Genetic Analysis of Streptomycin-Resistant (Sm^R) Strains of *Erwinia amylovora* Suggests that Dissemination of Two Genotypes Is Responsible for the Current Distribution of Sm^R *E. amylovora* in Michigan

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ABSTRACT

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Streptomycin-resistant (Sm^R) strains of the fire blight pathogen *Erwinia amylovora* were first isolated in southwest Michigan in 1991. Since that time, resistant strains have progressed northward to other apple-producing regions in the state. A total of 98.7% of Sm^R strains isolated between 2003 and 2009 in Michigan harbored the *strA-strB* genes on transposon Tn5393. *strA* and *strB* encode phosphotransferase enzymes that modify streptomycin to a nonbactericidal form. Mutational resistance to streptomycin, caused by a point mutation-mediated target-site alteration of the ribosomal S12 protein, occurred in 1.3% of *E. amylovora* strains from Michigan. Tn5393 was originally introduced to *E. amylovora* on the plasmid pEa34; thus, the first Sm^R strains isolated contained both pEa34 and the ubiquitous nonconjugative plasmid pEA29. More recently, we have observed Sm^R strains in which Tn5393 is present

on pEA29, suggesting that the transposon has moved via transposition from pEa34 to pEA29. Almost all of the strains containing Tn5393 on pEA29 had lost pEa34. Of 210 pEA29::Tn5393 plasmids examined, the transposon was inserted at either nucleotide position 1,515 or 17,527. Both of these positions were in noncoding regions of pEA29. Comparative sequencing of the housekeeping genes *groEL* and potentially variable sequences on pEA29 was done in an attempt to genetically distinguish Sm^R strains from streptomycin-sensitive (Sm^S) strains isolated in Michigan. Only 1 nucleotide difference within the total 2,660 bp sequenced from each strain was observed in 2 of 29 strains; multiple sequence differences were observed between the Michigan strains and *E. amylovora* control strains isolated in the western United States or from *Rubus* spp. Alterations in virulence observable using an immature pear fruit assay were detected in three of eight Sm^R strains examined. Our current genetic data indicate that only two Sm^R strain genotypes (strains containing pEA29::Tn5393 with Tn5393 inserted at either nucleotide position 1,515 or 17,527 on the plasmid) are responsible for the dissemination of Tn5393-encoded streptomycin resistance in Michigan, and that the Sm^R and Sm^S strains in Michigan compose a homogenous group.

Fire blight of apple, pear, and other plant species in the family *Rosaceae* is caused by the bacterium *Erwinia amylovora*, an organism that is capable of infecting flowers, fruit, vegetative shoots, woody tissues, and rootstock crowns. There are several distinct phases of the disease, including blossom blight, shoot blight, and rootstock blight (28). The diversity of host tissues combined with the limited number of management tools available to control the disease has made it difficult to stop or slow the progress of fire blight epidemics. Also, the increased planting costs of high-density apple blocks combined with the extreme fire blight susceptibility of many new scion cultivars and some apple rootstocks has resulted in significant financial losses for apple growers due to fire blight.

Reducing pathogen populations on flowers is critical to the control of the blossom blight phase of the disease and also in reducing inoculum levels for the shoot blight phase of the disease. The antibiotic streptomycin is the most effective bactericide commercially available in the United States for reducing the incidence of blossom blight. Streptomycin has been a mainstay for fire blight management in the United States since the 1950s

(24), and was utilized heavily for several decades. Unfortunately, this reliance on streptomycin for fire blight control resulted in the development of streptomycin-resistant (Sm^R) strains of *E. amylovora* in California by 1971 (25) and in Washington and Oregon by 1972 (9). Sm^R *E. amylovora* strains are now commonly detected in the western United States and Canada, and have been isolated in Michigan and New York and in other countries, including Israel (7,16,17,33–35).

