Prevalence of Streptomycin-Resistant *Erwinia amylovora* in New York Apple Orchards

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Abstract

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Resistance to streptomycin in *Erwinia amylovora* was first observed in the United States in the 1970s but was not found in New York until 2002, when streptomycin-resistant (SmR) *E. amylovora* was isolated from orchards in Wayne County. From 2011 to 2014, in total, 591 fire blight samples representing shoot blight, blossom blight, and rootstock blight were collected from 80 apple orchards in New York. From these samples, 1,280 isolates of *E. amylovora* were obtained and assessed for streptomycin resistance. In all, 34 SmR *E. amylovora* isolates were obtained from 19 individual commercial orchards. The majority of the resistant isolates were collected from orchards in Wayne County, and the remaining were from other counties in western New York. Of the 34 resistant isolates, 32 contained the streptomycin resistance gene pair *strA/strB* in the transposon Tn5393 on the nonconjugative plasmid pEA29. This determinant of

streptomycin resistance has only been found in SmR *E. amylovora* isolates from Michigan and the SmR *E. amylovora* isolates discovered in Wayne County, NY in 2002. Currently, our data indicate that SmR *E. amylovora* is restricted to counties in western New York and is concentrated in the county with the original outbreak. Because the resistance is primarily present on the nonconjugative plasmid, it is possible that SmR has been present in Wayne County since the introduction in 2002, and has spread within and out of Wayne County to additional commercial growers over the past decade. However, research is still needed to provide in-depth understanding of the origin and spread of the newly discovered SmR *E. amylovora* to reduce the spread of streptomycin resistance into other apple-growing regions, and address the sustainability of streptomycin use for fire blight management in New York.

Fire blight is a devastating disease of apple, pear, and many other rosaceous hosts caused by the bacterium *Erwinia amylovora*. *E. amylovora* overwinters in cankers formed during the apple-growing season and initiates infection in the spring, when the orchard temperatures warm and cankers begin to release ooze containing high numbers of bacteria (Beer and Norelli 1977; Vanneste 2000). The bacteria are then spread by insects, wind, and rain to flowers, which are highly susceptible to infection (Wael et al. 1990). Flower stigmas provide a nutrient-rich environment in which *E. amylovora* can colonize and multiply rapidly (Beer and Norelli 1977). Following rain or even dew, these bacteria are washed into the hypanthium and enter natural floral nectaries, which may result in blossom infection producing blackened and necrotic flower clusters (Rosen 1936; Sundin 2014). *E. amylovora* subsequently spreads internally throughout the vascular tissues, resulting in systemic infections that may kill limbs, the trunk, and the rootstock of the tree. Infection of susceptible rootstocks may result in death of the entire tree (van der Zwet 1969). The loss of entire trees and plantings has a tremendous impact on production. This is especially the case in moderately to highly susceptible apple cultivars, which are widely planted and in increasingly high demand (Bonn and van der Zwet 2000). To complicate matters, modern planting systems in New York are typically composed of tightly spaced (>1,000 trees/acre), small, vigorous trees of highly susceptible scion cultivars (Robinson 2008a,b; Robinson et al. 2013). Because high epiphytic populations of *E. amylovora* may lead to blossom blight, shoot blight, and, potentially, rootstock blight, protecting flowers from infection throughout bloom is essential for managing fire blight in orchards.

Blossom blight is typically managed by a combination of biological and chemical controls. Biological controls inhibit *E. amylovora* through antibiosis or competitive inhibition, preventing the buildup of high population levels on the stigma (Pal and Mc Spadden Gardener 2006; Johnson et al. 2009). Although many biological controls are fairly effective in controlling blossom blight in semiarid apple production regions in the western United States, they do not provide effective control of blossom blight in the humid, temperate production regions of the midwestern and northeastern United States (Sundin et al. 2009). Aside from the biological controls, conventional chemical controls for fire blight include copper and antibiotics. Copper is only effective for control of blossom blight when applied prior to infection and can cause deleterious effects to the developing fruit and foliage (Jamar and Lateur 2007; Montag et al. 2006). Hence, copper is usually not applied for blossom blight in orchards producing apple fruit destined for the fresh market. Oxytetracycline can provide effective blossom blight control in semiarid apple production regions such as those in the western United States (Stockwell et al. 2008) but the humid climate of the northeastern United States diminishes the effectiveness of oxytetracycline's bacteriostatic mode of action (van der Zwet et al. 2012). The most effective means of chemical control of blossom blight in the midwestern and northeastern United States is the aminoglycoside antibiotic streptomycin (Cox et al. 2013; Russo et al. 2008; Sundin et al. 2009). This is primarily due to streptomycin's bactericidal mode of action, its potential for slight curative activity, and the fact that there are no other products offering a similar cost effectiveness (Russo et al. 2008; Sundin et al. 2009; van der Zwet et al. 2012). The aminoglycoside antibiotic kasugamycin was registered for use by the Environmental Protection Agency (EPA) in 2015 (EPA registration number 66330-404) and can provide a similar level of control, but does not have curative activity or the same cost effectiveness as streptomycin (McGhee and Sundin 2011; Yoder et al. 2012).

