Sensitivity of *Erwinia amylovora* in Illinois Apple Orchards to Streptomycin, Oxytetracycline, Kasugamycin, and Copper

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Abstract

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In 2008 and 2009, severe fire blight (*Erwinia amylovora*) occurred in Illinois apple orchards, leading to speculation that streptomycin-resistant strains of *E. amylovora* might be present in some orchards. Statewide surveys were conducted in 2010, 2011, and 2012, and 117, 129, and 170, *E. amylovora* isolates were collected, respectively, from 20 counties. None of the 416 *E. amylovora* isolates tested were resistant to streptomycin (Agri-Mycin 17WP) at 50 mg/liter. Seven non-*E. amylovora* bacterial isolates were collected from *E. amylovora*-infected shoots that contained both a *strA-strB* streptomycin resistance gene and IS1133 on transposon Tn5393, which could be a potential source of streptomycin resistance for *E. amylovora* in Illinois in the future. Colony development of all 84 *E. amylovora* isolates tested was inhibited on Luria-Bertani medium amended with oxytetracycline at 50

mg/liter and kasugamycin (Kasumin 2L) at 100 mg/liter. Similarly, colony development of the 84 *E. amylovora* isolates was inhibited on casitone-yeast extract medium amended with copper sulfate at 0.16 mM. In 2011 and 2012, field trials were conducted to evaluate the efficacy of oxytetracycline (Mycoshield 17WP), kasugamycin (Kasumin 2L and ARY-4016-06), copper hydroxide (Kocide-3000 41.6DF), *Bacillus subtilis* (Serenade Max, QST713), and *Pseudomonas fluorescens* (Blight Ban A506) for management of fire blight in an apple orchard. Only kasugamycin (Kasumin 2L and ARY-4016-06) reduced blossom infection significantly. There was a significant interaction of kasugamycin (Kasumin 2L) with prohexadione calcium (Apogee 27.5DF) in reducing shoot blight incidence in the field in 2012.

Fire blight disease, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is a serious disease of pome fruit, especially apple (*Malus × domestica* Borkh.) and pear (*Pyrus* spp.), worldwide (3). Yield losses to fire blight and control costs of the disease in the United States are estimated at \$100 million per year (17).

E. amylovora overwinters at the edges of cankers of infected trees. In spring, when temperatures exceed 18.3°C, the bacteria rapidly multiply and become the primary inoculum (3,6). Rain splash, wind, and insects spread the bacteria onto the flowers, leaves, and shoots. Infection is initiated as the bacteria enter through natural openings and wounds, multiply, invade, and kill host tissue (3).

Control of fire blight requires year-round comprehensive management practices, including pruning, early-spring copper applications, and bactericide applications during bloom and following storm trauma to prevent the bacterium from entering through the nectarthodes or wounds and infecting the host tissue (3). Since the 1950s, streptomycin has been the primary bactericide used for fire blight control and has been highly effective. However, in 1971, the first detection of streptomycin-resistant (SmR) *E. amylovora* occurred in California (16). Subsequently, SmR *E. amylovora* was reported from the western United States, Michigan, Canada, and Israel, and has been isolated in New York (2,9,11,12,18,20). During 2003 to 2009, in Michigan, two genotypes of SmR isolates were collected: (i) *strA-strB* genes, encoding phosphotransferase enzymes modifying streptomycin to a nonbactericidal form, and (ii) a point mutation of ribosomal S12 protein, RspL (12). McGhee et al. (12) reported that *strA-strB* genes harbored on transposon Tn5393 were present in 98.7% of SmR isolates in Michigan, whereas RspL mutations dominated other regions with streptomycin resistance. This indicated that, in Michigan, SmR likely moved from SmR

non-target orchard bacteria via horizontal gene transfer on Tn5393 into *E. amylovora* (12).

Currently, registered streptomycin alternatives are considered less effective, unless SmR populations are present (23). However, recent laboratory and field data suggest that the antibiotic kasugamycin (Kasumin 2L; Arysta Life Science) is a suitable streptomycin alternative (1,13). Kasugamycin is an antibiotic in the same class of streptomycin but has a different mode of action (7,13).

In 2008 and 2009, fire blight infection was widespread and severe throughout Illinois, particularly in western parts of the state. Some growers hypothesized that SmR strains of *E. amylovora* might be present in Illinois. The objective of this study was to evaluate sensitivity of *E. amylovora* in Illinois to streptomycin, streptomycin alternatives, and copper.

Materials and Methods

Field survey and sample collection. Incidence and severity of blossom blight and shoot blight caused by *E. amylovora* were assessed in 24, 35, and 39 apple orchards in Illinois in 2010, 2011, and 2012, respectively. Surveyed apple orchards were located in 24 counties, including Boone, DeKalb, Kane, McHenry, and Winnebago in northern; Calhoun, Champaign, Jersey, Macoupin, Marshall, Peoria, Piatt, Putnam, Sangamon, and Woodford in central; and Clinton, Jackson, Jefferson, Madison, Marion, Pope, Randolph, St. Clair, and Union in southern Illinois (Fig. 1).

