Streptomycin Resistance in *Erwinia amylovora*

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ABSTRACT

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Streptomycin resistant (Str^r) *Erwinia amylovora* were detected in California pear orchards in areas where little or no streptomycin had been used for fireblight control. Both Str^r and streptomycin susceptible (Str^s) *E. amylovora* strains frequently were isolated from both healthy and diseased pear parts. Only strains resistant to high levels of streptomycin (100 μg/ml) were detected from the field during a 7-yr period. Culture media greatly affected the sizes of inhibition zones. Based on the Luria-Delbrück fluctuation test, the mutation rate for high-level Str^r (500 μg/ml) was 4.1×10^{-9}. Mutants with a low level of resistance (10 μg/ml) occurred at 0.1×10^{-9} but these were unstable and died after several transfers. Generation times for Str^r and Str^s *E. amylovora* strains ranged from 78 to 118 min with no significant difference between the two groups. Increasing the concentrations of streptomycin in media up to 1,000 μg/ml increased the generation times of Str^r strains but did not prevent growth. Virulence among Str^r and Str^s strains varied but there was no consistent difference between the two groups. The Str^r strains appeared to be relatively stable; they were detected in orchards 6 yr after termination of streptomycin application.

Streptomycin-resistant (Str^r) strains of *Erwinia amylovora* were detected in a number of California pear orchards in 1971 (7,9). They were first discovered in a Gridley, CA, orchard (Sacramento Valley area) in which fireblight was epidemic even though a streptomycin spray program had been successful in previous years. The grower employed up to 21 kg/ha of 17% streptomycin sulfate in 760 L of water every 2 days without success. Subsequent laboratory studies revealed that most isolates from the orchard were Str^r.

After Str^r *E. amylovora* were found in California, they also were found in Washington and Oregon (2). Presumably the Str^r strains originated from spontaneous mutation, but were not detected earlier either because streptomycin exposure was not sufficient to increase populations to a readily detectable level or because only minimal detection efforts were made.

After Str^r *E. amylovora* were found, it seemed important to determine level of resistance, frequency of mutation associated with resistance, pathogenicity, and their stability and longevity in orchards.

MATERIALS AND METHODS

Distribution and frequency of Str^r *E. amylovora* strains relative to streptomycin application. Plant parts infected with *E. amylovora* from various localities were examined from 1971–1973 for the occurrence of Str^r strains by culturing on Miller-Schroth (MS) selective medium (7) with and without varying concentrations of streptomycin sulfate (78.1% streptomycin base; Sigma Chemical Co., St. Louis, MO 63178). The incidence of Str^r strains in orchards was determined and compared with the history of streptomycin usage. Collections were made from *Pyrus communis* (L.) 'Bartlett' pear orchards in all major pear growing areas of the state. Infected *Pyracantha coccinea* Roem. from a southern California nursery also was examined.

Mixed flower populations of Str^r and Str^s strains of *E. amylovora* and levels of streptomycin resistance. In 1975, 200 healthy and 67 diseased flowers from a Glenn County pear orchard individually were washed in 10 ml of sterile distilled water (SDW) and 0.1 ml of the suspension was spread on MS agar with a bent glass rod. Plates were incubated for 96 hr at 28 C and those with up to 200 well-dispersed colonies of *E. amylovora* were replica-plated on MS media with increasing concentrations of streptomycin. The concentrations of streptomycin tested were 0, 10, 25, 50, 100, and 500 μg/ml.

Isolations from 28 insects collected in the same orchard were processed as above. The insects sampled were eight *Apis mellifera*, 10 syrphid flies, seven unidentified miscellaneous Diptera, and three ants.

Mutation rate of *E. amylovora* to streptomycin resistance. The Luria and Delbrück fluctuation test (4) was used to determine the theoretical mutation rate of *E. amylovora* and the possible existence of strains with different levels of resistance to streptomycin among Str^s strains FB32 and FB33. Nutrient broth and nutrient agar amended with 0.5% glucose were used as media. The concentrations of streptomycin sulfate used for detection of Str^r strains were 1, 5, 10, 20, 30, 50, 75, 100, 200, 400, 500, 600, 800, and 1,000 μg/ml.