Three genetic mechanisms are known to confer streptomycin resistance in *E. amylovora*. Almost all Sm^R strains of *E. amylovora* isolated from the western United States and only a few strains from Michigan are spontaneous mutants that possess a chromosomal mutation resulting in the resistance phenotype (24). The chromosomal mutation confers a phenotype of high resistance with a minimal inhibitory concentration of streptomycin of 2,000 µg ml⁻¹ (8). These mutations are point mutations in the *rpsL* gene encoding ribosomal protein S12, the bacterial protein target of streptomycin. Three mutations are known, all affecting codon 43 of *rpsL* which is AAA, specifying lysine in wild-type streptomycin-sensitive (Sm^S) strains. In the majority of highly resistant strains recovered from orchards, lysine-43 (AAA) is changed to arginine (AGA); other mutations result in changes of codon 43 to asparagine (AAT or AAC) or threonine (ACA) (8).

The other two known mechanisms of streptomycin resistance in *E. amylovora* involve the acquisition of streptomycin resistance

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genes via horizontal gene transfer. In Michigan, Sm^R *E. amylovora* from only a few orchards encoded an *rpsL* mutation at codon 43 while the majority of strains harbored a Sm^R transposon (Tn5393) on the plasmid pEa34 that was presumably acquired via horizontal gene transfer from other orchard epiphytic bacteria (6). Tn5393 is a 6.7-kb transposable element (7) that includes the *strA-strB* gene pair, two linked genes encoding phosphotransferase enzymes that modify streptomycin to a nontoxic form. In California, three isolates of *E. amylovora* from a single apple orchard were found to carry an 8.7-kb plasmid that was similar to or identical with the known Sm^R plasmid RSF1010 (29). RSF1010 is a small plasmid with a broad distribution among enteric bacteria that also encodes the *strA-strB* gene pair (41).

The *strA-strB* genes are widely disseminated among gram-negative bacterial pathogens and commensals from humans as a consequence of streptomycin use in clinical medicine beginning in the mid-1950s (36,41). The *strA-strB* genes are most commonly found on small plasmids such as RSF1010 or encoded on larger plasmids and harbored within the Tn5393 transposon (41). Among plant pathogens, variants of Tn5393 (Tn5393b and Tn5393c) are present in Sm^R *Pseudomonas syringae* and *Xanthomonas campestris* isolated in the United States, Argentina, Japan, and Korea (13,38,40). The main difference between the Tn5393 element from *E. amylovora* and Tn5393b or Tn5393c is a result of the insertion of IS1133, an insertion sequence element that is required for efficient expression of the *strA-strB* genes in *E. amylovora* (7,40).

The presence of transferrable streptomycin resistance genes in plant pathogen populations is of concern because gene transfer events could result in the rapid dissemination of resistance within growing regions. Also, in many cases, the *strA-strB* genes and Tn5393 are even more widely distributed in nontarget bacteria within orchards (27,44), resulting in a ready supply of resistance genes to any remaining Sm^S pathogen strains. Tn5393 was thought to have been originally transferred to *E. amylovora* on pEa34, a conjugative Sm^R plasmid that probably originated in *Pantoea agglomerans*, a common orchard epiphyte (6). Characterization of other Sm^R *E. amylovora* from Michigan recovered in 1990 to 1993 revealed the movement of Tn5393 in some cases to the indigenous ubiquitous plasmid pEA29, and also to the chromosome (22). These observations parallel those in other plant pathogens such as *Pseudomonas syringae* pvs. *papulans* and *syringae*, in which Tn5393b is present on several indigenous plasmids of different sizes in different strains and thought to be mobile within genomes (4,42).