Because of the aforementioned utility, streptomycin has been used widely in the United States for over 50 years, providing effective and necessary control of *E. amylovora* outbreaks. However, reports of

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streptomycin resistance have raised concern about the sustainability of this antibiotic for fire blight management. The first reports of streptomycin resistance occurred in California in 1971 followed by Washington and Oregon in 1972 (Coyier and Covey 1975; McManus and Jones 1994; Miller and Schroth 1972). Since these discoveries, streptomycin-resistant (SmR) *E. amylovora* has become established in apple-growing regions along the western coast of the United States and in certain apple-growing regions of Missouri and Michigan (McManus et al. 2002). SmR isolates of *E. amylovora* were not observed in New York until 2002 (Beer and Norelli 1976; Russo et al. 2008). In this instance, two SmR isolates of *E. amylovora* (NY17.1 and NY17.2) were recovered from fire blight outbreaks in two adjacent orchards in Wayne County (Russo et al. 2008). Eradication efforts, which included prompt removal of trees, were put in place in order to contain and destroy SmR *E. amylovora*-infected plant materials and prevent spread of these bacteria to other orchards (Russo and Aldwinckle 2009; Russo et al. 2008).

Currently, there are two known determinants of streptomycin resistance in *E. amylovora*. The first is the presence of a point mutation at codon 43 in the *rpsL* gene, which codes for the S12 ribosomal protein (Chiou and Jones 1995a). This single base-pair mutation, causing a substitution of lysine for arginine (K43R), alters the binding site of streptomycin, which would otherwise bind to the S12 protein and inhibit translation of mRNA to proteins (McManus et al. 2002). In a sensitive strain of *E. amylovora*, streptomycin binds to the S12 protein on the ribosome to block protein synthesis, killing the bacterium. This mechanism confers resistance in *E. amylovora* at streptomycin concentrations up to 2,500 µg/ml (Chiou and Jones 1995a). This mutation is persistent in populations, and is the most common mechanism found in SmR *E. amylovora* in the western United States (Moller et al. 1981). Strains with this type of resistance are rarely found in apple orchards east of the Mississippi River (McManus et al. 2002).

A second determinant of streptomycin resistance is the gene pair *strA/strB*. These tandem genes code for aminoglycoside-modifying phosphotransferase enzymes that modify streptomycin, making the antibiotic ineffective within the cell (Chiou and Jones 1995b). These genes are commonly found in many epiphytic bacteria, such as *Pseudomonas* sp., and reside on conjugative R plasmids (Burr et al. 1988). In *E. amylovora*, the *strA/strB* pair has been found on plasmid RSF1010 (Palmer et al. 1997) and within the transposable element Tn5393 on either a conjugative plasmid pEA34 or a nonconjugative plasmid pEA29 (McGhee et al. 2011; McManus and Jones 1994). The *strA/strB* gene pair in Tn5393 on plasmid pEA29, the basis of resistance for the isolates discovered in 2002 in New York, is the most common determinant of resistance in Michigan (Russo et al. 2008; McGhee et al. 2011; McManus and Jones 1994).

Since the discovery and eradication of SmR *E. amylovora* in 2002, there have been limited investigations regarding these bacteria in New York. Informal surveys from 2004 to 2006 were conducted for SmR *E. amylovora* in areas of western New York where fire blight developed, but such surveys failed to detect SmR *E. amylovora*, leading to the belief that eradication efforts were successful in containing the outbreak in New York. Despite these assurances, apple producers raise concerns about the effectiveness of streptomycin every year, and little is known about the prevalence of SmR *E. amylovora* in New York beyond the sites of original detection in 2002. Moreover, the genetic determinants responsible for resistance would need to be characterized for any new isolates of SmR *E. amylovora* recovered from New York apple orchards. In order to address these knowledge gaps, our goals were to examine fire blight outbreaks in New York apple orchards from 2011 to 2014 for the presence SmR *E. amylovora*, and to characterize determinants of streptomycin resistance for any SmR *E. amylovora* isolates recovered. The resulting information would help New York apple producers better assess the threat of streptomycin resistance development in *E. amylovora*, and adjust antibiotic use practices for resistance management.

Methods and Materials

Collection of fire blight samples and isolation of *E. amylovora*. From 2011 to 2014, samples of fire blight were collected from outbreaks at nurseries and production orchards across the apple-growing

regions of New York State. Collection efforts took place as a cooperative effort between the New York State Agricultural Experiment Station, Cornell cooperative extension, the Lake Ontario Fruit Program, New York State Integrated Pest Management, and Eastern New York Regional Fruit Program. Sample collection efforts were largely driven by reports from growers and cooperative extensions of instances of fire blight outbreaks within individual orchards of relevance to production sustainability. Samples consisted of blighted blossom clusters, shoot blight of first- and second-year scion, and blighted rootstocks. When fewer than 10 symptomatic trees were present, each tree was sampled. If more than 10 trees were sampled, one sample was collected for every 2 to 5 symptomatic trees. Usually (<3% of all samples), no more than one sample was taken per tree. Isolates representing multiple samples from the same tree were designated by a letter after the sample.