Susceptibility of *E. amylovora* strains to streptomycin. A representative assortment of Str^r and Str^s strains were tested for susceptibility to streptomycin by the standard antimicrobial disk susceptibility test (PST) developed by the National Committee for Clinical Laboratory Standards (NCCLS). For comparative purposes, the MS and Luria media (6) were used in addition to the Mueller-Hinton medium (MH) (Difco Laboratories, Detroit, MI 48201) recommended by NCCLS. The disks were impregnated with streptomycin at concentrations of 10, 25, 100, 500, or 1,000 μg/disk. Four strains each of Str^s and Str^r *E. amylovora* were used, three tests per strain at each concentration.

Generation times of Str^r and Str^s strains. The generation times of six Str^r strains (FB23, FB42, FB44, FB60, FB69, and FB78) and five Str^s strains (FB30, FB37, FB40, FB43, and FB45) were determined according to Miller (6) in nutrient broth supplemented with 0.5% glucose. The bacteria were cultured in side-arm flasks at 28 C and rotated at 250 rpm. The Str^r strains originally were isolated from Bartlett pear by plating diseased tissues on MS containing 100 μg/ml streptomycin sulfate. The generation times of Str^r strains FB12, FB42, and FB44 also were determined in the nutrient broth medium supplemented with streptomycin sulfate at 0, 1, 50, 100, 300, 600, and 1,000 μg/ml. Five replications were made for each generation time. The Str^r strains were stored in water.

Susceptibility of Str^r and Str^s strains to various antibiotics. Susceptibility of Str^r strains FB37, FB42, and FB44 and Str^s strains FB23, FB92, and FB105 to other antibiotics was tested with COLAB 11-160T and 11-160L multidisks (Colab Industries,

Glenwood, IL 60425). Bacteria were plated on MS and nutrient agar supplemented with 0.5% glucose. Disks were placed on the agar plate cultures immediately after bacteria were spread and zones of inhibition were measured after 2-day incubation at 28 C.

Pathogenicity of streptomycin-resistant and -susceptible *E. amylovora*. Str^r strains FB36 and FB40 and Str^s strains FB2, FB26, FB32, and FB33 of *E. amylovora* were inoculated individually into succulent cultivar Bartlett pear shoot tips in the field. Twenty shoot tips were inoculated with each strain by wounding them with a needle dipped into a suspension containing $\sim 10^9$ colony-forming units (cfu)/ml. The number of infections and distances that the disease progressed from site of inoculation (visual symptoms) were recorded after 3 wk. The same experiment was performed in the greenhouse as described above with Str^r strains FB37 and FB44, and Str^s strains FB23 and FB105. Five shoots were used per strain and disease readings were taken after 2 wk. The latter experiment was done a second time with less succulent shoots (15 shoots per strain) inoculated with Str^r strains FB37, FB42, and FB44 and Str^s strains FB23, FB71, and FB105.

Stability and longevity of Str^r strains in the field. The occurrence of Str^r strains in a Glenn County orchard was monitored over a 6-yr period beginning in 1972. Use of streptomycin was terminated in 1971. Healthy and infected flowers were washed in 10 ml of SDW and 0.1 ml of inoculum suspension dilutions were spread with a bent glass rod on MS media with and without streptomycin. Flower samples of varying ages were used throughout the study.

RESULTS

Distribution and frequency of Str^r strains relative to streptomycin usage. The occurrence of Str^r strains was widespread, ranging from northern to southern California (Table 1), and it appeared to be unrelated to the history of streptomycin usage (Table 2). Strains of Str^r *E. amylovora* were readily detected in areas where considerable, little, or no streptomycin had been used. However, they also were not detected in some orchards with a long history of streptomycin usage (Contra Costa County) (Table 1).

Mixed flower populations of Str^r and Str^s *E. amylovora* and levels of streptomycin resistance. *E. amylovora* was isolated from 175 of 200 apparently healthy flowers, all of 67 infected flowers, and from 17 of 28 insects. The replica platings indicated that both Str^r and Str^s *E. amylovora* frequently colonized the same flowers (healthy or diseased) and insects (Table 3). Only high-level (500 μg/ml) Str^r strains were detected.

