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Streptomycin Resistance of *Erwinia amylovora* Isolated from Apple (*Malus domesticus*) in Utah

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Abstract

Fire blight, caused by the bacterium *Erwinia amylovora*, causes devastating losses in multiple fruit and ornamental crops worldwide. It is the most important disease for apple and pear growers in Utah. Currently, the only effective management strategy is the application of streptomycin. In 2006, isolates resistant to streptomycin were detected in an apple orchard in central Utah for the first time. To determine the distribution of resistant isolates and the level of resistance, isolates of *E. amylovora* were collected between 2006 and 2012 from apple trees across the state and tested for resistance to streptomycin. Each isolate was initially screened against 0, 50, 100, and 1,000 ppm of streptomycin. Selected isolates resistant to 1,000 ppm were exposed to higher concentrations of streptomycin. The majority of resistant isolates were found in Utah Co., the largest apple and pear production area in the state. Resistant isolates tolerated up to 200,000 ppm of streptomycin. The resistance mechanism in all isolates obtained in Utah was identified as a mutation of codon 43 found in the *rpsL* gene.

Introduction

Fig. 1. Apple with small droplets of oozing *Erwinia amylovora*.

Fire blight is a disease of apple (*Malus domesticus*), pear (*Pyrus communis*), and other rosaceous plants, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al. The bacterium survives the winter in stem cankers and enter into new plant growth through natural openings or wounds. The most common point of ingress is through newly opened blossoms (20). Colonization of the blossoms by the bacterium leads to shoot blight and in severe cases causes death of the tree (Figs. 1 and 2). Applications of streptomycin have been a highly effective management tool, but in 1971 the first streptomycin-resistant isolates were discovered in California and one year later were found in Washington and Oregon (5,6,10,12). Resistant strains have also been documented in Missouri, Michigan, New York, British Columbia (Canada), Israel, Lebanon, and New Zealand (6,11,15,16,19). Fire blight has been an important disease consistently confronted by Utah apple and pear growers. In 2006, streptomycin-resistant *E. amylovora* isolates were first detected in an orchard in Utah Co., Utah (7). Growers in Utah still using streptomycin apply it based on a version of the Cougarblight forecasting system (17) adapted for Utah to reduce the amount of antibiotics used and limit the potential for development of resistance.

Fig. 2. Apple tree with a "shepherd's crook," a common symptom of fire blight.

There are at least two molecular mechanisms of streptomycin resistance reported in *E. amylovora*. Resistance can occur due to a single base pair mutation in the binding site for streptomycin as detected in Michigan (3). In 1995, Chiou and Jones (3) discovered a single base pair mutation in codon 43 of the *rpsL* gene, which accounted for the streptomycin resistance, where streptomycin binds to the S12 protein of the 30S ribosomal subunit that is encoded by the *rpsL* gene. Binding of streptomycin to the S12 protein prevents protein synthesis resulting in death of the bacterium. A mutation at this site prevents binding of the antibiotic, thus allowing for normal protein synthesis to occur. A second resistance mechanism identified in *Erwina amylovora* in New York State is by the bacterium acquiring the *StrA-StrB* gene complex that resides in a plasmid (14). These genes encode for two enzymes, aminoglycoside-3-phosphotransferase and aminoglycoside-6-phosphotransferase, which modifies streptomycin and confers a high level of resistance when present (2,4). The spread of the *StrA-StrB* gene complex is by bacterial conjugation, a plasmid mediated-transfer of genetic material through cell-to-cell contact (1). It is currently unknown which resistant mechanism occurs in Utah. The objectives of this study were to determine the frequency and distribution of streptomycin-resistant *E. amylovora* strains in Utah orchards and determine the molecular mechanism of resistance.