In each orchard, 50 asymptomatic flowers (2 flowers/tree) and 20 symptomatic shoots (1 shoot/tree) were collected for isolation of the bacterium (*E. amylovora*). No symptomatic flowers were observed. Each infected shoot was cut 20 cm below the visible necrotic tissue and pruners were dipped in 95% ethanol between cuts. Collected flowers and shoots were placed in plastic bags, kept on ice during transport, and stored at 4°C in the laboratory within 12 h of collection. In 2010, shoots were collected on 3 June and 22 July. In 2011, flowers were collected on 10 April and 16 May and shoots were collected on 7 June and 22 June. In 2012, flowers were collected on 23 March and 9 April and shoots were collected on 25 May and 8 June.

Isolation, purification, and maintenance of *E. amylovora*. Luria-Bertani (LB) medium was used for isolation and maintenance

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of *E. amylovora* (12). LB medium was amended with cycloheximide (50 mg/liter) to make LBch. LBch was amended with 50 mg/liter of streptomycin (Agri-Mycin 17WP; Nufarm Americas Inc.) to make LBcham.

All collected samples were processed within 36 h of collection. Five flowers were placed in a mesh bag (Agdia, Inc.) with 5 ml of sterilized 0.5× phosphate-buffered saline (PBS) buffer, on ice. Flower tissue was macerated for approximately 20 s or until tissue was well ground using a tissue homogenizer (Agdia, Inc.) attached to a drill press. For isolation of the bacterium from shoots, a 10- to 15-mm piece of shoot tissue was cut immediately below the visible necrotic lesion and bark was removed using sterile scalpel and tweezers. The shoot tissues were macerated as described for flowers. Then, underneath a sterile hood, extract from ground tissue was diluted (3:1) in sterilized 0.5× PBS buffer. Using a bacterial loop, the suspension was streaked to develop single colonies on LBch and LBcham. The cultures were incubated at 27°C for 48 h. White colonies characteristic of *E. amylovora* on LBch and all white colonies growing on LBcham were subcultured on LB; then, each colony was transferred to a cryogenic vial containing 15% glycerol. The vials were stored at -20 and -80°C for further studies.

PCR identification of *E. amylovora*. The identity of each *E. amylovora* isolate was confirmed by using a polymerase chain reaction (PCR). The PCR was conducted using primers AJ75 (5' CGC ATT CAC GGC TTC GCA GAT 3') and AJ76 (5' AAC CGC CAG GAT AGT CGC ATA 3') targeting ubiquitous plasmid pEA29 in *E. amylovora* (15). Using a sterile pipette tip, a colony was suspended in 100 µl of sterilized distilled water (SDW). Then, 0.5 µl of the bacterial suspension was added to 10 µl of DNA-free water, 1 µl of each primer AJ75 and AJ76 (10 pmol/µl), and 12.5 µl of GoTag Green Master Mix 2x (Promega Corporation) for a final reaction mixture volume of 25 µl. Cycling conditions for PCR (Model PCT-200; MJ Research Inc.) included initial denaturation at 94°C for 5 min; 37 cycles of denaturation for 1 min, annealing at 52°C for 2 min, and extension at 72°C for 2 min; followed by 15 min of final extension at 72°C, and was kept at 4°C until electrophoresis. The PCR product was run on 1% agarose gel containing ethidium bromide at 100 V for 60 min, and photographed under UV light. The bands were compared with a known streptomycin-sensitive *E. amylovora* isolate, MK1, and a streptomycin-resistant *E. amylovora* isolate, Ea88, supplied by George Sundin (Michigan State University). The process was repeated at least once for each isolate.

Virulence of *E. amylovora* isolates. An immature pear fruit assay (4) was used to determine virulence of 48 *E. amylovora* isolates from Illinois. Immature 'Seckel' pear fruit were surface disinfested in 75% ethanol, rinsed in SDW, and dried on a sterile lab bench. Using a sterile pipette, a 15-µl suspension (10^7 CFU/ml) of each isolate was inoculated into each pear fruit (approximately 2 mm deep). *E. amylovora* isolate MK1 and SDW were used as positive and negative controls, respectively. Inoculated and control pears were incubated in a moist chamber at 27°C in darkness. Diameters of necrotic lesions were measured perpendicularly 4 and 7 days post inoculation. Each virulence test had five replications (fruit) and the experiment was repeated once. The virulence tests were complimented with a tobacco hypersensitivity assay to determine the presence of type III effectors, as described by Kado (8).

Screening *E. amylovora* isolates for streptomycin sensitivity. In all, 416 *E. amylovora* isolates were cultured on LB medium and LB medium amended with streptomycin at 50 mg/liter (Agri-Mycin 17WP). The streptomycin-sensitive isolate MK1 and streptomycin-resistant isolate Ea88 were used as negative and positive controls, respectively. Colony development was recorded after 24 and 48 h. Each isolate was evaluated in two experiments, with three replications per experiment.

Of 416 *E. amylovora* isolates, 133 were further tested for streptomycin sensitivity by zone of inhibition. Each isolate was grown on LB medium for 48 h. Colonies were washed in SDW and the bacterial suspensions were adjusted to 10^7 CFU/ml using a

spectrophotometer (Smart Spec 3000; Bio-Rad). Using a sterilized bent glass rod, a 50-µl bacterial suspension was spread onto LB medium. A 12-mm filter disc was soaked in a 100 mg/liter solution of streptomycin (Agri-Mycin 17WP), briefly dried, and placed onto the agar surface, as described by Loper et al. (11) and Russo et al. (18). The streptomycin-susceptible isolate MK1 and streptomycin-resistant isolate Ea88 were used as positive and negative controls, respectively. Clear zones of inhibition were measured at 24 and 48 h. The width of zone (≥ 1 mm) with no bacterial colony was considered a measure of sensitivity of *E. amylovora* to streptomycin. Each isolate was tested twice, each test had three replications, and each replication had four filter discs.