Upon arrival in the New York State Agricultural Experiment Station fruit pathology laboratory, tissue samples were trimmed to obtain sections of symptomatic tissue (2 to 3 cm in length) around the margins of infection. Samples were agitated in a 10% bleach solution for 15 min for surface sterilization and subsequently rinsed three times with sterile distilled water. After sterilization, all bark was removed using sterile dissection tools and discarded. The internal tissues were dissected to obtain approximately 1-cm sections of cambium tissue. Sections were immediately plated on Crosse Goodman medium (CG; Crosse and Goodman 1973). If samples were oozing, the samples were also swabbed with a sterile cotton swab, suspended in sterile deionized water, and spread on CG. Plates were incubated at 28°C for 2 to 5 days until bacterial growth was visible around the tissue. Resulting bacterial growth was dilution streaked again on CG and grown for 2 days at 28°C to obtain single colonies. Single colonies with a cratered appearance on CG characteristic of *E. amylovora* (Crosse and Goodman 1973) were collected and stored at -80°C.

Identification of *E. amylovora* colonies. Polymerase chain reaction (PCR) amplification of plasmid pEA29 was used to confirm the identity for putative *E. amylovora* isolates with cratered colony appearance on CG. The pEA29 was shown to be a reliable marker for the identification *E. amylovora* because it is found ubiquitously in isolates collected worldwide; furthermore, laboratory strains cured of the plasmid show reduced virulence and were deemed unlikely to persist in nature (Bereswill et al. 1992; Chiou and Jones 1993; McGhee and Jones 2000). However, it is possible that, if *E. amylovora* isolates naturally lacking this plasmid exist in New York, these isolates may have been overlooked in this study. PCR amplification of pEA29 was conducted using primers previously developed by McManus and Jones (1995) (Table 1). PCR assays were performed in 25-µl reaction volumes and consisted of 12.3 µl of H₂O, 5 µl of 5× Green GoTaq Flexi Buffer (Promega Corp., Madison, WI), 1 µl each of forward and reverse primers (AJ75 and AJ76), 0.5 µl of 10 mM dNTP mix (Promega Corp.), 2.5 µl of 25 mM MgCl₂ (Promega Corp.), 0.2 µl of GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial suspension sample. Cycling parameters were 5 min at 94°C; followed by 35 cycles of 94°C for 30 s, annealing at 52°C for 30 s, and 72°C for 30 s; followed by a final extension at 72°C for 7 min. PCR products were separated using gel electrophoresis with 1% agarose gels in 1× Tris-acetate-EDTA buffer (44.5 mM Tris-acetate and 1 mM EDTA, pH 8.0) at 90 V for 60 min. The resulting amplified region was sequenced for a subset of colonies for confirmation of the presence of pEA29. PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research, Irvine, CA). Purified products were sequenced at the Cornell Biotechnical Resource center in Ithaca, NY using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems, Waltham, MA).

Streptomycin resistance screening of *E. amylovora* isolates. Isolates positively identified as *E. amylovora* were assessed for streptomycin resistance in vitro. Initially, single colonies of the isolates were grown in 1 ml of Luria-Bertani (LB) broth (Bertani 1952) at 28°C for 24 h. Following incubation, 100 µl of the solution was spread on LB media and allowed to dry. Autoclaved filter paper discs were soaked in streptomycin sulfate (Sigma-Aldrich, St. Louis) at 0,

100, and 2,500 µg/ml and placed on the LB containing the isolate lawns. Plates were incubated at 28°C for 48 h, and streptomycin resistance was evaluated by observing the presence or absence of a zone of inhibition around the streptomycin discs. The lack of a zone of inhibition at each concentration confirmed resistance at varying concentrations of streptomycin sulfate, and isolates with resistance phenotypes were retested to confirm resistance.

Isolates of *E. amylovora* with an SmR phenotype and the streptomycin sensitive (SmS) isolate Ea273 (Norelli et al. 1984), used as a positive control, were assessed for pathogenicity using immature pear fruit as previously described (Billing et al. 1960). Immature 'Bartlett' pear fruit (10 to 20 mm in diameter) were cut into 15-mm cross sections and wounded with sterile toothpicks that were touched to individual isolate colonies. Inoculated pear fruit cross sections were incubated on moist filter paper in petri dishes at 28°C for 48 to 96 h. Inoculated wound sites were observed for the presence of necrosis and bacterial ooze.

Identification of the *strA/strB* gene pair in SmR *E. amylovora* isolates. All SmR isolates and the SmS isolate Ea273 (negative control) were tested for the presence of the *strA/strB* gene pair using primers previously developed by Russo et al. (2008) (Table 1). PCR assays were performed in 25-µl reaction volumes and consisted of 12.3 µl of H₂O, 5 µl 5× Green GoTaq Flexi Buffer (Promega Corp.), 1 µl of forward and reverse primer (strA406-F and strA406-R for *strA* and strB403-F and strB403-R for *strB*), 0.5 µl of 10 mM dNTP mix (Promega Corp.), 2.5 µl of 25 mM MgCl₂ (Promega Corp.), 0.2 µl of GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial suspension sample, resulting in a 50-µl reaction. Cycling parameters were 5 min at 94°C; followed by 35 cycles of 94°C for 30 s, annealing for 30 s (56°C for *strA* primers and 53°C for *strB*), and 72°C for 30 s; followed by a final extension at 72°C for 7 min. PCR products were visualized using gel electrophoresis on a 1% agarose gel. For a subset of isolates, PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research). Purified products were then sequenced at the Cornell Biotechnical Resource center in Ithaca, NY using an ABI 3730xl capillary sequencer (Applied Biosystems).