Isolation and Molecular Identification of *E. amylovora*

Five to seven symptomatic shoots or 100 blossoms were randomly collected from apple trees from 21 orchards in the main apple-growing region of Utah (Cache, Box Elder, Davis, and Utah counties) (Fig. 3) during September 2010, and May and June of 2011 and 2012. Symptomatic apple shoots were finely chopped with a sterile razor blade allowing bacteria to stream into distilled water and loopfuls of the suspension were streaked onto CCT medium (8). Alternatively, when oozing was observed, ooze was directly collected from the stem using a bacteriological loop and streaked onto CCT medium. Blossoms from each tree were put into a re-sealable plastic bag, 50 ml of water were added, blossoms were gently rubbed to dislodge bacteria, and 100 µl of wash water were pipetted onto CCT medium and spread across the plate. After incubation at 28°C for 48 h, mucoid colonies were transferred to LB (Luria-Bertani) medium plates and grown for 24 h. The bacteria were then transferred to microcentrifuge tubes containing 0.5 ml sterile water and boiled for 3 to 4 min to release DNA. Polymerase chain reaction (PCR) was conducted to confirm the identity of the putative *E. amylovora* isolates. The total reaction volume for the PCR was 50 µl and each reaction consisted of 5 µl 10X buffer, 5 µl Q solution, 1 µl dNTPs, 1 µl each of *E. amylovora*-specific primers AMS1 (5'-GGCAGAAGTTGTGAGCA-3') and AMS2 (5'-AAACAGGTGCGCCGAATA-3') (9), 0.25 µl Taq, and 2 µl bacterial DNA. All reagents, except primers and water, came from Qiagen, Germantown, MD. Nuclease-free water was added to bring the total volume to 50 µl. A Techne 3000 thermocycler was used with the following protocol: 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 52°C

for 1 min, and 72°C for 1 min. The final extension was for 10 min at 72°C. The PCR products were visualized by gel electrophoresis in a 1% agarose gel stained with ethidium bromide. *Erwinia amylovora* isolates showed the expected 1,200-bp band. Eighty-three isolates of *E. amylovora* were isolated from 15 orchards in Utah Co. and two isolates from two orchards in Cache Co. In Davis Co., 15 isolates were collected from one orchard.