In addition, 10 of the 416 *E. amylovora* isolates were compared for bacterial multiplication in LB broth (Miller; Arcos Organics) amended with streptomycin (Agri-Mycin 17WP) at 0, 0.5, 1, 2, 3, 4, and 5 mg/liter and in nutrient broth (BD, Sparks, MD) amended with streptomycin (Agri-Mycin 17WP) at 0, 0.25, 0.5, and 1 mg/liter. The tests were conducted using sterile 24-well plates (351147; BD Falcon). From each bacterial suspension (10^8 CFU/ml), 20 µl was added to 2 ml of broth in each well and incubated on a shaker at 28°C for 18 h. Bacterial cell density was assessed using a spectrophotometer at an optical density at 600 nm (OD₆₀₀). The bacterial cell densities from the streptomycin-amended cultures were compared with unamended cultures and

Fig. 1. Circles show locations of Illinois apple orchards surveyed for streptomycin-resistant *Erwinia amylovora* during 2010 to 2012.

expressed as a percentage. Each isolate was tested twice with four replications.

Non-*E. amylovora* bacteria in Illinois orchards. While testing blossom and shoot samples for the presence of *E. amylovora*, 119 non-*E. amylovora* isolates were also collected that developed colonies with streptomycin at 50 mg/liter. All 119 non-*E. amylovora* isolates were screened for the presence of IS1133 region (associated with Tn5393) using primer set IS1133-F ('GCG TGA TGC AGT TCG CAT AGC') and IS1133-R ('CAT ACG CGG CCT ACC ATA GCT') (12). The IS1133 region of nine isolates was sequenced using primer IS1133-F, except the cycling parameters were modified as described below.

These nine non-*E. amylovora* isolates were also screened with primers strab01-F ('TGG TGT CCC GCA ATG CCG TC') and strab01-R ('CCC GGA TCG GGA GAA GGG CA') to amplify a portion of *strB* region on Tn5393. PCR cycling conditions included initial denaturing for 4 min at 94°C; 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C (IS1133 primer set) or 60°C (strab01 primer set) for 30 s, and extension at 72°C for 2.5 min; followed by a final extension at 72°C for 7 min. The PCR product was run in 1% agarose gel containing ethidium bromide at 100 V for 60 min, and photographed under UV light. The bands and sequences were compared with results produced by *E. amylovora* isolates MI 5-1 and W4 that are known to contain Tn5393 (12). The process was repeated twice for each isolate.

The PCR test was performed to amplify the 16S rRNA genes from the extracted DNA using the primers 27F (5' AGA GTT TGA TCM GGC TCA G 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3'), as described by Lane (10). PCR products were purified with the Wizard SV gel and PCR Clean-Up system (Promega Corporation). Purified 16S rRNAs were single-end sequenced from 27F at the University of Illinois Core DNA Sequencing Facility. Edited sequences were compared using the BLASTn database (<http://www.ncbi.nlm.nih.gov>).

Oxytetracycline and kasugamycin sensitivity. Of the 416 *E. amylovora* isolates, 84 were selected for in vitro evaluation of antibiotics. LB medium (pH 7) was amended with oxytetracycline (Mycoshield 17WP; NuFarm Americas Inc.) at 0, 50, 100, and 200 mg/liter. Also, two formulations of kasugamycin (Kasumin 2 L and ARY-4016-06; Arysta Life Science) were evaluated at 0, 25, 50, and 100 mg/liter in LB medium with bacterial suspensions streaked onto the agar surface. All experiments included unamended LB medium for controls. Colony development of *E. amylovora* was assessed after 24 and 48 h.

Ten *E. amylovora* isolates were further compared in nutrient broth (pH 7) amended with Kasumin 2L and ARY-4016-06 (kasugamycin at 0, 3, 5, and 10 mg/liter) for bacterial multiplication. This test was conducted as described previously for the streptomycin test. The test was conducted using sterile 24-well plates, with 20 µl from each bacterial suspension (10⁸ CFU/ml) added to 2 ml of amended nutrient broth in each well and incubated on a shaker at 28°C for 18 h. Bacterial cell density was assessed using a spectrophotometer at OD₆₀₀.

Copper sensitivity. The same 84 *E. amylovora* isolates were also evaluated for their sensitivity to copper sulfate using casitone-yeast extract (CYE) medium (1.7 g of casitone, 0.35 g of yeast extract, 2 g of glucose, and 15 g of agar in 1,000 ml of deionized water), as reported by Loper et al. (11). CYE medium was amended with 0.08, 0.16, and 1.1 mM copper sulfate (Cuprofix Ultra 40DF; United Phosphorus). A 10-µl aliquot of bacterial suspension from each isolate was spotted onto the agar surface in each 110-mm-diameter petri plate. All experiments included unamended agar plates for controls. Each isolate was tested twice, with three replications (plates) per test. Colony development was assessed after 48 and 72 h.