Determination of the Tn5393 insertion site on pEA29 pair SmR *E. amylovora* isolates. All SmR isolates with the *strA/strB* gene pair and two SmR isolates (DM1 and M15-1, positive controls; McGhee et al. 2011) were tested for the presence and insertion of Tn5393 in pEA29 using primers Tn5393E and AJ1717 (pEA29 position 1,515) and AJ507 and AJ339 (pEA29 position 17,527), as described by McGhee et al. (2011). PCR assays were performed in 25-µl reaction volumes and consisted of 12.3 µl of H₂O, 5 µl of 5× Green GoTaq Flexi Buffer (Promega Corp.), 1 µl of forward and reverse primer, 0.5 µl of 10 mM dNTP mix (Promega Corp.), 2.5 µl of 25 mM MgCl₂ (Promega Corp.), 0.2 µl of GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial suspension sample, resulting in a 50-µl reaction. Cycling parameters were identical to those reported for the two sets of primer pairs in McGhee et al. (2011). PCR products were visualized using gel electrophoresis on a 2% agarose gel. For a subset of isolates, PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research).

S12 ribosomal protein *rpsL* gene identification and sequencing in SmR *E. amylovora* isolates. Isolates confirmed to have a streptomycin resistance phenotype that would allow them to grow in the presence of streptomycin sulfate at 2,500 µg/ml were examined for the presence of mutations at codon 43 in the *rpsL* gene. Using primers previously developed by Russo et al. (2008) (Table 1), a portion of the *rpsL* gene containing codon 43 was amplified and sequenced. PCR assays consisted of 24.6 µl of H₂O, 5 µl of 5× Green GoTaq Flexi Buffer (Promega Corp.), 2.0 µl each of forward and reverse primer (rpsL212-F and rpsL212-R), 1.0 µl of 10 mM dNTP mix (Promega Corp.), 5.0 µl of 25 mM MgCl₂ (Promega Corp.), 0.4 µl of GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 5.0 µl of bacterial suspension sample resulting in a 50-µl reaction. Cycling parameters were 5 min at 94°C; followed by 35 cycles of 94°C for 30 s, annealing at 53°C for 30 s, and 72°C for 30 s; followed by a final extension at 72°C for 7 min. PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research). Purified products were sequenced at the Cornell Biotechnical Resource center in Ithaca, NY using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems).

Results

Collection of fire blight samples and isolation of *E. amylovora*.

From 2011 to 2014, samples were collected from a total of 80 commercial orchards with fire blight outbreaks. The majority of the samples were from orchards in Wayne, Monroe, Niagara, Ontario, Orleans, and Tompkins Counties in western New York. By comparison, only 19 of the 591 samples were from Albany, Clinton, Orange, Suffolk, and Ulster Counties in eastern New York. Of the 591 samples collected, 97 were blighted blossoms, 415 were shoot blight of first- or second-year scions, and 74 were blighted rootstocks. In addition, there were five samples collected from the coleopterian *Xylosandrus germanus* or its galleries that appeared to be oozing with fire blight. Across all years, samples were predominantly from 'Gala', 'Gingergold', 'Honeycrisp', 'SnapDragon', and 'RubyFrost' apple. From the samples, 1,384 bacterial isolates were obtained and 1,280 were confirmed to be *E. amylovora*. Single colonies of isolates produced the characteristic cratered appearance on CG and, when PCR was performed using primers AJ75 and AJ76, all isolates produced an 840-bp band, indicative of the presence of the ubiquitous, nonconjugative plasmid pEA29.

Streptomycin resistance screening and pathogenicity of *E. amylovora* isolates. Of the 1,280 isolates, 34 displayed an SmR phenotype on CG, in that a zone of inhibition failed to develop around filter papers discs with streptomycin concentrations of 100 µg/ml. For 2 of the 34 isolates (306b and 189a), a zone of inhibition failed to develop around filter papers discs with streptomycin concentrations of 2,500 µg/ml (Table 2). All 34 SmR isolates of *E. amylovora* isolates and Ea273 were found to produce necrotic lesions and bacterial ooze within 48 to 96 h on immature pear fruit confirming pathogenicity (Table 2).

Of the 34 SmR isolates of *E. amylovora*, 20 were recovered from orchards in Wayne County and 5 were from orchards in Ontario County (Tables 2 and 3). The remaining isolates were recovered from

Table 1. Primers used in this study to confirm the identity of *Erwinia amylovora* isolates, and to determine the presence of the gene pair *strA/strB* and amplify the *rpsL* gene for sequencing

Target ^a	Primer designation	Sequence (5'–3') ^b	Source
pEA29	AJ75	CGTATTCACGGCTTCGCAGAT	McManus and Jones 1995
	AJ76	AACCGCCAGGATAGTCGCATA	...
<i>strA</i>	strA406-F	TGACTGGTTGCCTGTCTCAGAG	Russo et al. 2008
	strA406-R	CGGTAAGAAGTCGGGATTGA	...
<i>strB</i>	strB403-F	ATCGCTTTGCAGCTTTGTGT	Russo et al. 2008
	strB403-R	CGTTGCTCCTCTTCTCCATC	...
<i>rpsL</i>	rpsL212-F	CGTACGCAAAGTTGCAAAAA	Russo et al. 2008
	rpsL212-R	GGATCAGGATCACGGAGTGT	...

