Evaluation of Streptomycin, Oxytetracycline, and Copper Resistance of *Erwinia amylovora* Isolated from Pear Orchards in Washington State

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ABSTRACT

Loper, J. E., Henkels, M. D., Roberts, R. G., Grove, G. G., Willett, M. J., and Smith, T. J. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington State. Plant Dis. 75:287-290.

One hundred and thirty-eight pathogenic strains of *E. amylovora* were isolated from fire blight cankers of pear trees from 44 orchards in the major pear-growing regions of Washington State. Ninety-eight strains, isolated from 38 of the orchards sampled, were resistant to streptomycin (1 mg/ml). Streptomycin-resistant strains of *E. amylovora* were ubiquitous in all of the major pear-growing regions of Washington. None of the strains tested were resistant to oxytetracycline (25 μg/ml) or $CuSO_4$ (0.16 mM). Nevertheless, spontaneous mutants with tolerance to 0.16 mM $CuSO_4$ were observed at a frequency of 10^{-6}–10^{-7} mutant colonies per wild type colony in most strains.

Fire blight disease of pear (*Pyrus communis* L.), which is caused by *Erwinia amylovora* (Burrill) Winslow et al, was first observed in the Pacific Northwest region of the United States in the early 1900s (5). Low levels of fire blight are commonly observed on pear in Washington, but severe disease, which threatens scaffold branches or entire trees, is rare. Mean temperatures above the published threshold conducive to blossom infection (15,16,18) are rare during primary bloom of pear in Washington (4). A severe fire blight epidemic occurred in the spring of 1988, however, when unusually warm temperatures and heavy rains coincided with primary bloom (19). Two consecutive days of mean temperatures above 18 C and 4 days above 15 C were recorded in orchards of the Yakima Valley (G. G. Grove, *unpublished*), a major pear-growing region of the state. The fire blight epidemic of 1988 resulted in great economic losses to growers throughout Washington, attributable to reductions in fruit yield and loss of scaffold branches or trees to disease. Streptomycin, oxytetracycline, and fixed copper compounds are applied commonly to pear for fire blight disease control, but the sensitivities of indigenous *E. amylovora* to these compounds are unknown. Streptomycin-resistant strains of *E. amylovora* have been reported in many regions of the United States, including California (11), Missouri (12), and the Yakima Valley of Washington (5). The presence of such strains in other pear-growing regions of Washington has not been reported. The present study was initiated to evaluate the resistance of strains isolated from diseased pear trees in Washington State to streptomycin, oxytetracycline, and copper. Preliminary results of this study have been published (6).

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MATERIALS AND METHODS

Isolation of *Erwinia amylovora*. Pear branches bearing active fire blight cankers were collected in October 1988 from 44 orchards located in the major pear-growing regions of Washington (Fig. 1). Up to four branches with visible cankers were sampled from different locations in each orchard. Peridermal sections of water-soaked cankers were streaked across the surface of CCT agar, the differential medium of Ishimaru and Klos (7). A representative colony, which conformed to the pulvinate, striate appearance of *E. amylovora* on CCT, was selected from each plate, purified on CCT, and promptly lyophilized in skim milk solution. One hundred and thirty-eight strains of *E. amylovora* were isolated in this manner. Pathogenicity of all strains was confirmed on apple seedlings by a modification of the technique of Ritchie and Klos (9) described previously (10).

Preparation of inoculum for sensitivity tests. *E. amylovora* and *Pseudomonas* spp. were routinely cultured at 28 C on Luria Bertani medium (LB) (8) and nutrient agar (Difco Laboratories, Detroit, MI) amended with glycerol (1% w/v), respectively. Individual colonies from 48-hr cultures were suspended in sterile distilled water and adjusted to a cell density of approximately 10^8 colony-forming units (cfu) per milliliter (A_{640} = 0.10–0.15) for use in antibiotic sensitivity tests.

Streptomycin and oxytetracycline sensitivity. One-tenth of a milliliter of a bacterial suspension was spread on the surface of LB medium. A sterile filter paper disk (12.7 mm diameter) infiltrated with 50 μl of streptomycin sulfate at 10, 100, 500, or 1,000 μg/ml or oxytetracycline at 10, 25, 50, or 100 μg/ml, was placed on the agar surface. The radius of each inhibition zone was measured after plates were incubated for 72 hr at 28 C. Strain A506 of *P. fluorescens* Migula and strain Cit7 of *P. syringae* van Hall, with known resistance and sensitivity, respectively, to both streptomycin and oxytetracycline (S. Lindow, *personal communication*), were included as controls in all experiments. Sensitivity was determined from duplicate plates in each of two independent experiments.