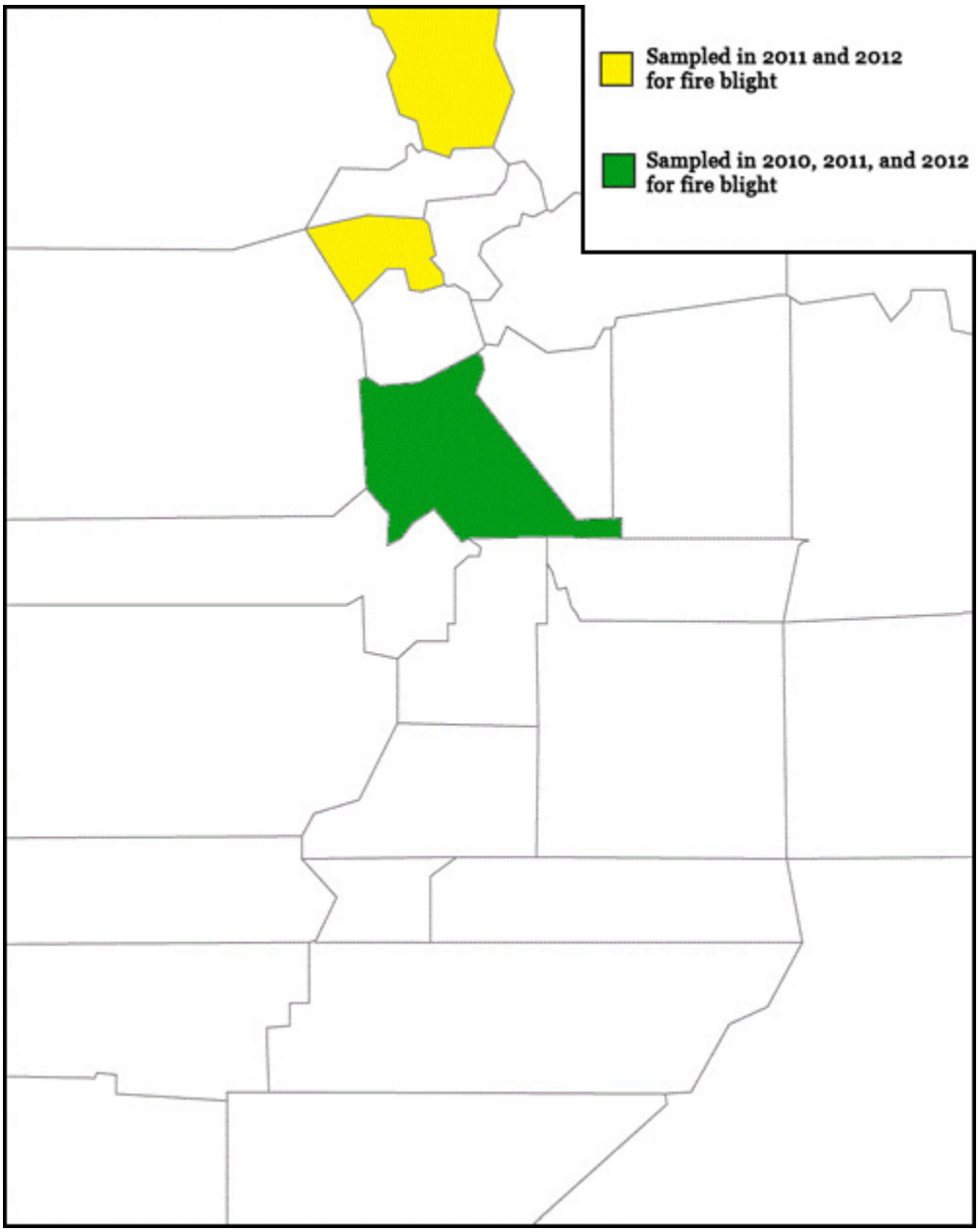

Fig. 3. Counties in Utah in which orchards were sampled for fire blight in 2010, 2011, and 2012. Orchards in yellow counties were sampled 2011 and 2012. Orchards in green colored county were sampled in all three years.

Streptomycin Resistance Testing

Confirmed *E. amylovora* isolates were tested for streptomycin resistance. The bacteria were grown in LB agar plates for 24 h. Bacteria were harvested from the medium and suspended in sterile tap water. Bacterial concentration was adjusted to 1×10^8 colony-forming units (CFU)/ml using a spectrophotometer. One hundred microliters were spread on to LB plates that

had a 5-mm hole punched into the medium in the center of the plate with a cork borer. Streptomycin was dissolved in sterile water and 65 µl of 0 (water control), 100 ppm or 1,000 ppm streptomycin sulfate solutions (MP Biomedical, Solon, OH) were added to each well. 100 ppm of streptomycin sulfate is the concentration used by growers in the field. Each isolate was screened against the above concentrations. Strains were evaluated for streptomycin resistance after plates were incubated for 24 h at 28°C. A zone of inhibition indicated the isolate was sensitive and the absence of a zone indicated resistance to streptomycin (Fig. 4). Sixty-six percent of orchards in Utah Co. and the orchard in Davis Co. contained streptomycin-resistant strains of *E. amylovora* (Table 1). The Cache Co. isolates were sensitive. This is the first report of streptomycin resistance in *E. amylovora* in Utah outside of Utah Co. Of the 18 orchards with fire blight, three had only resistant isolates, eight had mixed populations, and six had only sensitive isolates. Streptomycin had never been used in the orchards that had only sensitive isolates except for one orchard in Cache Co. This is very interesting, because all of the orchards in Utah Co. with only sensitive strains are closely located (1-2 miles) near orchards with streptomycin resistant strains. Streptomycin resistant strains easily could be transported by wind or bees and other insects from one orchard to another. But to date, use of streptomycin remains a viable management tool in those orchards with only sensitive strains. All resistant isolates were resistant at 1,000 ppm and four isolates tested were resistant at 200,000 ppm. In addition to the isolates collected from 2010-2012, stored isolates from 2006 and 2007 from Utah Co. were included for streptomycin resistance testing. Resistant isolates recovered from storage showed the same resistance level as the newly collected strains.