^a Plasmid or gene target amplified by the PCR primer set.

^b Primer sequence in the 5' to 3' direction for the forward and reverse primer.

orchards in Monroe, Orleans, Tompkins, and Niagara Counties in western New York (Fig. 1). These isolates were recovered from 19 individual orchards in which SmR *E. amylovora* was isolated from 4 to 100% of samples (Tables 2 and 3). Isolates of SmR *E. amylovora* were recovered from 20 cultivars of apple, with three or more SmR isolates recovered from 'Idared', RubyFrost, and 'McIntosh' apple. Nearly all of the isolates ($n = 28$) were obtained from blighted shoots that developed in the current year's scion tissue (Table 2). Three isolates were obtained from blossom clusters, two from rootstock blight samples, and one from the coleopterian *X. germanus*.

Identification of the *strA/strB* gene pair and Tn5393 insertion site on pEA29 in SmR *E. amylovora* isolates. Amplification of the Tn5393 region containing the *strA/strB* gene pair revealed the presence of a 406- and 403-bp band in 32 of the SmR isolates of *E. amylovora*. These bands were not present for SmS isolate Ea273 and the two SmR isolates 306b and 189a, which grew in the presence of streptomycin at 2,500 $\mu\text{g/ml}$. Sequencing of these regions confirmed the identity of the 406- and 403-bp bands (GenBank accession numbers KT899306 and KT899307) to be the *strA* and *strB* genes previously described for SmR isolates of *E. amylovora* (Russo et al. 2008). The Tn5393 insertion site for all 32 isolates

occurred at position 17,527 on pEA29, as shown by the presence of an expected 396-bp band (Fig. 2).

S12 ribosomal protein *rpsL* gene identification and sequencing in SmR *E. amylovora* isolates. Amplification of the region containing codon 43 of the S12 ribosomal protein yielded a 212-bp band for all 34 SmR isolates of *E. amylovora* and Ea273, the SmS control. Sequencing of the 212-bp band confirmed the identity of the 212-bp region to be a portion of the *rpsL* gene of *E. amylovora*. Sequences of each resistant isolate were compared with the sequence of the sensitive isolate Ea273 (GenBank accession number KT899305). Two isolates, 306b (GenBank accession number KT899304) and 189a, were found to have a point mutation at codon 43 (Table 2). In both isolates, this mutation resulted in an amino acid change from lysine to arginine (K43R). The remaining 32 isolates and the SmS isolate Ea273 did not have a mutation present in this region.

Discussion

In total, 34 SmR isolates of *E. amylovora* were found in 19 individual orchards across six counties in western New York (Tables 2 and 3). Although 19 samples were collected from orchards in five counties in eastern New York, SmR *E. amylovora* was not found

Table 2. Characteristics of streptomycin-resistant (SmR) *Erwinia amylovora* isolates collected from apple orchards in New York from 2011 to 2014

Year, isolate	Orchard ^a	County	Cultivar ^b	Tissue ^c	SmR (%) ^d	pEA29 ^e	Pathogenic ^f	<i>strA/strB</i> ^g	<i>rpsL</i> ^h
2011									
161	W1	Wayne	'Idared'	Shoot	12	+	+	+	—
162	W1	Wayne	'Rome'	Shoot	12	+	+	+	—
173	W1	Wayne	SnapDragon	Shoot	12	+	+	+	—
174	W1	Wayne	'RubyFrost'	Shoot	12	+	+	+	—
175	W1	Wayne	'McIntosh'	Shoot	12	+	+	+	—
176	W1	Wayne	'Red Delicious'	Shoot	12	+	+	+	—
177	O1	Ontario	'Idared'	Shoot	50	+	+	+	—
178	M1	Monroe	'Idared'	Shoot	33	+	+	+	—
179	W2	Wayne	'Rhode Island Greening'	Shoot	100	+	+	+	—
2012									
316	M2	Monroe	'RubyFrost'	Shoot	17	+	+	+	—
301	N1	Niagara	'SweeTango'	Shoot	50	+	+	+	—
230	O2	Ontario	'Idared'	Shoot	40	+	+	+	—
313	O2	Ontario	'Twenty ounce'	Rootstock	40	+	+	+	—
306a	O3	Ontario	'Lady'	Shoot	25	+	+	+	—
306b	O3	Ontario	'Lady'	Shoot	25	+	+	—	K43R
249	Or1	Orleans	'Aztec Fuji'	Blossom	15	+	+	+	—
278	Or1	Orleans	'Cameo'	Shoot	15	+	+	+	—
254	W2	Wayne	'M.26 RS'	Shoot	100	+	+	+	—
292	W5	Wayne	'Idared'	Shoot	20	+	+	+	—
189	W4	Wayne	Gingergold	Shoot	25	+	+	+	—
189a	W4	Wayne	Gingergold	Shoot	25	+	+	—	K43R
189b	W4	Wayne	Gingergold	Shoot	25	+	+	+	—
2992d	W3	Wayne	'RubyFrost'	Shoot	45	+	+	+	—
3002d	W3	Wayne	Gala	Shoot	45	+	+	+	—
3002e	W3	Wayne	Gala	Shoot	45	+	+	+	—
2013									
465	M3	Monroe	'Royal Court'	Blossom	4	+	+	+	—
321	Or2	Orleans	M.9	Rootstock	17	+	+	+	—
436	T1	Tompkins	'McIntosh'	Blossom	22	+	+	+	—
439	T1	Tompkins	'McIntosh'	Shoot	22	+	+	+	—
330	W7	Wayne	'Jonagold'	Shoot	8	+	+	+	—
333	W7	Wayne	'Jonagold'	Shoot	8	+	+	+	—
345	W8	Wayne	'Pink Lady'	Shoot	9	+	+	+	—
508	W6	Wayne	'Idared'	Shoot	17	+	+	+	—
374	W9	Wayne	'Macoun'	<i>Xylosandrus germanus</i>	5	+	+	+	—