Copper sensitivity. CYE medium, which had limited cupric ion binding capacity, was composed of 1.7 g of casitone (Difco), 0.35 g of yeast extract (Difco), 2 g of glucose, and 15 g of purified agar (Difco) per liter (20). Autoclaved CYE was cooled to 50 C and amended with filter-sterilized $CuSO_4$ to final concentrations of 0.00, 0.02, 0.04, 0.08, 0.16, and 1.10 mM. Bacterial suspensions were spotted on the agar surface in 10-μl droplets. Bacterial growth was evaluated after 72 hr of incubation at 28 C. Strains A1513 and A1487 of *P. syringae* (1), with known copper tolerance and sensitivity, respectively, were included as controls in all experiments. The frequency with which spontaneous copper-tolerant mutants were detected among cells of a copper-sensitive strain was calculated from colony numbers on CYE medium either unamended or amended with 0.16 mM $CuSO_4$. Aliquots (0.1 ml) of a dilution series of a bacterial suspension were spread on surfaces of CYE, and plates were incubated at 28 C for 3 days. Pathogenicity of representative copper-tolerant derivatives of two strains was compared with that of parental strains on immature pear fruit. Fruit were wounded, inoculated with bacterial cells, and incubated at room temperature and 100% RH for 3–4 days. The presence of water-soaked lesions and bacterial ooze was evidence for pathogenicity.

Grower survey. A survey was mailed to each grower managing one or more of the 44 orchards that were sampled in this study. Questions addressed the pear varieties grown; orchard age; the history of streptomycin, copper, and oxytetracycline applications; and the severity of fire blight in 1988. Disease severity was categorized as follows: 1) no fire blight; 2) mild fire blight (strikes were visible, but there was no substantial loss of fruit yield and scaffold branches were not lost where strikes were pruned out); 3) moderate fire blight (fruit yield was reduced and some scaffold branches were removed but no trees were lost); 4) severe fire blight (scaffold branches and up to 10% of the trees were lost); and 5) very severe fire blight (more than 10% of trees were lost to fire blight). Streptomycin application was categorized by the number of years as follows: 1) 0 yr, 2) 1 yr, 3) 2–5 yr, 4) 5–10 yr, and 5) 10 or more years.