Mutation rate of *E. amylovora* to streptomycin-resistance. The Luria-Delbrück fluctuation test to determine mutation rates showed two levels of resistance, a low level of resistance to 10 μg/ml with a mutation rate of 0.1×10^{-9} and a high level to 500 μg/ml at 4.1×10^{-9}. Some colonies (approximately 10^{-8}) were resistant to streptomycin at low levels (1 μg/ml) but died within days and could not be transferred. These were not streptomycin-dependent strains.

Susceptibility of *E. amylovora* isolates to streptomycin. The Str^r mutants that were isolated from pear-growing regions were "completely resistant" to streptomycin, as defined by the NCCLS. No zone of inhibition was detected around disks impregnated with 10, 25, or 100 μg streptomycin per disk on Luria, MH, or MS media. At the high concentrations of 500 and 1,000 μg per disk, zones of approximately 2 and 4 mm and 5 and 8 mm were detected about disks on MH and MS, respectively. No zones appeared around the disks in Luria's medium.

The largest zones of inhibition occurred on MH medium followed in decreasing order by MS and Luria's media. For example, the mean zones of inhibition (millimeter diameter, including the disk) of eight different Str^s strains about disks impregnated with 10 μg streptomycin were 24.8, 21.0, and 16.6 mm on MH, MS, and Luria's media, respectively. The relationship was consistent at the four concentrations of streptomycin sulfate that were tested.

TABLE 1. Frequency of streptomycin-resistant *Erwinia amylovora* (Str^r) strains sampled in several California counties

Location	Year	Samples (no.)	Str^r Samples (no.)	Str^r Samples (%)
Glenn County	1971	18	9	50
Butte County	1971	34	32	96
Sutter County	1971	12	2	18
Yolo County	1971	13	4	30
Contra Costa County	1974–1977	327	0	0
Orange County[a]	1972	5	5	100

[a] Orange County is in southern California and samples were from pyracantha plants. All other locations are in northern California and those samples were from Bartlett trees in pear orchards.

TABLE 2. Occurrence of streptomycin-resistant *Erwinia amylovora* (Str^r) strains in California Bartlett pear orchards in relation to history of streptomycin usage

Streptomycin use in orchards (yr)	Diseased samples (no.)	Streptomycin-resistant samples (no.)	Streptomycin-resistant samples (%)
6–10	11	6	55
4–5	21	14	67
7	250	0	0
5[a]	50	0	0
3	3	3	100
2	28	19	58
1	14	5	36
1	77	0	0
0[a]	5	5	100

[a] Samples of diseased tissues were from a Walnut Creek, CA Bartlett pear orchard, and from a southern California pyracantha nursery, respectively. All other diseased samples were from the Sacramento Valley, CA, area.

TABLE 3. Colonization of individual flowers and insects with both streptomycin-resistant (Str^r) and streptomycin-susceptible (Str^s) *Erwinia amylovora*

Sample	Total (no.)	*E. amylovora* isolated (no.)	Plates replicated[a] (no.)	Replicated plates with both Str^r and Str^s *E. amylovora* (no.)	Replicated plates with both Str^r and Str^s *E. amylovora* (%)
Healthy flowers	200	175	147	89	60.5
Diseased flowers	67	67	30	15	50
Insects	28	17	10	9	90

[a] Not all plates were replicated on the Miller-Schroth medium containing 0, 10, 25, 50, 100, 500, or 1,000 μg/ml streptomycin sulfate due to excessive numbers or poor dispersal of colonies on the original isolation plates.

TABLE 4. Generation times of streptomycin resistant *Erwinia amylovora* (Str^r) strains in media with varying concentrations of streptomycin

Streptomycin concentrations (g/ml)	Generation times of strains[b]: FB12 (min)	FB12B (min)	FB42 (min)	FB44 (min)
0	140	135	118	100
1	...	...	116	105
50	150	145	137	106
100	170	160	161	138
300	185	190	300	155
600	245	235	590	237
800	300	270	...	...
1,000	325	275	640	270

[a] The generation times were calculated from four replications for each strain grown in nutrient broth plus 0.5% glucose.