^a Orchard designation, with the letter indicating the county from which the orchard was planted and the number indicating a specific grower operation.

^b *Malus × domestica* cultivar scion or rootstock from which the tissue sample was collected.

^c Type of apple tissue from which the isolate was collected.

^d Percentage of samples from which SmR *E. amylovora* was isolated in each orchard.

^e Presence (+) or absence (–) of the nonconjugative plasmid pEA29 ubiquitous in *E. amylovora*.

^f Isolate pathogenicity on immature pear fruit: (+) isolate produced necrosis and ooze or (–) isolate produced neither ooze nor necrosis

^g Presence (+) or absence (–) of the *strA/strB* gene pair responsible for conferring resistance to streptomycin.

^h Presence (K43R) or absence (–) of the K43R mutation in the *rpsL* gene.

in the eastern apple production regions of the state. Given that the use of streptomycin for fire blight management practices is fairly consistent throughout the state (K. Cox, unpublished data), the absence of SmR in eastern New York may suggest that the emergence of streptomycin resistance in western New York after 2002 may be due to local transport of asymptomatic propagative materials or localized spread of isolates in regionalized storms over the last decade. However, such patterns of emergence cannot be used as evidence or an explanation for the spread SmR *E. amylovora*.

Although the fire blight collection taskforce made efforts to scout all of the major commercial production operations throughout the state, by no means do our efforts represent a structured, systematic sampling survey effort. Of the fire blight outbreaks that were observed by the survey team, they primarily were found on blocks planted to RubyFrost, Gala, Gingergold, SnapDragon, and Honeycrisp but

Table 3. Summary of survey information for orchards where streptomycin-resistant (SmR) *Erwinia amylovora* was detected

Orchard ^a	Year	County, city	Total isolates ^b	SmR isolates ^c
W1	2011	Wayne, Huron	41	6
	2012	Wayne, Huron	1	1
	2013	Wayne, Huron	No fire blight	...
	2014	Wayne, Huron	10	0
O1	2011	Ontario, Phelps	1	1
	2012-14	Ontario, Phelps	No fire blight	...
M1	2011	Monroe, Webster	2	1
	2012-14	Monroe, Webster	No fire blight	...
W2	2011	Wayne, Sodus	3	1
	2012-14	Wayne, Sodus	No fire blight	...
M2	2012	Monroe, Brockport	6	1
	2013	Monroe, Brockport	13	0
	2014	Monroe, Brockport	No fire blight	...
N1	2012	Niagara, Appleton	2	1
	2013	Niagara, Appleton	No fire blight	...
	2014	Niagara, Appleton	30	0
O2	2012	Ontario, Phelps	7	2
	2013-14	Ontario, Phelps	No fire blight	...
O3	2012	Ontario, Geneva	7	2
	2013-14	Ontario, Geneva	No fire blight	...
Or1	2012	Orleans, Kendall	16	2
	2013	Orleans, Kendall	No fire blight	...
	2014	Orleans, Kendall	5	0
W5	2012	Wayne, Macedon	6	1
	2013	Wayne, Macedon	3	0
	2014	Wayne, Macedon	35	0
W4	2012	Wayne, Wolcott	4	3
	2013	Wayne, Wolcott	No fire blight	...
	2014	Wayne, Wolcott	80	0
W3	2012	Wayne, Williamson	17	3
	2013-14	Wayne, Williamson	No fire blight	...
M3	2013	Monroe, Hilton	30	1
	2014	Monroe, Hilton	10	0
Or2	2013	Orleans, Medina	5	1
	2014	Orleans, Medina	25	0
T1	2013	Tompkins, Lansing	7	2
	2014	Tompkins, Lansing	No fire blight	...
W7	2013	Wayne, Wolcott	23	2
	2014	Wayne, Wolcott	No fire blight	...
W8	2013	Wayne, Sodus	21	1
	2014	Wayne, Sodus	No fire blight	...
W6	2013	Wayne, Williamson	5	1
	2014	Wayne, Williamson	No fire blight	...
W9	2013	Wayne, Sodus	24	1
	2104	Wayne, Sodus	13	0

^a Orchard designation with the letter indicating the county from which the orchard was planted and the number indicating a specific grower operation.