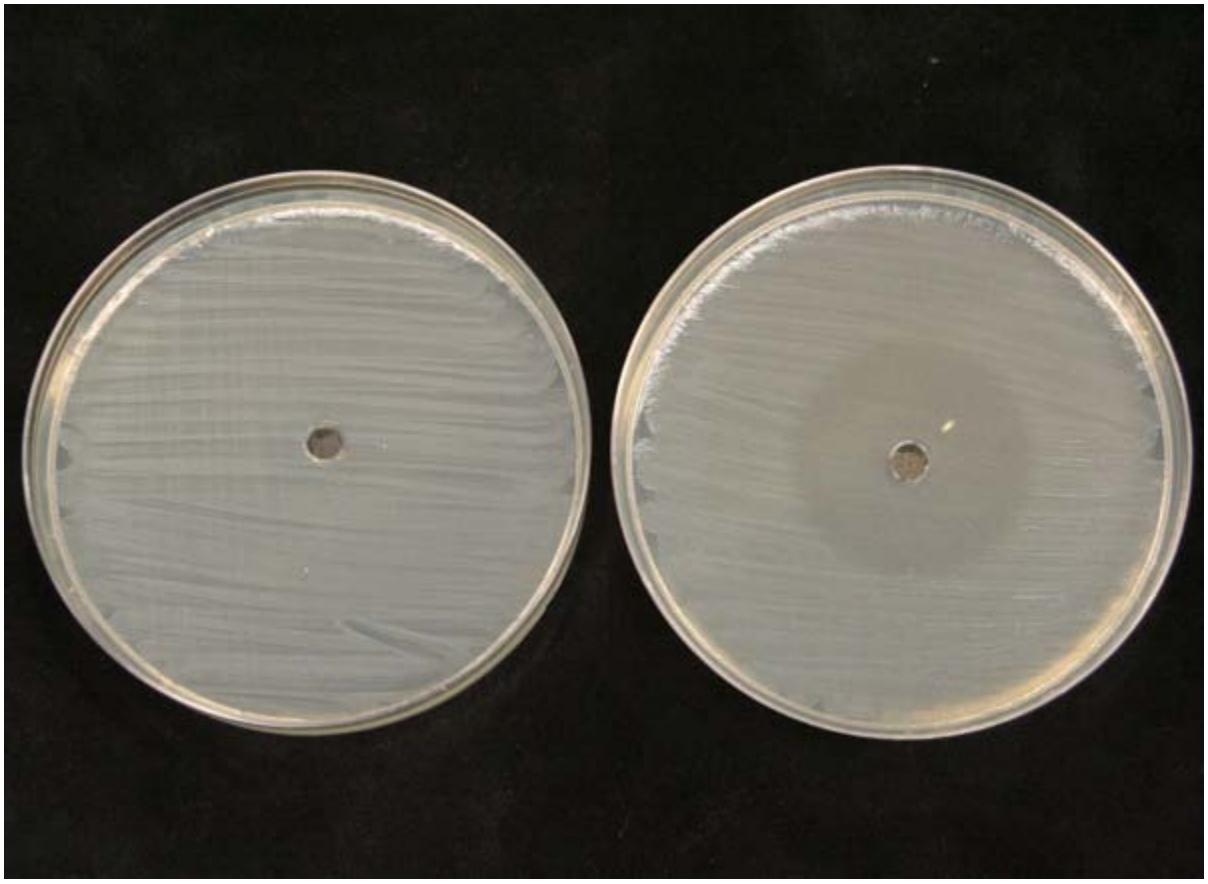

Fig. 4. *Erwinia amylovora* strain resistant (left) and sensitive (right) to 10,000 ppm streptomycin.