Of the 84 *E. amylovora* isolates, 11 were further evaluated for their sensitivity to various copper formulations with metallic copper at 0 and 14 mg/liter. CYE medium was amended with copper sulfate (Cuprofix Ultra 40DF), copper hydroxide (Kocide 3000 40.1DF; Dupont); cuprous oxide (Nordox 75 WG; Nordox Indus-

tries AS), and a mixture of copper hydroxide and copper oxychloride (Badge X2 45DF; Isagro USA). Colony development was assessed after 48 and 72 h.

Field experiments. Field experiments were conducted in 2011 and 2012 to evaluate potential streptomycin alternative compounds for management of fire blight. The experiments were conducted on a 16-year-old block of 'Jonathan' apple trees at the University of Illinois Fruit Research Farm, Urbana. Inoculum of *E. amylovora* was prepared from 48-h-old colonies of the isolate (2A) that had been collected from the same orchard. Bacterial suspensions in 0.5× PBS buffer at 10⁸ CFU/ml (in 2011) and 5 × 10⁶ CFU/ml (in 2012) were used. The bacterial suspension was transported on ice and applied when a MARYBLT disease forecasting logger (Watchdog 400; Spectrum Technologies) indicated high risk of fire blight infection. All treatments and inoculations were applied using a gas-powered backpack mist sprayer (SR400; Stihl) at 1,400 liters/ha. Data were collected on four sides of each tree from the upper, middle, and lower canopy. In total, 120 flower clusters and 120 shoots were examined on each tree when it was possible. In 2012, the number of flower clusters per tree ranged from 6 to 120 (mean 70 clusters) per tree.

The 2011 experiment was performed in a randomized complete block design with eight treatments, and each treatment had two application times of 24 h pre- and 24 h post inoculation. Each treatment had three replications for each application time, with 1 tree for each replication (48 trees in the experiment). The treatments included (i) an uninoculated control, (ii) an inoculated control, (iii) streptomycin (Agri-Mycin 17WP) at 100 mg/liter, (iv) oxytetracycline (Mycoshield) at 200 mg/liter, (v) kasugamycin (Kasumin 2L) at 100 mg/liter, (vi) kasugamycin (ARY-4016-06) at 100 mg/liter, (vii) *Pseudomonas fluorescens* A506 (BlightBan A506) at 369 mg/liter, and (viii) *Bacillus subtilis* QST713 (Serenade Max) at 525 mg/liter.

Cuprofix Ultra 40D (7.8 kg/ha) and dormant oil were applied to all trees on 31 March 2011. Growth development stages of apple trees were full pink (18 April), 1% bloom (20 April), 40 to 60% bloom (25 April), and petal fall (11 May). The trees were inoculated with the pathogen twice (on 26 April and 8 May) to ensure that adequate inoculum was present. Pre-inoculation treatments (treatments iii to viii) were applied on 25 April and 7 May and post-inoculation treatments (treatments iii to viii) were applied on 27 April and 9 May. All plots received an additional spray of the respective test product on 29 April, a timing that was independent of the pre- and post-inoculation timings sprays. As a result, all test trees received a total of three fire blight control sprays during bloom. Blossom infection was evaluated on 12 May and 23 May and shoot infection was evaluated on 10 June, 30 June, and 27 July.

The 2012 experiment was a split-plot in a randomized complete block design with 11 main plots (antibiotics and biocontrol agents), each with two subplots of with or without growth regulator prohexadione calcium (Apogee 27.5DF; BASF Corporation) at 1,200 mg/liter. Each treatment had four replications (trees). The main plot treatments included (i) inoculated control, (ii) copper hydroxide (Kocide 3000) at 240 mg/liter + mancozeb (Dithane 75DF, Dow AgroSciences LLC) at 2,700 mg/liter, (iii) streptomycin (Agri-Mycin 17WP) at 100 mg/liter, (iv) oxytetracycline (Mycoshield) at 200 mg/liter, (v) oxytetracycline (Mycoshield) at 200 mg/liter+ copper hydroxide (Kocide 3000) at 240 mg/liter, (vi) kasugamycin (Kasumin 2L) at 100 mg/liter, (vii) kasugamycin (ARY-4016-06) at 100 mg/liter, (viii) *P. fluorescens* A506 (BlightBan A506 71) at 369 mg/liter, (ix) *B. subtilis* (Serenade Max QST713) at 525 mg/liter, (x) kasugamycin (Kasumin 2L) at 100 mg/liter, and (xi) streptomycin (Agri-Mycin 17WP) at 100 mg/liter. Treatments ii to vii were applied 24 h pre- and 24 h post-inoculation; treatments viii and ix were applied 24 h pre-inoculation only; and treatments x and xi were applied 24 h post-inoculation only. All 11 treatment applications included a nonionic surfactant, Regulaid (Kalo, Inc.), at 1.25 ml/liter.