^b Total number of *E. amylovora* isolates. "No fire blight" indicates that an orchard did not develop fire blight to sample.

^c Number of SmR *E. amylovora* isolates collected from symptomatic trees for each year of the survey.

isolates of SmR *E. amylovora* were rarely found on these cultivars. It may be that RubyFrost, Gala, Gingergold, SnapDragon, and Honeycrisp apple are rather susceptible to fire blight and growers with these cultivars should take additional care with fire blight management practices. It should be noted that all plantings of RubyFrost and SnapDragon were no more than 1 year old. Hence, apparent sensitivity of RubyFrost and SnapDragon may have been masked by overly vigorous production in the establishment years.

Aside from cultivar trends, the majority of the SmR *E. amylovora* isolates were recovered shoot blight samples. Shoot blight samples made up the majority of fire blight samples observed and collected by the survey team because blossom blight and rootstock blight were rarely observed by comparison. Shoot blight samples were all collected in late June through July (data not shown), when most of the streptomycin use had ceased. Shoot blight is typically initiated either by internal migration of *E. amylovora* to the growing shoot tips or by trauma to growing shoots, which become infected by *E. amylovora* spread from oozing cankers and infected flower clusters (van der Zwet et al. 2012). Hence, SmR *E. amylovora* isolates collected from shoot blight samples may not have emerged from local streptomycin control failures or streptomycin overuse during bloom. Alternatively, SmR *E. amylovora* found in shoot blight samples may have resulted from the limited streptomycin applications often made to protect against fire blight during storms that occur within the period of shoot elongation. Although data from the current study cannot prove that applications of streptomycin for shoot blight management lead to the development of streptomycin resistance, it cannot be discounted because several producers with SmR *E. amylovora* isolated from blighted shoots often reported using streptomycin after bloom for shoot blight management.

Of the SmR *E. amylovora* isolates recovered, a total of 32 contained the *strA/strB* gene pair located on the Tn5393 transposon on pEA29. This *strA/strB* gene pair itself has been shown to confer a streptomycin resistance in several bacterial species commonly found on the surfaces of apple fruit (Burr et al. 1988, 1993). The *strA/strB* gene pair has been reported to confer resistance to SmR isolates of *E. amylovora* from Michigan and California at concentrations of 100 to 200 µg/ml in vitro (Chiou and Jones 1991; Chiou and Jones 1993; Palmer et al. 1997; Russo et al. 2008). In agreement with the previous reports, all 34 SmR isolates displayed resistance to streptomycin at a minimum of 100 µg/ml in vitro. This level of resistance is particularly relevant for the management of fire blight given that agricultural applications of streptomycin are applied at a target rate of 100 µg/ml (100 ppm). Hence, any grower operations with these isolates would not be able to fully manage blossom blight using applications of streptomycin and would need to rely on kasugamycin, copper, or other biological controls.

Aside from the implications regarding the level of resistance, the presence of the *strA/strB* gene pair in the Tn5393 transposon in *E. amylovora* may shed some light on the origin of streptomycin resistance. This gene pair transposon combination has only been found on conjugative (pEA34) and nonconjugative (pEA29) plasmids in isolates from Michigan and New York (Chiou and Jones 1993; McManus and Jones 1994; Russo et al. 2008). The *strA/strB* gene pair has been shown to confer resistance in *E. amylovora* in California but the gene pair was on plasmid RSF1010 (Palmer et al. 1997). In the current study, all 32 isolates had the *strA/strB* gene pair in Tn5393 on pEA29. The isolates also belong to the lineage from Michigan with the 17,527-nucleotide position insertion site for Tn5393 on pEA29 (McGhee et al. 2011). Because the gene pair transposon combination resides on a plasmid that has shown to be nontransmissible between strains of *E. amylovora*, the isolates from the current study must be physically dispersed to new locations (McGhee and Jones 2000). Hence, these 32 SmR *E. amylovora* isolates are likely to have been transported to or spread among locations in western New York instead of local emergence due to the ingress of resistance plasmids from local epiphytic bacterial species with streptomycin resistance. Local physical distribution of isolates would further explain the lack of SmR *E. amylovora* in apple operations in eastern New York. Indeed, half of the SmR *E. amylovora* isolates

with the *strA/strB* gene pair were obtained from orchards in Wayne County, within 160 km of the location where the SmR *E. amylovora* isolates were discovered in 2002 (Russo et al. 2008). Planting material is rarely shared outside the neighboring production operations

and statewide dispersal by storms is unlikely. The localization of SmR isolates in western New York raises questions about the success of the eradication efforts in 2002 and the possibility of additional acquisitions of trees from southwest Michigan in 2002, as described by

Fig. 1. New York State counties with orchards from which streptomycin-resistant (SmR) isolates of *Erwinia amylovora* were identified in 2011 to 2014. **A**, Counties with 7 or more, 3 to 9, or 1 to 2 orchards with SmR *E. amylovora* are shown in dark gray, medium gray, or light gray, respectively. Counties with star symbols had orchards with fire blight outbreaks and were surveyed but did not have SmR *E. amylovora*. **B**, Close-up of the five counties displaying the approximate location of the orchard designated by a letter for the county and numerical identifier.