The development of antibiotic resistance in populations of bacterial pathogens of humans or animals typically involves the movement of specific resistance genes via horizontal transfer from resistant to sensitive individuals (10). The resistance genes are usually encoded on transposons or integrons that can also be associated with conjugative plasmids (12). In many cases, nontarget bacteria co-located in habitats containing the pathogen are the first organisms to acquire resistance genes following antibiotic selection (15). These nontarget bacteria then transfer the resistance genes to the pathogen, leading to the rapid, almost simultaneous evolution of bacterial antibiotic resistance in multiple locations. A scenario similar to that observed in human and animal pathogens has been observed with Tn5393-mediated transfer of streptomycin resistance in agricultural situations, in which nontarget bacteria inhabiting plant leaves and soil in orchard or nursery locations sprayed with streptomycin are implicated in the transfer of Tn5393 to the pathogens *E. amylovora* and *P. syringae* (7,27,44). In plant-pathogenic bacteria, conjugative plasmids are the most common source of antibiotic and copper resistance determinants and for the intraspecies spread of these determinants (24,37).

Prior to 2003, Sm^R *E. amylovora* strains in Michigan were only detected in orchards in southwest Michigan and in two isolated orchards in Newaygo County. However, the knowledge that

Tn5393 was present in saprophytic bacteria in Michigan apple orchards sprayed with streptomycin (7) raised the possibility that gene transfer to Sm^S *E. amylovora* strains could result in new outbreaks of Sm^R *E. amylovora* in other regions of Michigan. Furthermore, if new transfers of Tn5393 from nontarget bacteria to *E. amylovora* or among *E. amylovora* strains were selected following even one streptomycin application, that would preclude the use of the most effective blossom blight material within the state. In contrast, if the introduction of Tn5393 to Sm^S *E. amylovora* was a rare event, then it would be possible that streptomycin could still be used effectively in regions where Sm^S *E. amylovora* strains prevail, if use was limited to a small number of yearly applications.

Since the first isolation of Sm^R *E. amylovora* in southwest Michigan, Sm^R strains have not been detected in other apple-growing regions in the state that are geographically separated from known orchards containing Sm^R strains by land which is not planted with apple or pear (G. W. Sundin, unpublished data). Our more recent surveys of Michigan apple orchards, beginning in 2003, resulted in the detection of Sm^R *E. amylovora* strains in new growing regions. We hypothesized that the origin of these strains would be through either the novel development or acquisition of resistance or the movement of preexisting Sm^R strains. We also hypothesized that we could distinguish and group the existing Sm^R *E. amylovora* strains using genetic and virulence approaches and identify progenitor Sm^S strains in the *E. amylovora* population. Our objectives in this study were to determine the extent of distribution of Sm^R *E. amylovora* in Michigan, the origin of Sm^R strains detected in new growing regions, and whether Sm^R strains could be distinguished using genetic and virulence analyses.

MATERIALS AND METHODS

Bacterial strains, growth media, and oligonucleotide primers. The *E. amylovora* strains used in this study, and their relevant characteristics, are listed in Table 1. Bacteria were routinely cultured on Luria-Bertani medium (LB). Cycloheximide (50 µg ml⁻¹) was added to LB (LBc) to prevent fungal contamination for bacterial isolations from apple tissue and streptomycin (100 or 2,000 µg ml⁻¹) or oxytetracycline (25 µg ml⁻¹) was added to LB (LBsm and LBtc, respectively) for resistance screening. The oligonucleotide primers used in this study and their sequence are listed in Table 2.

Sampling for *E. amylovora* and determination of streptomycin resistance. Surveys for streptomycin resistance in Michigan commercial apple and pear orchards were conducted in 2003 to 2009. Previously, Sm^R isolates of *E. amylovora* were detected in orchards located in southwest Michigan (Berrien and Van Buren Counties), with resistance also detected in a few isolated orchards in Newaygo County (7). In our surveys, we sampled orchards in southwest Michigan that were located on the borders of or outside of the geographic "pocket" of orchards where resistance was detected before. In addition, we sampled orchards in other locations throughout central and western Michigan, including the Fruit Ridge growing area (Ionia, Kent, Montcalm, and Ottawa Counties) and in Oceana County.

