Orchards and the number of times they were sampled each year, in parentheses, were as follows: 1989—A (1) and B (1); 1990—A (1), B (3), C (2), D (3), E (1), and F (2); 1991—A (1), B (3), C (2), D (3), E (1), and F (1); 1992—A (1) and C (1).

Blossom samples were collected during April and May each year and consisted of approximately 30 blossom clusters. To recover bacteria, 30 g of blossoms were placed in beakers with 250 ml of sterile phosphate buffer (15) and shaken at 150 rpm on a rotary shaker for 1 to 2 h at 22 ± 1°C. Serial dilutions were spread on duplicate plates of *Pseudomonas* agar F (PAF) (Difco Laboratories, Detroit, Mich.) amended with 40 µg of cycloheximide per ml. After 72 h, colonies that fluoresced under long UV light at 350 nm were purified (maximum of five colonies per plate) on nutrient agar and tested for cytochrome oxidase activity (8) and pathogenicity to pear fruitlets (25). Strains that were fluorescent, cytochrome oxidase negative, and pathogenic to pear fruitlets were subcultured three times on PAF, then stored in sterile distilled water with 0.02% glycerol at 5°C. Strains were tested for antibiotic resistance within 3 months after recovery.

Resistance to copper, oxytetracycline, and streptomycin. Copper sensitivity of isolates was determined by spotting 10 µl of an aqueous bacterial suspension of each isolate (approximately 10^6 cfu per ml based on a standard dilution and verified by dilution plating on PAF) onto casitone–yeast extract–glycerol medium (CYE) (1) modified by decreasing the casitone from 1.7 to 1.0 g/liter. Strains also were spotted onto modified CYE amended with cupric sulfate to obtain concentrations of 0.25, 0.5, 1.0, and 2.0 mM. Plates were incubated for 2 days at 21°C, then the presence or absence of growth was determined. Three replicates of each copper concentration were included in one experiment for each isolate.

Purified isolates were tested for resistance to oxytetracycline and streptomycin by spreading 100 µl of aqueous suspen-

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sions of each strain (approximately 10^6 cfu/ml) onto PAF. After the bacterial suspension was absorbed on the surface of the PAF, sterile blank Bacto concentration disks (6.35 mm diameter, Difco) were dipped in suspensions of oxytetracycline (Mycoshield, Merck & Co., Inc., Whitehouse Station, N.J.) or streptomycin sulfate (Agri-mycin 17, Merck & Co., Inc.) containing 50, 100, 250, and 500 µg a.i./ml, blotted to remove excess moisture, then placed equidistantly apart on the surface of the PAF. Three replicate plates, each containing the four antibiotic concentrations of oxytetracycline or streptomycin and a water control disk, were prepared for each strain. Inhibition zones, the distance from the edge of the disk to the bacterial lawn, were measured after 2 days of incubation at 22°C. Whenever the inhibition zone was ≤1 mm, the strain was recorded as resistant to the antibiotic.

RESULTS

A total of 323 strains of *P. s.* pv. *syringae* that were fluorescent, cytochrome oxidase negative, and pathogenic were collected from six pear orchards from 1989 through 1992. All 323 were tested for resistance to copper, but three strains were subsequently lost and could not be tested for resistance to oxytetracycline or streptomycin. In addition to these pathogenic strains, many strains were recovered that were negative in the pear fruitlet pathogenicity test. The nonpathogenic strains were not tested for antibiotic resistance.

Resistance to copper. Of 323 strains of *P. s.* pv. *syringae,* 8, 25, 75, and 99% did not grow on CYE medium amended with 0.25, 0.5, 1.0, and 2.0 mM $CuSO_4$, respectively (Table 1). Strains from the abandoned orchard (A) were more sensitive to copper than those from the other orchards, and 46% did not grow on CYE amended with 0.5 mM $CuSO_4$ or higher (Table 1). Even in this orchard, however, 4% of the strains grew on CYE amended with 1 to 2 mM $CuSO_4$.

Copper was not used immediately prior to or during the study in orchards A, B, and C, and these orchards had the highest percent of *P. s.* pv. *syringae* copper-sensitive strains (grew on nonamended CYE but not on copper-amended CYE). However, the percentage of strains that grew at 1.0 mM $CuSO_4$ was considerably higher in orchards B and C than in orchard A.