Table 1. Number of streptomycin resistant *Erwinia amylovora* isolates from each orchard by county surveyed in Utah from 2010-2012.

County (UT)	Orchards	Total no. of *Erwinia amylovora* isolates	No. of streptomycin resistant isolates[x]	Percent streptomycin resistant isolates[x]
Utah	1	8	0	0
	2	5	2	40
	3	4	3	75
	4	1	0	0
	5	1	0	0
	6	5	5	100
	7	4	4	100
	8	3	1	33
	9	7	2	29
	10	3	2	67
	11	4	1	25
	12	27	18	67
	13	5	3	60
	14	3	0	0
	15	3	0	0
Cache	1	1	0	0
	2	1	0	0
Davis	1	11	2	19
Total	19	96	43	45

[x] Isolate was considered resistant when no halo developed at 100 ppm. 100 ppm is the streptomycin sulfate concentration used by growers.

Identification of Resistance Mechanism

The isolates that showed resistance to streptomycin were tested for the presence of a mutation in the *rpsL* gene and the presence of the *StrA-StrB* gene complex, the two resistance mechanisms occurring in *E. amylovora* in other states (14). The PCR reaction mixtures were the same as described above using primers rpsl F and rpsl R (14) and StrA-StrB primer sets (14).

The PCR products obtained for the *rpsL* gene were extracted using the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD) following manufacturer's instructions. The products were sequenced at The University of Arizona Genetics Core facility in Tucson, AZ. The obtained *rpsL* sequences of resistant and sensitive isolates were aligned using the CLUSTAL W program to determine the presence of a mutation. All resistant isolates had a mutation in codon 43 that resulted in the change of lysine to arginine (Fig. 5). This mutation has been reported in resistant isolates in other states (3). PCR products were obtained for the *StrA* gene from one *E. amylovora* isolate, but no PCR product for the *StrB* gene. The PCR product for *StrA* was sequenced and matched *StrA* sequences in GenBank. The mutation in the *rpsL* gene is highly stable and does not seem to have a cost of fitness. The resistant isolates grow as vigorously on streptomycin amended Luria-Bertani (LB) plates as on non-amended plates. In addition, these isolates are still dominant in many of the sampled orchards up to seven years after the application of streptomycin was discontinued. Moller et al. (13) still detected streptomycin resistant *E. amylovora* strains in California pear orchards even after the grower discontinued the use of streptomycin for ten years. The results indicate that streptomycin is not a viable option for growers in Utah, as 61% of the tested orchards contained streptomycin resistant strains. Based on results elsewhere, resistant strains tend to survive even in the absence of selection pressure of applying streptomycin. Many fungi have a cost of fitness

when they obtain resistance to fungicides and are outcompeted by sensitive strains after the use of the fungicide has ceased. For example, Suzuki et al. (18) could not detect resistant *Pyricularia oryzae* strains four years after the use of the fungicide they were resistant to was discontinued. In contrast, streptomycin resistant *E. amylovora* strains, once established, remain dominant for many years.

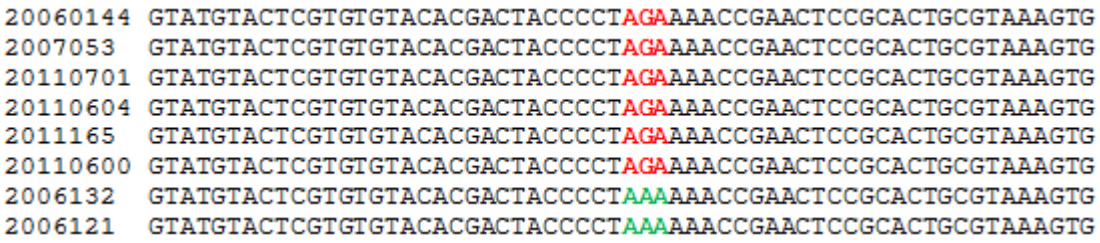

Fig. 5. Mutation in the rpsL gene at codon 43. The resistant strains have the triplet AGA (highlighted in red) that encodes for arginine and the sensitive strains have AAA (highlighted in green) which codes for lysine.