[b] Strains FB12A and FB12B were laboratory-derived mutants resistant to streptomycin, and FB42 and FB44 were Str^r strains obtained from infections in the field and isolated on a medium containing 100 μg/ml streptomycin.

Generation times of Str^r and Str^s *E. amylovora* strains. Generation times among Str^r and Str^s strains were not significantly different; those of the Str^s strains ranged from 78 to 110 min and those of the Str^r strains 86 to 118 min, averaging 94 and 96 min, respectively.

When Str^r strains were cultured in media containing streptomycin, the generation times increased proportionately with increased concentrations of streptomycin (Table 4), up to 640 min for FB42 on media containing 1,000 μg/ml of streptomycin. The generation times of the high-level Str^r strains obtained as laboratory mutations and those isolated from the field were similar and within the expected range of variation.

Susceptibility of Str^r and Str^s strains to various antibiotics. Str^r and Str^s strains did not differ in susceptibility to various antibiotics in the multidisk sensitivity tests. There were, however, minor variations among strains. Both groups of strains were sensitive to the following amounts of antibiotics contained in the disks: ampicillin, 10 μg; tetracycline, 10 μg; chloramphenicol, 5 μg; and erythromycin, 2 μg. The following antibiotics were not inhibitory: colistin, 2 μg; neomycin, 5 μg; polymycin, 50 units; novobiocin, 5 μg; methicillin, 5 μg; penicillin G, two units; phenethicillin, three units; and lincomycin, 2 μg.

Pathogenicity of Str^r and Str^s strains. Pathogenicity tests showed considerable variation in virulence among both Str^r and Str^s strains (Table 5). However, there was no apparent difference between the two groups of strains. Although FB2 did not infect young shoots of orchard trees, pathogenicity tests by stab inoculations of green pear fruits and flowers in the laboratory showed that it was pathogenic.

Stability and longevity of Str^r strains. Isolations from flowers in 1973 revealed that 68.4% of the healthy flowers and 50% of the flower infections contained Str^r (Table 6). Although the percentage of Str^r bacteria in orchards declined between 1973 and 1977, 16% of the infections in 1977 still harbored Str^r *E. amylovora* even though streptomycin applications terminated in 1971.

TABLE 5. Virulences of *Erwinia amylovora* streptomycin resistant and streptomycin sensitive strains

Sources and streptomycin reactions of strains	Mean length diseased shoot (cm)[a]
Orchard trees:	
Streptomycin-susceptible;	
FB2	0
FB26	6.3
FB32	17.4
FB33	3.0
Streptomycin-resistant;	
FB36	17.4
FB40	7.7
Greenhouse-grown trees:	
Streptomycin-susceptible;	
FB23	10.0[b]
FB105	9.3[b]
FB23	4.8
FB71	4.7
FB105	2.6
Streptomycin-resistant;	
FB37	14.6[b]
FB44	14.0[b]
FB37	2.8
FB42	1.8
FB44	2.7

[a] Pear shoots (five and 20 shoots per strain for greenhouse and orchard experiment, respectively), were inoculated at the tips with a needle that was dipped into a suspension of bacteria containing ~10^9 colony-forming units per milliliter. Length of infected shoots was determined by macroscopic observations.

[b] Shoot tips were more succulent than those inoculated at a later date.

DISCUSSION

The widespread occurrence of Str^r *E. amylovora* strains in California was predictable and consistent with current knowledge on aminoglycoside resistance in bacteria. The diversity of the Str^r strains with respect to virulence, generation times, and colony morphologies indicates that they arose by mutation from a heterogeneous assortment of strains rather than from one resistant strain which spread from an epicenter. There were areas, however, such as in Contra Costa County, in which Str^r strains have not been found.