Cuprofix Ultra 40DF (7.8 kg/ha) and dormant oil were applied to all trees on 13 March 2012. Growth development stages of apple

trees in 2012 were full pink (23 March), 1% bloom (26 March), 40 to 60% bloom (28 March), and petal fall (12 April). In total, Apogee (0.925 kg/ha of prohexadione calcium) at 3.363 kg/ha in two sprays (1.68 kg/ha in each spray) plus Regulaid (1.25 ml/liter) were applied on 29 March (60 to 80% bloom) and 12 April (petal fall), using water at 1,400 liters/ha for each application. The trees were inoculated on 30 March and 3 April. Treatments were applied on 29 March (24 h pre-inoculation), 31 March (24 h post inoculation), 2 April (24 h pre-inoculation), and 4 April (24 h post inoculation). Additional applications of BlightBan A506 (treatment viii) and Serenade Max (treatment ix) were made on 28 March. Blossom infection was evaluated on 17 April, 24 April, and 1 May and shoot infection was evaluated on 8 May, 22 May, 5 June, and 3 July.

Data analysis. All statistical analyses were performed using SAS 9.3 (SAS Institute Inc.). Homogeneity of variances was tested using the Brown-Forsyth test and normality was observed. Percent cell multiplication data from liquid-amended media (streptomycin) were analyzed using analysis of variance (ANOVA) and means were separated by least significant difference in PROC GLM at $\alpha = 0.05$. All other data were analyzed using ANOVA in PROC MIXED. Percent cell multiplication data from liquid amended media (kasugamycin) were separated at $\alpha = 0.05$ using macro pdmix800 (19) to indicate mean separation. Pear pathogenicity and field data were square root transformed [$\sqrt{(x + 1/6)}$] before analysis. In 2012, blossom data were analyzed as a covariate to account for uneven blossom development in the orchard. Dunnett's test was used to compare blossom infection of field treatments to the inoculated control.

Results

Occurrence of fire blight in Illinois apple orchards. No blossom blight was observed during this study. *E. amylovora* was isolated from 2 of 214 and 1 of 221 flower samples in 2011 and 2012, respectively. Shoot blight was observed in 22 of 24, 26 of 35, and 32 of 39 of orchards in 2010, 2011, and 2012, respectively. Central Illinois had the highest and northern Illinois had the lowest incidence of shoot blight. Calhoun County in central Illinois had the highest shoot blight incidence and severity in all 3 years.

Streptomycin sensitivity of *E. amylovora* in Illinois. Overall, 416 *E. amylovora* isolates were collected from 39 individual orchards in 20 counties during 2010 and 2012. None of the 416 *E. amylovora* isolates tested was streptomycin (Agri-Mycin 17WP)-resistant at 50 mg/liter. Differences in the streptomycin sensitivity of isolates from different counties were observed (Table 1). Isolates from Boone ($P = 0.0014$) and Champaign ($P = 0.0028$) Counties were less sensitive to streptomycin than the state mean. In contrast, isolates from Calhoun ($P = 0.0143$), Madison ($P = 0.0002$), and Union ($P = 0.0056$) Counties were more sensitive to streptomycin than the state mean.

Results from 10 individual isolates in amended LB broth supported the results of the tests on LB plates. For streptomycin at 1 mg/liter, the rates of cell multiplication of all 10 *E. amylovora* isolates was significantly ($P = 0.05$) reduced when compared with the streptomycin-resistant control. Calhoun isolate 6081 was the most sensitive isolate, with 0% cell multiplication for streptomycin at 3 mg/liter, whereas the Boone isolate 7101 was the least sensitive isolate, with 16.1% cell multiplication for streptomycin at 5 mg/liter, compared with the control. In amended nutrient broth, none of the 10 tested *E. amylovora* isolates multiplied with streptomycin at 0.25, 0.5, or 1 mg/liter.

Virulence of *E. amylovora* isolates. All 48 isolates of *E. amylovora* tested produced lesions on immature Seckel pear fruit and a hypersensitive reaction on tobacco leaves. *E. amylovora* was recovered from the necrotic lesions of inoculated pear fruit. Isolate 7711 from Woodford County produced the largest (8.9 mm in diameter) lesions. Overall, there were no significant differences ($P = 0.19$) in diameters of the necrotic lesions produced by isolates from different counties, except isolates from St. Clair County that produced significantly ($P = 0.009$) larger necrotic lesions (4.5 mm in diameter) than the state mean of lesions (3.5 mm) on the pear fruit.

Oxytetracycline and kasugamycin sensitivity. Colony development of all 84 *E. amylovora* isolates was inhibited on LB medium amended with oxytetracycline at 50 mg/liter. Also, colony development of all 84 *E. amylovora* isolates was inhibited with kasugamycin (Kasumin 2L) at 100 mg/liter. However, kasugamycin at 100 mg/liter in ARY-4016-06 inhibited colony development of only 35 of the 84 isolates. Kasumin 2L with kasugamycin at 50 mg/liter inhibited colony development of 29 of 84 the isolates; whereas kasugamycin at 50 mg/liter in ARY-4016-06 inhibited colony development of only 1 of the 84 isolates.

Kasugamycin of both Kasumin 2L and ARY-4016-06 at 5 mg/liter in nutrient broth reduced cell multiplication of all 10 *E. amylovora* tested (Table 2) but neither Kasumin 2L nor ARY-4016-06 completely inhibited cell multiplication. For kasugamycin at 10 mg/liter in nutrient broth, however, cell multiplication of 6 of 10 and 3 of 10 was completely inhibited by Kasumin 2L and ARY-4016-06, respectively.