In orchards D, E, and F, a single application of fixed copper was made at bud burst each year. Most strains of *P. s.* pv. *syringae* in these orchards grew at $CuSO_4$ concentrations of 0.25 to 1.0 mM. Considerable variability, however, occurred in copper sensitivity, especially at the 1.0 mM concentration, at which only 5% of the strains from orchard E grew but 33 to 36% from orchards D and F grew.

Resistance to oxytetracycline. Strains of *P. s.* pv. *syringae* resistant to 50, 100, 250, and 500 µg of oxytetracycline per ml were recovered from six, three, two, and one of the six orchards, respectively (Table 1). Nevertheless, over 70 and 90% of the strains in all six orchards were sensitive to 50 and 250 µg of oxytetracycline per ml, respectively. Of the 320 strains tested for oxytetracycline resistance, 317 were sensitive to 500 µg of oxytetracycline per ml, and the three resistant strains were from orchard C, in which oxytetracycline was applied 15 times during the 3 years of sampling in this orchard. Average sizes of zones of inhibition for sensitive strains were 4.2, 6.4, 9.0, and 12.0 mm from the edge of the disk to the edge of the bacterial colony for 50, 100, 250, and 500 µg of oxytetracycline per ml, respectively. Inhibition zones for resistant strains were always less than 1 mm and averaged 0.6 mm.

Resistance to streptomycin. Strains of *P. s.* pv. *syringae* resistant to 50 µg of streptomycin per ml occurred in all six orchards, and resistance to 500 µg of streptomycin per ml was detected from four of the six orchards (Table 1). In orchard D, where streptomycin was applied once each year at about 100 µg a.i./ml, 33% of the strains were resistant to this concentration. In orchards sprayed less frequently with streptomycin (B, E, and F) or not at all (A), strains of *P. s.* pv. *syringae* resistant to 100 µg/ml ranged from 0 to 24% (Table 1). Streptomycin was applied four times, once, and once, in 1988, 1989, and 1990, respectively, in orchard C, and 76% of the strains of *P. s.* pv. *syringae* were resistant to 100 µg/ml. Average zones of inhibition were 4.1, 6.3, 8.6, and 11.1 mm for 50, 100, 250, and 500 µg of streptomycin per ml, respectively, for sensitive strains and averaged less than 0.2 mm for resistant strains.

Resistance to multiple antibiotics. Whereas resistance to single antibiotics occurred in 27, 2, and 28% of the strains of *P. s.* pv. *syringae* to copper (≥1 mM), oxytetracycline (250 µg/ml), and streptomycin (100 µg/ml), respectively, resistance to more than one antibiotic was less frequent (Fig. 1). Of the 320 strains of *P. s.* pv. *syringae*, 25 were resistant to both copper and streptomycin, and 3 of these were resistant to copper, streptomycin, and oxytetracycline (Fig. 1). None of the copper-sensitive strains were resistant to both streptomycin and oxytetracycline. Strains were exposed to single antibiotics in our tests rather than all combinations of antibiotics simultaneously.

DISCUSSION

Strains of *P. s.* pv. *syringae* resistant to copper, oxytetracycline, and streptomycin were common in pear orchards in the Mid-Columbia region of Oregon and Washington. Copper and streptomycin are often used for control of blossom blast, and all three antibiotics are used for control of fire blight caused by *Erwinia amylovora.* Thus, it appears that lack of control of blossom blight and canker caused by *P. s.* pv. *syringae* may be related to antibiotic resistance. In addition, other factors may be involved, such as endophytic populations that are not in contact with the antibiotics (24) and the recently reported phenomenon that wounds may enhance resistance of plant pathogenic bacteria at infection sites and eliminate the effect of copper compounds (5).