In all, 5 to 10 apple or pear shoots showing fire blight symptoms (necrosis, wilting, and ooze production) were collected from each orchard and transported to the laboratory on ice. Infected succulent tissue (≈2 g) was dissected from each infected shoot, placed in 10 ml of 0.5× phosphate-buffered saline (PBS) solution on ice, and ground using a PT 10-35 polytron (Brinkmann, Westbury, NY) for 10 s. The blade was sterilized between processing each individual shoot. Homogenate was serially diluted in 0.5× PBS and appropriate dilutions were plated on LBc. Plates were incubated at 25°C for 48 h before 50 individual colonies with typical *E. amylovora* colony morphology were selected

(when available). The colonies were replica plated onto LB and LBsm, and the plates were scored for growth on the LBsm plates and the numbers recorded. Final identification of colony isolates was performed using identification primers (23) for *E. amylovora* targeting the ubiquitous plasmid pEA29. The Sm^R and Sm^S strains *E. amylovora* CA11 and Ea110, respectively, were used as controls.

Genetic analysis of Sm^R *E. amylovora* isolates. The genetic mechanism of streptomycin resistance was determined by plating each isolate onto LB medium amended with streptomycin at either 100 or 2,000 µg ml⁻¹. Isolates capable of confluent growth on LB amended with streptomycin at 2,000 µg ml⁻¹ have been previously shown to contain a chromosomal mutation in the *rpsL* gene that confers high-level resistance (8). Isolates capable of growth on LB amended with streptomycin at 100 µg ml⁻¹ but not on LB amended with streptomycin at 2,000 µg ml⁻¹ have been previously shown to harbor the *strA-strB* streptomycin resistance genes that are most commonly located within the transposable element Tn5393 (7). For *E. amylovora* isolates suspected of

carrying Tn5393, the genomic location of the transposon was first assessed by sequencing using oligonucleotide primer AJ507 (Table 2), a sequencing primer directed outward from the transposon. Because the complete sequence of pEA29 is known (20), the determination of transposon location was straightforward. Sequencing was conducted at the Michigan State University Genome Technology Research Facility using ABI dye-terminator chemistry and an ABI 3730 genetic analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was conducted using BLASTN version 2.2.18 at the National Center of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/blast>).

For screening of multiple isolates suspected of carrying Tn5393, a series of polymerase chain reactions (PCRs) were used to determine the location and orientation of the transposon within the *E. amylovora* genome. Sequences of the oligonucleotide primers used in this study are shown in Table 2. Primer pairs AJ530 and AJ531 or Is1133-F and Is1133-R were used to confirm the presence of Tn5393. Primer pair AJ509 and AJ528 was used

TABLE 1. *Erwinia amylovora* strains used in this study and their relevant characteristics

<i>E. amylovora</i>	Streptomycin phenotype ^a	Origin, source, year isolated ^b	Reference
Ea110	S	Michigan, apple, 1977	32
EL01	S	East Lansing, Michigan, 1994	7
K2, RB07, MK1, GH9, DR5, RL3, RRP12, L14	S	Michigan, apple, 1995	22
DP11	S	Michigan, pear, 1995	22
CA11	R	SW Michigan, crabapple, 1991	7
DM1	R	SW Michigan, apple, 1994	22
S5	R	SW Michigan, apple, 1994	7
BCN20	R	SW Michigan, apple, 1995	22
HS10	R	SW Michigan, apple, 1995	22
MI5-1	R	SW Michigan, apple, 2002	This study
SWWC75M	R	SW Michigan, apple, 2003	This study
GR4C-1	R	Fruit Ridge, Michigan, apple, 2003	This study
GR5B-1	R	Fruit Ridge, Michigan, apple, 2004	This study
CV1E, R5F, SCH2F	R	Fruit Ridge, Michigan, apple, 2005	This study
SB1-9	R	Oceana Co. Michigan, pear, 2006	This study
KR	R	Fruit Ridge, Michigan, apple, 2007	This study
MA-1, MC-5, W4, BBA-8, RM5	R	SW Michigan, apple, 2007	This study
Ea88	R	Washington, pear, 1995	22
CA1R	S	California, apple, 1995	22
Ea273	S	New York, apple	3
MR1	S	Michigan, <i>Rubus</i> sp., 1995	22

^a S = sensitive, minimum inhibitory concentration (MIC) < 100 µg ml⁻¹; R = resistant, MIC > 100 µg ml⁻¹. Streptomycin phenotypes and MICs for some strains have been reported previously.