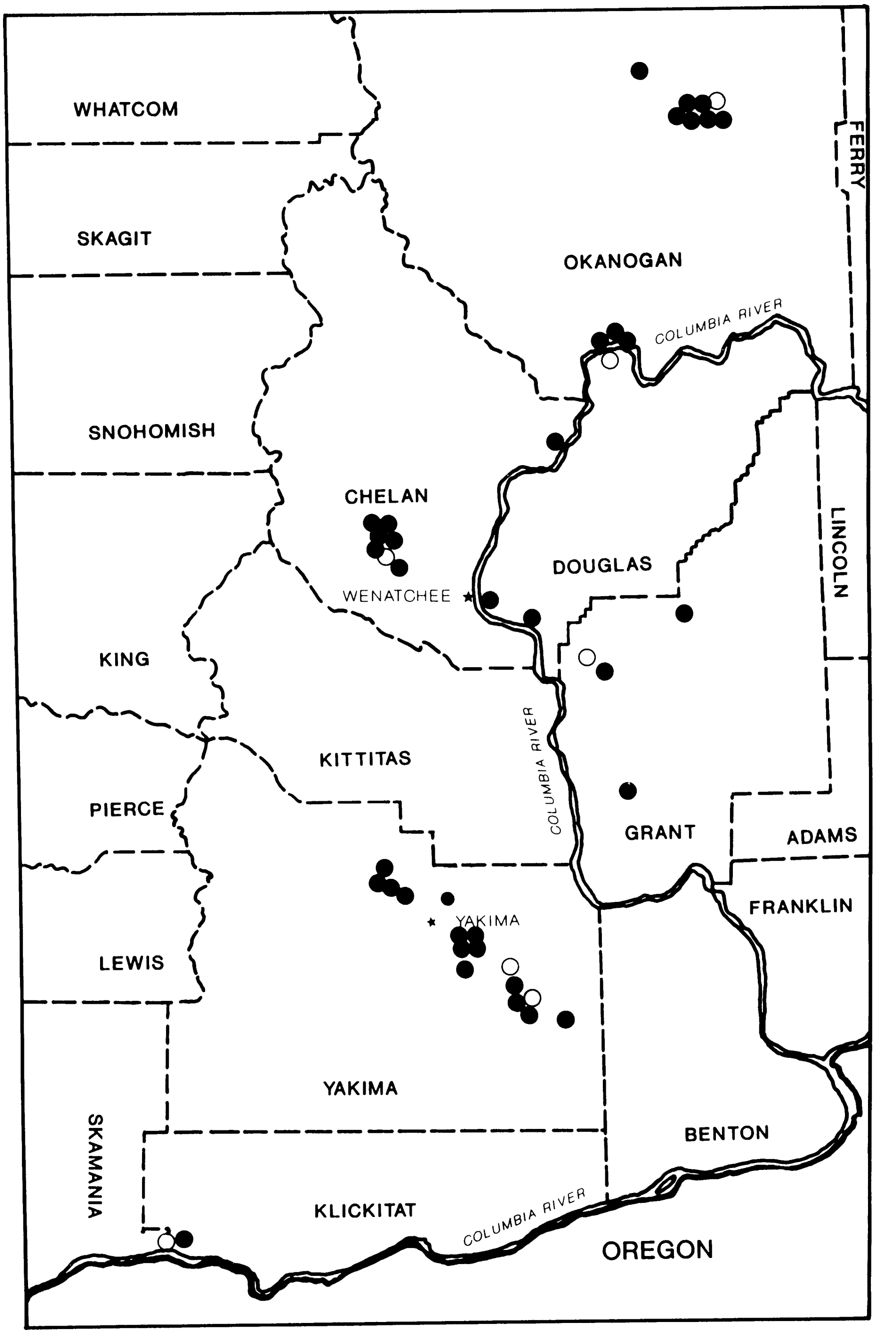

Fig. 1. Orchards in Washington State sampled for *Erwinia amylovora*. Only streptomycin-sensitive *E. amylovora* were isolated from orchards represented with an open circle (○). Streptomycin-resistant *E. amylovora* were isolated from orchards represented with a closed circle (●).

RESULTS

Streptomycin sensitivity. Ninety-eight of the 138 strains of *E. amylovora* isolated from fire blight cankers in pear orchards in Washington were resistant to streptomycin at 1,000 μg/ml. Streptomycin-resistant strains were isolated from 38 of the 44 orchards sampled (Fig. 1). Resistant strains of *E. amylovora* and strain A506 of *P. syringae* were not inhibited by streptomycin at any concentration tested. Forty strains of *E. amylovora* were sensitive to streptomycin. Radii of inhibition zones of sensitive strains ranged from 0 to 2, 2 to 5, and 4 to 6 mm surrounding disks with streptomycin at 100, 500, and 1,000 μg/ml, respectively. Radii of inhibition zones of the streptomycin-sensitive strain Cit7 of *P. syringae* ranged from 4 to 10, 9 to 12, and 10 to 16 mm surrounding disks with streptomycin at 100, 500, and 1,000 μg/ml, respectively. All strains of *E. amylovora* or *P. syringae* were resistant to streptomycin at 10 μg/ml.

Oxytetracycline sensitivity. The 138 strains were sensitive to oxytetracycline at 25 μg/ml or greater. Radii of inhibition zones ranged from 0 to 2, 1 to 5, 1 to 7, and 3 to 8 mm among strains tested at 10, 25, 50 and 100 μg/ml, respectively. Radii of inhibition zones of the oxytetracycline-sensitive strain Cit7 of *P. syringae* ranged from 0 to 2, 4 to 6, 6 to 9, and 8 to 11 mm among experiments at 10, 25, 50, and 100 μg/ml, respectively. No inhibition of the resistant strain A506 of *P. syringae* was observed at any oxytetracycline level tested.

Copper sensitivity. The 138 strains were sensitive to copper and did not grow on CYE medium containing $CuSO_4$ concentrations of 0.16 mM or greater. In contrast, strains A1513 and A1487 of *P. syringae* grew at $CuSO_4$ concentrations of 1.10 mM and 0.16 mM, respectively. Spontaneous mutants of *E. amylovora* with tolerance to 0.16 mM $CuSO_4$ were observed at a frequency of 10^{-6}–10^{-7} mutant colonies per wild type colony. Colonies of spontaneous mutants appeared larger and more mucoid than those of parental strains on LB and CYE media. Spontaneous mutants and parental strains were pathogenic on immature pear fruit.

Grower survey. Pear growers managing 32 of the 44 orchards sampled responded to the grower survey. Trees in the 32 orchards were of Bartlett, Anjou, Bosc, and Asian varieties and ranged from 2 to 35 yr in age. Streptomycin-resistant strains were isolated from 28 of the 32 orchards represented in the grower survey. Seventy-seven of the 99 strains isolated from the 32 orchards of the survey were resistant to streptomycin, while 22 were sensitive. Streptomycin-resistant strains were isolated from orchards where streptomycin had been applied for more than 10 yr and from those where streptomycin had not been used for the past 5 yr (Table 1). Resistant strains were isolated from two orchards where, to the growers' knowledge, streptomycin had never been applied.