Copper sensitivity. All 84 isolates tested developed colonies on CYE medium amended with 0.08 mM copper sulfate. At 0.16 and 1.1 mM copper sulfate, none of the isolates developed colonies. Also, copper sulfate, copper hydroxide, cuprous oxide, and copper hydroxide/copper oxychloride with metallic copper at 14 mg/liter inhibited colony development of all 11 *E. amylovora* isolates on CYE medium.

Occurrence of non-*E. amylovora* bacteria with Tn5393. All of the 119 non-*E. amylovora* isolates collected developed colonies on LB medium amended with streptomycin (Agri-Mycin 17WP) at 50 mg/liter. Seven of these isolates were confirmed to contain both IS1133 and *strB* (Fig. 2) on Tn5393. Three isolates (5897, 7234, and 5174) were identified as *Pantoea agglomerans* and one isolate (6114) as *Pseudomonas graminis* based on 16s rRNA sequences (450 to 600 bp) when compared in BLASTn.

Field experiments. In 2011, due to high inoculum density of *E. amylovora*, severe blossom blight and shoot blight developed in the inoculated trees. Only three treatments, including post-inoculation

Table 1. Streptomycin-sensitivity of *Erwinia amylovora* populations in Illinois apple orchards during 2010 to 2012

County	Number of isolates		Radius (mm) ^y	P value ^z
	Collected ^w	Tested ^x		
Boone	14	7	2.4–	0.0014
Calhoun	87 + 1	25	3.5+	0.0143
Champaign	32	9	2.6–	0.0028
Clinton	8	1	3.0	0.8560
Jackson	22	7	3.1	0.9626
Jefferson	6	2	2.9	0.4742
Jersey	45	14	3.2	0.6900
Kane	2 + 1	1	3.2	0.9031
Macoupin	15	5	3.2	0.8728
Madison	13	3	4.4+	0.0002
Marion	4	2	3.1	0.9806
Marshall	28	13	3.1	0.6079
Peoria	10	4	3.2	0.9780
Piatt	3	2	3.8	0.0964
Pope	1	...	...	...
Putnam	15	5	2.6	0.0256
Sangamon	11	4	3.4	0.5290
St. Clair	64	19	3.3	0.3828
Union	24 + 1	5	3.9+	0.0056
Woodford	9	5	2.6	0.0442
Total	416	133	3.3	...

^w In total, three isolates (indicated by +1), were collected from blossoms.

^x Isolates were tested in three separate years and combined for analysis.

^y Zone of inhibition radius. Bacterial suspension (50 μ l of 10^7 CFU/ml) was spread on Luria-Bertani agar; then, a 12-mm filter disk was soaked in a solution of streptomycin (Agri-Mycin 17WP) at 100 mg/liter, briefly dried, and placed on the agar surface. Zone of inhibition was measured after 48 h. – = less sensitive to streptomycin than the state mean and + = more sensitive to streptomycin than the state mean at $P = 0.02$.

^z Data were analyzed using PROC MIXED and the LSMEAN of each county was compared with the LSMEAN of all counties combined.

Table 2. In vitro effects of kasugamycin on *Erwinia amylovora* multiplication in nutrient broth^y

<i>E. amylovora</i> isolate		Bacterial cell density (%) ^z					
		Kasugamycin (Kasumin 2L) (mg/liter)			Kasugamycin (ARY 4016-06) (mg/liter)		
County	Code	3	5	10	3	5	10
Boone	6951	98.9 bc	55.8 c	0.0 e	78.7 b–d	37.5 cd	11.3 d
Calhoun	6081	100.7 a–c	1.0 e	0.0 e	60.7 b–e	21.1 de	0.0 e
Calhoun	7651	52.4 e	1.1 e	0.0 e	46.4 de	10.1 e	0.0 e
Champaign	4802	72.0 d	9.4 d	0.0 e	38.0 e	6.9 e	0.0 e
Jersey	6002	95.6 b	67.9 bc	0.0 e	83.9 b	51.6 bc	20.7 c
Sangamon	84d	97.8 b	85.9 a	37.7 a	82.0 b	69.5 b	59.9 a
St. Clair	89a	87.6 c	73.4 b	12.7 c	73.2 cd	69.2 b	58.5 a
Union	5232	94.9 b	70.3 b	0.0 e	81.7 b	70.8 b	55.4 a
Woodford	7711	116.6 a	102.1 a	24.0 b	117.7 a	98.4 a	56.9 a
Woodford	165e	94.6 b	73.5 b	3.7 d	79.4 bc	51.1 c	37.7 b
Mean	...	96.1	61.1	7.1	78.8	51.5	31.1

^y Nutrient broth was adjusted to pH 7.^z Bacterial cell density was measured with a spectrophotometer with an optical density at 600 nm at 18 h and expressed as a percentage of cell density in unamended control (100%). Data were analyzed using PROC MIXED with macro pdmix800. In each column, values with a letter in common are not significantly different from each other ($P = 0.05$). **Fig. 2.** Positive identification of *strA-B* genes in seven non-*Erwinia amylovora* bacterial isolates from Illinois apple orchards, using primers strab01-F ('TGG TGT CCC GCA ATG CCG TC') and strab01-R ('CCC GGA TCG GGA GAA GGG CA'). Lane 1, DNA ladder; lane 2, negative control (Mk1); lane 3, 5897 (Jersey), lane 4, 7234 (McHenry), lane 5, 5374 (no band) (Jackson), lane 6, 6114 (Calhoun), lane 7, 5814 (St. Clair), lane 8, 6004 (Jersey), lane 9, 4264 (Calhoun), lane 10, 5174 (Union); lanes 11 and 12, positive controls (W4 and MI 5-1, respectively); and lane 13, water control.