^b SW = southwest Michigan region, including Berrien, Cass, and Van Buren Counties, Fruit Ridge = Grand Rapids Fruit Ridge region, including Ionia, Kent, Montcalm, Newaygo, and Ottawa Counties. Year of isolation is listed, if known.

TABLE 2. Oligonucleotide primers used in this study and their relevant characteristics

Primer	Sequence (5'–3')	Target	Source or reference
AJ75	CGCATTCACGGCTTCGCAGAT	<i>Erwinia amylovora</i> ID primer, targets pEA29	22
AJ76	AACCGCCAGGATAGTCGCATA	<i>E. amylovora</i> ID primer, targets pEA29	22
AJ44	GAAAGCAAAAGCTAAAACAGGAGCT	<i>E. amylovora rpsL</i>	8
AJ45	GGAATTCGGCCTTACTTAACGGAGA	<i>E. amylovora rpsL</i>	8
AJ507	GATCAAGCAGGTGCGACAGAC	Tn5393, 3' end (forward primer)	This study
AJ339	TGACGACTGAAATCCGCAAAAAC	Base pair 17 858 on pEA29 (reverse primer)	This study
Tn5393E	CCATGAAGCTGCGCGAGGTC	Tn5393, 5' end (reverse primer)	This study
AJ1717	CCCGATATGTGATGCTGATC	Base pair 27063 on pEA29, (forward primer)	This study
IS1133-F	GCGTGATGCAGTTCGCATAGC	Insertion sequence IS1133 on Tn5393	This study
IS1133-R	CATACGCGGCCTACCATAGCT	Insertion sequence IS1133 on Tn5393	This study
AJ530	GGAAGTGCCTGGGCTACA	<i>mpA</i> gene on Tn5393	This study
AJ531	GCTAGATCGCGTTGCTCCTCT	<i>mpA</i> gene on Tn5393	This study
pEa34-F	CGTTTATCGGATGCCTTTGAGTC	pEa34	This study
pEa34-R	AGTCGTGATTCCATTTTATTTT	pEa34	This study
groEL-A	GAAAGTKGCCTCTAAAGCGAAYGA	<i>groEL</i> gene, partial	20
groEL-B	GCMACRCCACCAGCAACC	<i>groEL</i> gene, partial	20
msrA-F	GCATCCGGCCCCGTGTCTTC	<i>msrA</i> gene on pEA29	This study
msrA-R	CTTGTTCTGTCCATTCTGTGTTA	<i>msrA</i> gene on pEA29	This study
AJ486	AACCGACTAAAACGATTGACCT	<i>msrA</i> gene on pEA29	19
AJ301	ATTTACAGCCCGTCATTG	parA pseudogene on pEA29	This study
gp-F	CAGGGATGATTTTCGCTTACTTAG	bp 25,920 on pEA29	This study
gp-R	TCACTCCACATAAGAAAACCTGCTA	bp 27,876 on pEA29	This study

to screen for the presence or absence of plasmid pEA34, a conjugative plasmid previously found in *E. amylovora* and thought to be the original source of the *strA-strB* genes. *E. amylovora* strain Ca11 was used as a positive control. Primer pairs Tn5393E and AJ1717 (pEA29 position 1,515) and AJ507 and AJ339 (pEA29 position 17,527) were used to screen for integration of the transposon into known insertion sites on plasmid pEA29 (base pairs 1,515 and 17,527, respectively). For PCR experiments characterizing the insertion site on plasmid pEA29, *E. amylovora* strains MI5-1 and DM1 were used as positive controls. All PCR mixtures (50 μ l) consisted of 1 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3), 1.5 mM MgCl₂, 0.1% Tween 20, 200- μ M concentrations of each deoxynucleotide triphosphate, 20 pM each primer, 2.5 U