Acknowledgements

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Literature Cited

1. Amabile-Cuevas, C. F., and Chicurel, M. E. 1992. Bacterial plasmids and gene flux. Cell 70:189-199.
2. Burr, T. J., and Huang, T. C. 1999. Characterization of plasmids that encode streptomycin-resistance in bacterial epiphytes of apple. J. Appl. Microbiol. 86:741-751.
3. Chiou, C., and Jones, A. L. 1995. Molecular analysis of high level streptomycin resistance in *Erwinia amylovora*. Phytopathology 85:324-328.
4. Chiou, C., and Jones, A. L. 1995. Expression and identification of the *strA-strB* gene pair from streptomycin-resistant *Erwinia amylovora*. Gene 152:47-51.
5. Chiou, C., and Jones, A. L. 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. Phytopathology 81:710-714.
6. Coyier, D. L., and Covey, R. P. 1975. Tolerance of *Erwinia amylovora* to streptomycin sulfate in Oregon and Washington. Plant Dis. Rep. 59:849-852.
7. Evans, C. K. 2008. Survey results of *Erwinia amylovora* in Utah for resistance to streptomycin and investigation comparing Kasugamycin (Kasumin) to streptomycin and oxytetracycline for control of fire blight. Acta Horti. 793:433-437.
8. Ishimaru, C., and Klos, E. J. 1984. New Medium for detection of *Erwinia amylovora* and its use in epidemiological studies. Phytopathology 74:1342-1345.
9. Kim, W. S., Hildebrand, M., Jock, S., and Geider, K. 2001. Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. Microbiology 147:2951-2959.
10. 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.
11. McManus, P. S., Stockwell, V. O., Sundin, G. W., and Jones, A. L. 2002. Antibiotic use in plant agriculture. Annu. Rev. Phytopathol. 40:443-465.
12. Miller, T. D., and Schroth, M. N. 1972. Monitoring epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62:1175-1182.
13. Moller, W. J., Schroth, M. N., and Thompson, S. V. 1981. The scenario of fire blight and streptomycin. Plant Dis. 65:563-568.
14. Russo, N. L., Burr, T. J., Breth, D. I., and Aldwinckle, H. S. 2008. Isolation of streptomycin resistant isolates of *Erwinia amylovora* in New York. Plant Dis. 92:714-718.
15. Shaffer, W. H., and Goodman, R. N. 1985. Appearance of streptomycin-resistant *Erwinia amylovora* in Missouri apple orchards. (Abstr.) Phytopathology 75:1281.
16. Sholberg, P. L., Bedford, K. E., Haag, P., and Randal, P. 2001. Survey of *Erwinia amylovora* isolates from British Columbia for resistance to bactericides and virulence on apple. Can. J. Plant Pathol. 23:60-67.

17. Smith, T. J. 1993. A predictive model for forecasting fire blight of pear and apple in Washington State. Acta Horti. 338:153-157.
18. Suzuki, F., Yamaguchi, J., Koba, A., Nakajima, T., and Arai, M. 2010. Changes in fungicide resistance frequency and population structure of *Pyricularia oryzae* after discontinuance of MBI-D fungicides. Plant Dis. 94:329-334.
19. Thomson, S. V., Gouk, S. C., Vanneste, J. L., Hale, C. N., and Clark, R. G. 1993. The presence of streptomycin resistant isolates of *Erwinia amylovora* in New Zealand. Acta Hortic. 338:223-230.
20. Vanneste, J. L. 2000. Page 1-6 in: Fire blight: The disease and its causative agent, *Erwinia amylovora*. J. L. Vanneste, ed. CAB Intl., Wallingford, UK.