Russo et al. (2008). Indeed, direct reports to the authors from producers in 2011 suggest that others may have participated in the acquisition of such trees in 2002.

Interestingly, isolates 436 and 439, collected from the same orchard, had a slightly larger band for the pEA29 PCR amplicon with primer AJ75 and AJ76 (data not shown). Upon sequencing the product along with one of typical amplicon size, the pEA29 sequence revealed a variant, which was identical to isolate Ea356 (GenBank accession HF560643) isolated from *Cotoneaster* from Germany in 1979 (Zhao 2014). Although the presence of the *strA/strB* gene pair in Tn5393 on pEA29 is believed to have a Michigan origin (McGhee et al. 2011; Russo et al. 2008), the presence of Tn5393 on a sequence variant of pEA29 from Europe suggests the possibility of another transposition. Such a discovery warrants further investigation to better determine the origin and movement of these 32 isolates.

Although the 32 SmR *E. amylovora* isolates from this study containing the *strA/strB* gene pair in the Tn5393 transposon on pEA29 are consistent with a Michigan or western New York origin, the two isolates with the K43R *rpsL* mutation from this study may suggest additional introductions or spontaneous development of resistance within New York orchards. *E. amylovora* isolates with the K43R *rpsL* mutation are most commonly found in California and Washington (Chiou and Jones 1991; Loper et al. 1991; Moller et al. 1981; Schroth et al. 1979;). Although isolates with the K43R *rpsL* have been found in apple production operations in the eastern United States, they are rarely recovered (McManus et al. 2002). Apple production operations in western New York do order trees from nurseries in Washington State, but the trees from which isolates 306b and 189a were isolated were not obtained from the western United States and could represent one of these rare recoveries. The owners of the orchards from which these isolates were recovered reported fairly standard streptomycin use practices to the authors. Hence, the origin of isolates with the K43R *rpsL* mutation is still perplexing.

Given the possibility of multiple introductions and the presence of different resistant determinants, a more in-depth genetic analysis should be conducted to determine the origin of New York isolates and to investigate the movement of isolates throughout the state. Despite the information presented in this study, there is inherent difficulty in comparing these current isolates arising from the extremely limited diversity in the genome of *E. amylovora* (Sebaihia et al. 2010; Smits et al. 2010). Traditional diversity studies involving techniques such as random amplified polymorphic DNA, pulse-field gel electrophoresis, variable number of tandem repeats analyses, and ribotyping have shown difficulties in distinguishing strains (Jock et al. 2002; Kim and Geider 1999; McGhee and Sundin 2012; Momol et al. 1997). To investigate movement of isolates on a local and regional scale, a method that allows a high degree of differentiation must be employed. Clustered

regularly interspaced short palindromic repeats (CRISPR) spacer analysis has recently been of interest in exploring strain diversity and tracking in several human-pathogenic bacteria, including *Salmonella enterica*, *Escherichia coli*, and *Yersinia pestis* (Almendros et al. 2014; Barros et al. 2014; Shariat et al. 2013). This method has also been used to explore diversity in *Erwinia amylovora* and was hypothesized to allow differentiation of isolates on a regional scale (McGhee and Sundin 2012). We are currently constructing CRISPR spacer profiles for New York SmR and SmS *E. amylovora* isolates to investigate diversity and strain tracking.

In summary, streptomycin resistance is an emerging threat to the New York apple industry. A single isolated event in 2002 has become a greater issue over the past decade. Currently, SmR *E. amylovora* isolates are present throughout three of the six major apple-growing counties of New York. Although kasugamycin presents a viable antibiotic alternative to streptomycin for control of fire blight, it does not offer the same cost effectiveness. Hence, streptomycin is still widely used and resistance to streptomycin still poses a serious threat to the New York apple industry. To make matters worse, the most popular and highest-value apple cultivars in the state are highly susceptible to *E. amylovora*. It is imperative that we continue to improve our understanding of this new threat in order to generate appropriate responses to mitigate the spread of streptomycin resistance in New York and prevent further spread of SmR *E. amylovora* to additional apple-growing regions of the United States.

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Fig. 2. Amplification of the Tn5393 insertion site in pEA29 at nucleotide position 17,527 in streptomycin-resistant (SmR) *Erwinia amylovora* from western New York using primers AJ507 and AJ339 from McGhee et al. (2011). Shown are agarose gels with the expected the 397-bp amplicon for the AJ507 and AJ339 primer pair spanning the 3' end of Tn5393 at nucleotide position 17,527 to nucleotide position 17,922 in pEA29. Both gels have Quick-Load 100-bp DNA Ladder (New England Bio-Labs) to denote amplicon size. Lanes in the gel on the left are loaded with amplicons of SmR *E. amylovora* isolates collected from western New York in 2011 and 2012, while lanes in the right gel are loaded with amplicons of SmR *E. amylovora* isolates collected from western New York in 2012 and the two SmR-positive control isolates DM1 and MI5-1 (McGhee et al. 2011). Isolate MI5-1 has a Tn5393 insertion at position 1,515 on pEA29, and does not make the expected amplicon for primers AJ507 and AJ339.

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