of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and either 0.1 to 100 ng of template DNA or 10 μ l of bacterial culture diluted to an optical density at 600 nm of 0.1 to 0.5. Cycling parameters were 94°C for 4 min; followed by 40 cycles of 94°C for 30 s, annealing temperatures of 56°C for 1 min (pEA29 ID primers [21] and Tn5393 at 17,527 on pEA29), 60°C for 30 s (Tn5393 at 1,515 on pEA29), 55°C for 30 s (Tn5393 ID primer sets), and 54°C for 30 s (pEA34 ID); followed by a 72°C extension for 1 to 5 min and a final extension time of 7 min. All products were visualized on a 1% agarose gel in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer stained with ethidium bromide.

Comparative sequencing analysis of *E. amylovora* strains. Three genomic locations for comparative sequence analysis were

Fig. 1. Genetic map of the ubiquitous plasmid pEA29 of *Erwinia amylovora* indicating the known insertion points of Tn5393 at nucleotide (nt) 1,515 and 17,527 in Michigan strains. A genetic map of Tn5393 is also shown, indicating genes involved in transposition (*tnpA*, *tnpR*) and streptomycin resistance (*strA*, *strB*). The location of two sequences (*msrA* gene and the *par* pseudogene) on pEA29 that were analyzed in comparative sequencing experiments is also shown.

TABLE 3. Sampling analysis of streptomycin-resistant (Sm^R) *Erwinia amylovora* strains in different growing regions of Michigan (2003 to 2009), with strains distinguished by level of resistance in moderately resistant (MR) and highly resistant (HR) strains

Year	No. of orchards	Region ^b	No. of strikes sampled	No. of <i>E. amylovora</i> strains ^a			
				Sm ^R	Sm ^S	MR ^c	HR
2003	23	SW	117	27	75	27	0
	6	FR	24	9	15	9	0
2004	5	SW	46	21	17	14	7
	7	FR	76	55	8	53	2
	1	WC	12	0	11	0	0
2005	17	FR	96	40	27	36 ^d	0
2006	2	FR	20	5	15	1 ^d	0
	10	WC	70	29	35	19 ^d	0
2007	26	SW	155	29	87	29	0
	3	FR	16	14	0	5 ^d	0
	10	WC	60	0	52	0	0
2009	16	SW	94	22	79	22	0
	2	FR	10	10	0	10	0

^a Sm^S = streptomycin susceptible.
^b Regions sampled: southwest (SW) = Berrien, Cass, and Van Buren Counties; Fruit Ridge (FR) = Ionia, Kent, Montcalm, Newaygo, and Ottawa Counties; and west central (WC) = Oceana County.
^c Minimal inhibitory concentration of streptomycin for MR strains is 1,000 to 2,000 μ g ml⁻¹ and for HR strains is >2,000 μ g ml⁻¹. All MR strains contained Tn5393 and all HR strains encoded chromosomal mutations in the *rpsL* gene.
^d Not all Sm^R strains were analyzed for MR or HR phenotype.

chosen as follows. The *groEL* sequence encodes a housekeeping gene commonly used in bacterial comparisons and has been used in comparisons of *E. amylovora* strains with the related species *E. pyrifoliae* (18,20). Because the *E. amylovora* species is known to be relatively homogeneous (26,31), we chose two additional locations on pEA29 for sequence analysis that we thought could potentially be more variable. One location was within the *msrA* gene and the other encompassed a region containing a *parA* pseudogene (Fig. 1). These regions were chosen because we hypothesized that plasmid-encoded genes may be more variable than chromosomal genes, and that a putative pseudogene would not be under selection pressure and could possibly accumulate mutations at a faster rate than other coding sequences.