Streptomycin, oxytetracycline, and copper were applied in 1988 to 18, 22, and 23 orchards, respectively, of the 32 surveyed. Every orchard was treated with one or more of these compounds. The number of streptomycin applications varied from one to five among the 18 treated orchards. Streptomycin-resistant strains were isolated from 12 of the 14 orchards where streptomycin was not applied in 1988.

Fire blight disease was more severe in the orchards located in the southern regions of the state than in the northern, according to the ratings given by indi-

Table 1. Recovery of streptomycin-resistant *Erwinia amylovora* from pear orchards in relation to history of streptomycin usage

Streptomycin use in orchards (yr)	Streptomycin-resistant strains (no./total)	
	Strains	Orchards
Over 10	18/23	6/7
5-10	16/21	6/7
2-5	37/45	12/13
0[a]	5/10	5/5

[a] Growers managing three of the five orchards in this category knew only that streptomycin had not been sprayed for the past 5 yr.

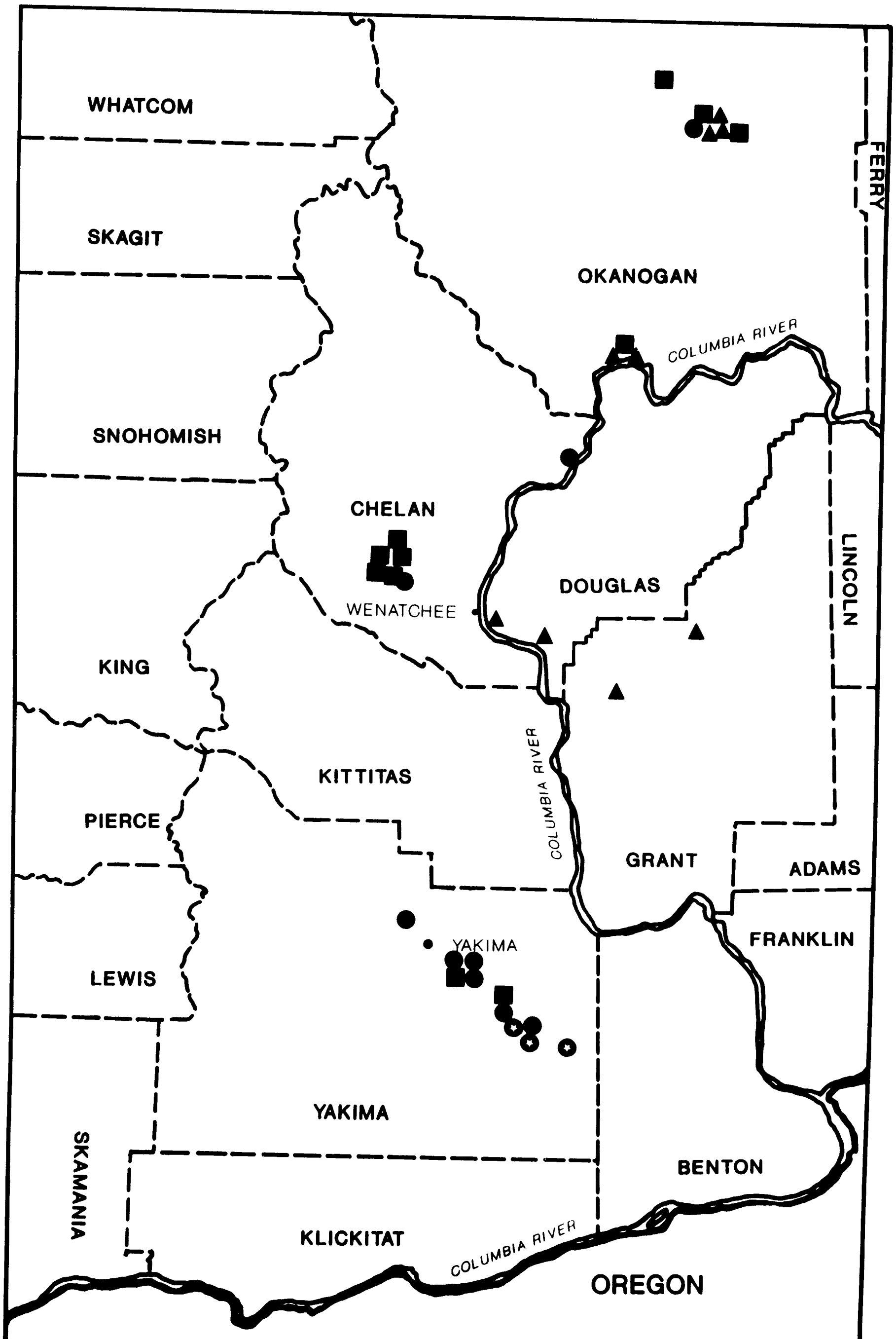

Fig. 2. Orchards in Washington State included in the grower survey. Fire blight disease severity was rated by individual growers managing each orchard indicated. Categories of disease severity were: ◉ = very severe, ● = severe, ■ = moderate, and ▲ = mild.