The finding of a substantial population of Str^r *E. amylovora* strains in an orchard 6 yr after termination of streptomycin treatment was surprising. Resistant mutants theoretically are considered to have less survivability than the parent species whose wild type is sensitive (3). However, information on the ecology of drug-resistant bacteria primarily concerns those with extra-chromosomal elements of importance in clinical situations.

The ranges of virulence in Str^r and Str^s *E. amylovora* strains were essentially the same. Accordingly, no present evidence indicates that mutation to drug resistance in bacteria is accompanied by increased virulence in animals (5). Schnitzer and Grunberg (8) summarized the influence of drug resistance on the virulence of *Staphylococcus, Diplococcus, Streptococcus, Pasteurella,* and *Neisseria* and reported that 26 strains among these genera showed less virulence on becoming resistant, 12 strains showed no change, and none showed an increase.

Growth rates varied considerably among various *E. amylovora* strains, but no overall difference was detected between Str^r and Str^s strains. Growth rates of resistant bacteria apparently may increase,

TABLE 6. Incidence of streptomycin-resistant (Str^r) and streptomycin-susceptible (Str^s)[a] *Erwinia amylovora* strains in a Glenn County, California, Bartlett pear orchard after streptomycin applications were discontinued in 1971

Year	Type of sample	Flowers (no.)	Flowers with *E. amylovora* (Y)	Flowers with Str^r *E. amylovora* (X)	% Str^r of flower sample
1973	healthy flowers (individual)[b]	95	80	65	68.4
	flower infections	20	20	15	75
1974	healthy flowers (individual)	60	48	14	23.3
	flower infections	15	15	5	33.3
1975	healthy flowers (individual)	45	32	7	15.6
	flower infections	42	42	6	14.3
1976	healthy flowers (individual)	62	0	0	0
	flower infections	10	10	1	10
2 Jun 77	healthy flowers (combined)	200	yes[c]	0	0
2 Jun 77	flower infections	43	43	0	0
12 May 77	healthy flowers (combined)	200	yes[c]	0	0
12 Jun 77	flower infections	25	25	4	16

[a] Flowers with Str^s strains are calculated by subtracting columns X from Y.

[b] Flowers wither were processed individually by washing them in ¦ ml of sterile distilled water (SDW) and plating 0.1 ml of Miller-Schroth (MS) media or were processed in bulk by adding 0.5 ml SDW per flower and plating 0.1 ml on MS media.

[c] *Erwinia amylovora* was present but the number of colonized flowers was not determined because the sample was processed in bulk.

decrease, or remain the same depending on the drug (8).

In general, bacteria resistant to aminoglycoside antibiotics are single-step, high-level mutants with altered ribosomes (10). All of the Str^r strains isolated from infected pears during 7 yr in California were resistant to high levels of streptomycin. Coyier and Covey (2), however, reported finding in nature strains of *E. amylovora* with intermediate sensitivity, as did Bennett and Billing (1) in laboratory derived mutants. Examples of low, intermediate, or high levels of streptomycin resistance in bacteria are known (8). In isolations to detect spontaneous Str^r mutants in pure cultures, we also obtained some that were resistant to low levels of streptomycin (approximately 10 μg/ml). Str^r mutants resistant at 1 μg/ml were common, but these did not survive long in culture and were considered to be metabolic cripples. It is not uncommon to obtain laboratory-derived resistant strains with low level resistance that are genetically unstable and grow poorly as a result of multiple mutations (3). Comparison of reported streptomycin susceptibility levels, as with *E. amylovora*, is often difficult because laboratories frequently do not use recommended performance standards in antibiotic disk susceptibility tests. Our comparative tests in different media showed a marked effect of media constituents on the size of *E. amylovora* inhibition zones.

The data on Str^r strains in *E. amylovora* indicate that resistance is caused by a chromosomal mutation. The absence of multiple drug resistance and collateral sensitivity is consistent with a ribosomal alteration as opposed to some permeability block that may result in cross resistance to several aminoglycoside antibiotics (3). Standard tests that reveal the presence of plasmids associated with the Str^r character (N. J. Panopoulos, *personal communication*) further indicated that Str^r in *Erwinia amylovora* is caused by a chromosomal mutation.

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