Table 1. Resistance to copper sulfate, oxytetracycline, and streptomycin of strains of *Pseudomonas syringae* pv. *syringae* collected from pear orchards in the Mid-Columbia region of Oregon and Washington from 1989 through 1992

		Percentage of strains					Strains resistant (%)[d]							
		Copper sulfate in CYE (mM)[c]					Oxytetracycline (µg/ml)				Streptomycine (µg/ml)			
Orchard[a]	Number of strains[b]	0.0	0.25	0.5	1.0	2.0	50	100	250	500	50	100	250	500
A	74	12	34	50	1	3	22	7	3	0	32	18	3	1
B	37	16	16	21	45	2	8	0	0	0	54	24	14	11
C	66	12	6	49	33	0	27	9	8	5	76	76	59	50
D	55	4	16	47	33	0	29	11	0	0	49	33	25	18
E	38	0	16	79	5	0	3	0	0	0	26	0	0	0
F	50	2	6	56	36	0	10	0	0	0	16	0	0	0

[a] Orchard A received no antibiotics. Orchards B and C received no copper, while orchards D, E, and F were sprayed with copper once each spring. Orchard C was sprayed with oxytetracycline 19 and with streptomycin 6 times from 1988 through 1992. Other orchards received one to three antibiotic applications per year (see Methods for detailed antibiotic schedules).

[b] Orchards B and D had 38 and 57 strains, respectively, in the copper sulfate test.

[c] Modified casitone–yeast extract–glycerol agar. Copper sulfate concentrations are maximum at which strains grew.

[d] Radius of inhibition zone ≤1 mm.

Copper was not applied in orchards A, B, and C during the study, but resistance to 1 mM copper appeared much greater in orchards B and C than in orchard A. Commercial orchards adjoining orchards B and C did receive copper applications, and resistant strains may have moved into the sampled orchards from the sprayed orchards. In contrast, orchard A was isolated from other commercial orchards by more than 20 km. The one application of copper to orchards D, E, and F appeared to result in a decrease in the percentage (0 to 4%) of strains that were sensitive to 0.25 mM copper, compared with 12 to 16% sensitive strains in orchards A, B, and C.

Strains of *P. s.* pv. *syringae*, even those isolated from the abandoned orchard, expressed greater resistance to copper than strains recovered from almond and citrus orchards in California that received several copper applications annually (1). This difference in expression of copper resistance may be due to ecotype differences in *P. s.* pv. *syringae*. Gross et al. (7) reported that bacteriocin production is a useful tool in differentiating strains of *P. s.* pv. *syringae*. They found that strains from fruit trees exhibited relatively specific bacteriocin patterns, with just four groups (6C, 8B, 8F, and 13) containing nearly 90% of all the strains. Interestingly, the predominance of typical bacteriocin group 14 strains in California orchards and the apparent absence of similar strains in the Pacific Northwest suggest that two different ecotypes of *P. s.* pv. *syringae* exist in these two different geographic locations (7).

High levels of copper resistance of *P. s.* pv. *syringae* also occur in Michigan in cherry orchards where 92% of the strains grew on 1.0 to 2.0 mM copper sulfate (23). Copper-resistant strains were detected in eight orchards for two consecutive years, indicating survival from season to season. In addition, conjugative transfer of copper resistance was demonstrated and provides evidence that pathovars of *P. syringae* have potential for developing resistance to antibiotics under selection pressure in the field.

Oxytetracycline is applied to pear trees at the rate of 200 µg/ml for control of fire blight (17). Thus, strains of *P. s.* pv. *syringae* are exposed to this antibiotic as nontarget bacteria. Of 138 strains of *E. amylovora* from major pear-growing regions of Washington State, none were resistant to oxytetracycline (25 µg/ml) or copper (0.16 mM) (9). In our survey of *P. s.* pv. *syringae*, we found strains resistant to 250 µg of oxytetracycline per ml in two of the six orchards. In orchard C, where oxytetracycline was applied 15 times over 3 years, three strains were recovered that were resistant to 500 µg/ml.

Strains of *P. s.* pv. *syringae* resistant to 500 µg of streptomycin per ml were found in four of the six orchards in this study. Resistance to streptomycin is very stable in *P. s.* pv. *syringae*, and plasmids encoding copper and streptomycin resistance were highly stable for over 200 generations of growth, suggesting that streptomycin-resistant strains of *P. s.* pv. *syringae* will persist in orchards independently of the bactericidal spray regime (22).