Agri-Mycin 17WP ($P = 0.0537$), pre-inoculation Kasumin 2L ($P = 0.0537$), and post-inoculation ARY-4016-06 ($P = 0.0234$), significantly reduced blossom infection compared with the control.

In 2012, all treatments reduced blossom infection compared with control plots. However, significant blossom blight reduction was observed in the plots that received four antibiotic treatments, including pre- or post-inoculation Agri-Mycin 17WP ($P = 0.0485$), post-inoculation Agri-Mycin 17WP ($P = 0.0951$), pre- or post-inoculation ARY 0416-06 ($P = 0.0698$), and pre- or post-inoculation Kasumin 2L ($P = 0.1081$) (Table 3).

In 2011 and 2012, shoot infection (observed in summer) was not significantly ($P = 0.10$) reduced by bloom-time treatments to control the pathogen. In 2012, however, treatments with growth regulator Apogee reduced shoot blight significantly ($P = 0.001$) when compared with plots that did not receive Apogee (Table 4). Plots that received both Apogee and Kasumin 2L (pre- and post-inoculation) had significantly less ($P = 0.0009$ and $P = 0.03$, respectively) shoot infection than Kasumin 2L or Apogee alone (Table 4).

Discussion

To our knowledge, this was the first statewide survey of the occurrence of fire blight in Illinois and testing *E. amylovora* isolates for resistance to streptomycin. In this study, *E. amylovora* was isolated from only 3 of 435 flower samples tested during 2011 and 2012. None of the tested flowers showed fire blight symptoms. The results support our observations in the past 13 years that blossom

blight is not common in apple orchards in Illinois. The reasons for the absence of blossom blight in Illinois are (i) streptomycin is still effective against fire blight in Illinois, (ii) almost all apple growers apply streptomycin during bloom; and (iii) temperature and moisture conditions in Illinois are not favorable for production and dispersal of *E. amylovora* populations at bloom.

Although blossom infection is not a common phase of fire blight in Illinois apple orchards, fruit blight, shoot blight, canker, and root-stock blight occurs widely in the state. One possible explanation for the higher incidence of shoot blight is that rain storms are common during April and May in Illinois. We believe that injury caused by windstorms on new shoots is the main factor in initiating shoot blight, because streptomycin applications have ceased by the end of the bloom period. Similar results have been reported by previous investigators (14). Further epidemiological studies are required to determine factors affecting shoot blight in Illinois apple orchards.

Detection of Tn5393 in bacteria in orchards that have received streptomycin applications has been reported from other apple-growing areas (12,21,22). This study is the first confirmation of bacterial isolates containing *strA-strB* paired with IS1133 in the Illinois orchards. The insertion sequence IS1133 is required for expression of *strA-strB* resistant genes via promoting transcription of streptomycin resistance genes (5,12,21,22). The finding in this study indicates that there is a possibility of acquisition of Tn5393 streptomycin resistance into Illinois *E. amylovora* populations.

Table 3. Incidence of blossom blight (caused by *Erwinia amylovora*) on 'Jonathan' apple trees following applications of antibiotics and biocontrol agents in Urbana, IL in 2012

Treatment ^w	Active ingredient (rate)	Application time ^x	Blossom infection (%) ^y	P value ^z
Control	...	...	18.7 a	...
Agrimycin 17WP	Streptomycin (100 mg/liter)	Pre/Post	1.9 e	0.0485
Agrimycin 17WP	Streptomycin (100 mg/liter)	Post only	2.5 e	0.0951
Mycoshield	Oxytetracycline (200 mg/liter)	Pre/Post	5.7 c	0.7564
Mycoshield + Kocide-3000 41.6DF	Oxytetracycline (200 mg/liter) + copper hydroxide (240 mg/liter)	Pre/Post	5.6 c	0.8199
Serenade Max	<i>Bacillus subtilis</i> QST713 (525 mg/liter)	Pre only	4.0 d	0.3769
Blight Ban A506	<i>Pseudomonas syringae</i> A506 (369 mg/liter)	Pre only	4.8 cd	0.9881
Dithane 75DF + Kocide-3000 41.6DF	Mancozeb (2,700 mg/liter) + copper hydroxide (240 mg/liter)	Pre/Post	5.9 c	0.8561
Kasumin 2L	Kasugamycin (100 mg/liter)	Pre/Post	2.4 e	0.1081
Kasumin 2L	Kasugamycin (100 mg/liter)	Post only	7.3 b	0.8778
ARY 0416-06	Kasugamycin (100 mg/liter)	Pre/Post	1.8 e	0.0698
LSD (<i>P</i> = 0.05)	...	...	1.23	...

^w All trees were inoculated with *E. amylovora* isolate 2A (5×10^6 CFU/ml) on 30 March and 3 April. Treatments included Regulaid (125 ml per 100 liters) and were applied at 1,400 liters/ha.

^x Pre = treatment was applied 24 h before inoculation and Post = treatment was applied 24 h after inoculation.