vidual growers of the survey (Fig. 2). All of the orchards with very severe fire blight and six of the nine orchards with severe fire blight were located in the Yakima Valley.

DISCUSSION

Streptomycin-resistant strains of *E. amylovora* were ubiquitous in pear-growing regions of Washington in 1988. In 1972, streptomycin resistance was observed in the Yakima Valley but not in other regions of Washington (5). It is clear that streptomycin-resistant strains have become more prevalent in Washington over the 16 yr between these studies. Streptomycin-resistant strains of *E. amylovora* undoubtedly have been selected by the common usage of streptomycin throughout the pear-growing regions of Washington for the past two decades. In contrast to the findings of Coyier and Covey (5) but consistent with those of Schroth et al (11), no intermediate levels of streptomycin resistance were observed among the strains evaluated in this study.

Streptomycin-resistant strains of *E. amylovora* were recovered from 88% of the orchards that were sampled in 1988, including some orchards with no recent history of streptomycin usage. This observation is consistent with that of Schroth et al (11), who found no relationship between recent streptomycin usage and resistance among strains of *E. amylovora* obtained from pear orchards in California. Streptomycin-resistant strains of *E. amylovora* were isolated 5 and 6 yr after termination of streptomycin usage in orchards of Washington (Table 1) or California (11), respectively. The occurrence of streptomycin-resistant strains in orchards with no recent history of streptomycin usage suggests that resistant and sensitive strains were similar in fitness in the absence of selection pressure. Accordingly, no consistent differences in growth rate or virulence were observed between streptomycin-resistant and streptomycin-sensitive strains of *E. amylovora* obtained from California pear orchards (11).

Resistance to high levels of oxytetracycline or copper was not observed among the strains of *E. amylovora* evaluated in this study. In comparison with known copper-tolerant and oxytetracycline-resistant *P. syringae*, all strains of *E. amylovora* were sensitive to these compounds. Spontaneous mutants of *E. amylovora*, with tolerance to 0.16 mM $CuSO_4$, were observed consistently. The copper-sensitive *P. syringae* strain A1487 was also tolerant of 0.16 mM $CuSO_4$. Because copper sprays effectively control copper-sensitive strains of *P. syringae* on plant surfaces (1), it is unlikely that the copper tolerance of the spontaneous mutants of *E. amylovora* is sufficient to preclude disease control by standard copper-containing bactericides. The occurrence of spontaneous and low-level copper tolerance among all strains of *E. amylovora* tested may indicate a potential for the development of resistance to copper compounds. Tolerance to copper ions has been observed among many different bacterial species from diverse habitats (17), including phytopathogenic bacteria (1,2,13). Mechanisms of resistance include exclusion of copper ions from the cell by the production of copious quantities of extracellular polysaccharides (3) and detoxification of copper ions (14). The mucoid colony morphologies of the spontaneous copper-tolerant mutants of this study are consistent with the exclusion mechanism of copper resistance.

Streptomycin, oxytetracycline, and copper were applied commonly for fire blight control in 1988. Nevertheless, 24 of the 32 growers who responded to the survey reported that they had lost substantial fruit yield to fire blight (Fig. 2). Nine of the 11 growers in the Yakima Valley reported that their orchards had been severely damaged by fire blight. The coincidence of weather conducive to fire blight and full bloom was more common in the Yakima Valley than in northern regions of the state (19). In northern regions where fire blight occurred but was generally less severe (Fig. 2), weather conducive to fire blight coincided with secondary (rattail) bloom but not with full bloom of pear. Although the independent estimations of 32 individual growers did not provide a definitive comparison of disease severity among the orchards of this study, it is clear that many were dissatisfied with the achieved level of fire blight control. Poor disease control may have been attributable to extreme disease pressure, poor timing of chemical applications, or poor persistence of chemicals on plant surfaces because of adverse weather conditions. Resistance of endemic populations of *E. amylovora* may explain the lack of control obtained with streptomycin but not with oxytetracycline or copper.

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