In addition to resistance to each of the individual antibiotics, we found 25 strains that were resistant to both copper (1 mM) and streptomycin (100 µg/ml). These are the two most commonly used antibiotics for control of blossom blight and fire blight. Only one previous study reports on strains of *P. s.* pv. *syringae* resistant to both copper and streptomycin (21). In that study, five of the 32 resistant strains were isolated from ornamental pear. Strains of *P. cichorii* and *Xanthomonas campestris* pv. *vesicatoria* also have been reported resistant to both copper and streptomycin (16,18). We found that three copper-resistant strains but none of the copper-sensitive strains also were resistant to streptomycin and oxytetracycline. To our knowledge, this is the first report demonstrating indigenous strains of *P. s.* pv. *syringae* resistant to copper, streptomycin, and oxytetracycline.

Copper resistance (Cu^r) determinants have been localized to plasmid DNA in all phytopathogenic bacteria studied (3,4,20, 23). The copper-inducible plasmid-borne operon (*cop*) of *P. syringae* contains four genes, all of which are required for expression of full copper resistance (2,13, 14). The streptomycin resistance (Sm^r) determinants of *P. s. papulans*, *P. s.* pv. *syringae*, and *X. c. vesicatoria* recently have been shown to be homologous to *strA-strB* genes of the broad-host-range entobacterial plasmid RS1010, and linkage of Cu^r and Sm^r determinants on conjugative plasmids has been demonstrated (21). It was postulated that use of other antibiotics such as tetracycline may select for additional gene transfer events and result in further development of antibiotic-resistant determinants. Oxytetracycline hydrochloride induced copper resistance in three pathovars of *P. syringae* when added to bacterial cells suspended in water (5).

Recently, streptomycin and oxytetracycline have been tank mixed for fire blight control in the Mid-Columbia region. In orchards where copper resistance is minimal and, thus, resistance to oxytetracycline and streptomycin is less common, this combination may be the most effective treatment available for control of blossom blast. However, additional field studies are needed to confirm the degree of effectiveness of the antibiotic combination.

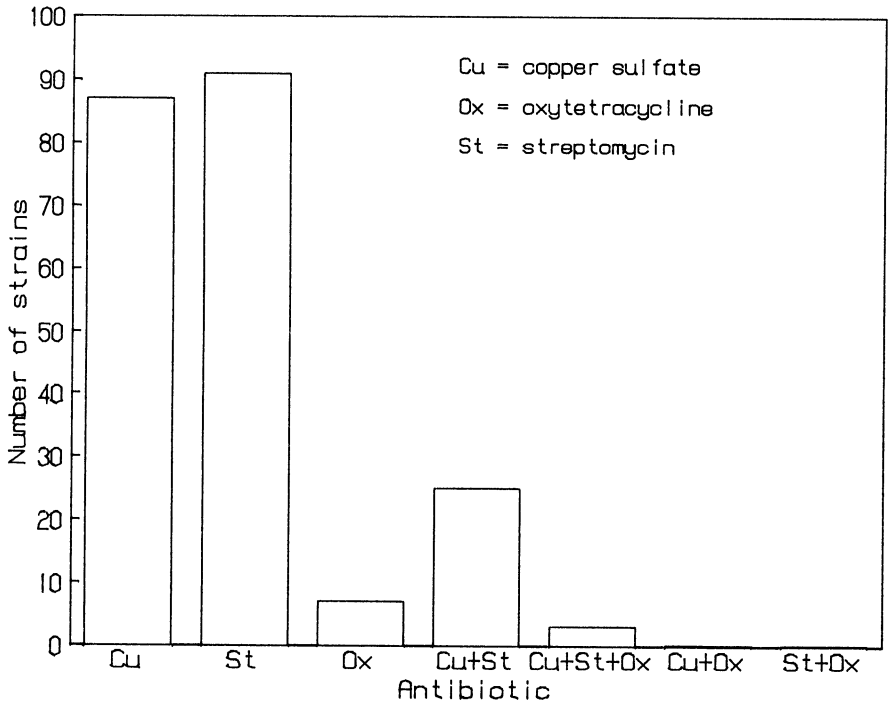

Fig. 1. Number of strains of *Pseudomonas syringae* pv. *syringae* resistant to copper sulfate (≥1 mM), oxytetracycline (250 µg/ml), streptomycin (100 µg/ml), or to more than one antibiotic.

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