^y Infected blossom clusters. Infection of blossoms was assessed on 1 May 2012. The number of clusters per tree tested varied from 6 to 120 (average 70 clusters per tree). Values with a letter in common are not significantly different according to Fisher's protected least significant difference (LSD) (*P* = 0.05).

^z Dunnett comparison to control. Original counted cluster data were transformed [$\sqrt{(x + 1/6)}$] and analyzed as a covariate in PROC MIXED.

Table 4. Severity of shoot blight (caused by *Erwinia amylovora*) on 'Jonathan' apple trees following application of antibiotics, biocontrol agents and prohexadione calcium (Apogee) in Urbana, IL in 2012

Treatment ^x	Active ingredient (rate)	Time ^y	Number of infected shoots ^w		P value ^z
			Without	With	
Control	...	...	14.3 d	15.5 b	0.9285
Agrimycin 17WP	Streptomycin (100 mg/liter)	Pre/Post	8.5 ef	5.5 f	0.6368
Agrimycin 17WP	Streptomycin (100 mg/liter)	Post only	7.0 fg	3.5 g	0.2216
Mycoshield	Oxytetracycline (200 mg/liter)	Pre/Post	17.0 c	10.8 c	0.2992
Mycoshield + Kocide-3000 41.6DF	Oxytetracycline (200 mg/liter) + copper hydroxide (240 mg/liter)	Pre/Post	18.5 c	9.8 cde	0.2733
Serenade Max	<i>Bacillus subtilis</i> QST713 (525 mg/liter)	Pre only	13.9 d	8.0 e	0.2870
Blight Ban A 506	<i>Pseudomonas syringae</i> A506 (369 mg/liter)	Pre only	31.3 a	18.5 a	0.1882
Dithane 75DF+ Kocide-3000 41.6DF	Mancozeb (2,700 mg/liter) + copper hydroxide (240 mg/liter)	Pre/Post	9.3 e	10.3 cd	0.7899
Kasumin 2L	Kasugamycin (100 mg/liter)	Pre/Post	23.3 b	3.5 g	0.0009
Kasumin 2L	Kasugamycin (100 mg/liter)	Post only	23.5 b	8.5 de	0.0388
ARY-0416-06	Kasugamycin (100 mg/liter)	Pre/Post	5.5 g	4.5 fg	0.4526
LSD (<i>P</i> = 0.05)	...	...	2.26	1.95	...

^w Without = no prohexadione calcium (Apogee) was applied and With = of 3.363 kg/ha of Apogee (0.925 kg/ha of prohexadione calcium) in two sprays (1.68 kg/ha in each spray) plus Regulaid (1.25 ml/liter) were applied on 29 March (60-80% bloom) and 12 April (petal fall), using water at 1,400 liters/ha for each application. On 22 May, 120 shoots were evaluated in each tree. Each value represents a mean of four trees and the mean of 480 shoots. Values with a letter in common are not significantly different according to Fisher's protected least significant difference (LSD) (*P* = 0.05).

^x All trees were inoculated with *E. amylovora* isolate 2A (5×10^6 CFU/ml) on 30 March and 3 April. Treatments included Regulaid (125 ml/100 liters) and were applied at 1,400 liters/ha.

^y Application time. Pre = treatment application 24 h before inoculation and Post = treatment application 24 h after inoculation.

^z Data were transformed [$\sqrt{(x)}$] and analyzed in PROC MIXED. *P* values of treatment $\times$ Apogee is shown.

Sholberg et al. (20) reported that 18 of 42 *E. amylovora* isolates tested in British Columbia developed colonies in the presence of 0.16 mM copper sulfate on CYE medium. They also reported that 2 of 42 isolates developed colonies at 0.32 mM copper sulfate on CYE. In our study, all 84 Illinois *E. amylovora* isolates tested were sensitive to copper sulfate at 0.16 mM. The study also showed that all four copper compounds tested prohibited cell multiplication of *E. amylovora* with metallic copper at 14 mg/liter. Thus, the results of our study indicate that copper is still an effective compound in managing *E. amylovora* in early spring in Illinois.

Determining streptomycin alternatives for management of fire blight in pome fruit is essential. In 2011 and 2012 field trials, kasugamycin significantly reduced blossom infection. The results indicated that kasugamycin could be a suitable streptomycin alternative for management of fire blight disease of apple in Illinois. Further studies are needed to determine effectiveness of kasugamycin-prohexadione calcium applications for management of fire blight of apple. Oxytetracycline (Mycoshield) did not significantly reduce blossom blight infection. Similarly, biocontrol agents Serenade Max and BlightBan A506 alone did not effectively reduce blossom blight. These results are similar to the multi-year, multi-location observations reported by Sundin et al. (23). Thus, neither

oxytetracycline (Mycoshield) nor biocontrol agents Serenade Max and BlightBan A506 may be considered effective streptomycin alternatives for control of fire blight in Illinois.

Results from the 2010 to 2012 surveys indicated that no streptomycin-resistant *E. amylovora* is currently present in Illinois. However, streptomycin-resistant strains could be introduced from other states or develop in the state. Growers should be vigilant when ordering nursery stock from states with streptomycin-resistant populations of *E. amylovora*, because the streptomycin resistance occurrence in New York was related to imported nursery stock (18).

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