Partial *groEL* sequence were amplified from the *E. amylovora* chromosome using primers *groEL*-A and *groEL*-B (Table 2) as previously described (20). The *msrA* gene from pEA29 was amplified using primer set *msrA*-F and *msrA*-R. A region on pEA29 located between base pairs 27,876 and 25,920 that contained a *parA* pseudogene was amplified using primers *gp*-F and *gp*-R. Cycling parameters for *msrA* and the *parA* pseudogene region of plasmid pEA29 were as follows: 94°C for 4 min; followed by 40 cycles of 94°C for 30 s and annealing temperatures of 50°C (*msrA*) or 54°C (*parA* pseudogene) for 30 s; followed by a 72°C extension for 1 min 30 s and a final extension time of 7 min. Reaction mixes were identical to those listed above. In all PCR reactions, *E. amylovora* Ea273 was used as a positive control. Resulting PCR products were visualized on a 1% agarose gel in 0.5× TBE buffer stained with ethidium bromide.

PCR products were purified from reaction mixes using the QIAquick PCR purification kit (Qiagen Sciences, Valencia, CA) prior to sequencing, as previously described. Confirmation of sequence differences was made by visually examining chromatograms using Finch TV version 1.4.0 (Geospiza, Seattle). Primers *groEL*-A and *groEL*-B were used to sequence the *groEL* PCR product. Primers AJ486 and *msrA*-F were used to sequence the *msrA* gene, and primers *gp*-F and AJ301 were used to sequence the *parA* pseudogene PCR product. Alignment and comparison of sequences was performed using the Lasergene (version 7.2.0) suite of programs (DNASTAR, Inc., Madison, WI).

Comparative virulence assays. Experiments were conducted to compare the virulence of *E. amylovora* strains varying in resistance or sensitivity to streptomycin and in mode of resistance. Virulence assays using 10 replicate immature pear fruit per strain per experiment were performed essentially as described previously (2), except a 5-μl inoculum dose (1×10^8 CFU/ml) was used. Following inoculation, pear fruit were incubated in a humidified chamber for 5 days at 28°C. Daily measurements of necrotic lesion size were taken at days 1 to 5 after inoculation. Two replicate experiments were conducted for each strain. Statistical analyses of lesion size data were done using a one-way analysis of variance; data values for each day were analyzed separately. Mean separation ($P < 0.05$) was accomplished using Fisher's protected least significant difference test. We used the M-STAT-C 4.0 statistical research program (<https://www.msu.edu/~freed/mstatc.htm>) for all statistical analyses of data.

Fig. 2. Locations of orchards in the counties of Berrien, Cass, and van Buren in southwest Michigan in which streptomycin-resistant (Sm^R) (●) or streptomycin-sensitive (Sm^S) (□) *E. amylovora* strains were detected in 2003 to 2009. The geographic “pocket” of orchards harboring Sm^R *E. amylovora* is indicated as orchards within the drawn half-circle. Two orchards outside of the pocket in which Sm^R *E. amylovora* strains were detected in 2009 are indicated by the arrow. Eight orchards resampled in 2009 in which only Sm^S *E. amylovora* strains have been isolated over multiple years are designated with ◇. In addition, the 12 orchards within the pocket in which Sm^R *E. amylovora* strains were detected prior to 2003 are shown (▲) for historical context.

RESULTS

Detection of Sm^R *E. amylovora* in new apple-growing regions in Michigan. Prior to 2003, Sm^R *E. amylovora* strains had only been isolated in southwest Michigan (Berrien and Van Buren Counties) and in two orchards in Newaygo County that were geographically isolated from other apple orchards (6). The surveys we conducted from 2003 to 2009 resulted in the processing of 796 total fire blight strikes and analysis of 682 *E. amylovora* isolates, confirmed by PCR analysis using the pEA29 identification primers. Surveys in southwest Michigan, including Cass County, resulted in the detection of Sm^R *E. amylovora* in 6/23, 1/5, 11/35, and 2/16 orchards in 2003, 2004, 2007, and 2009 respectively (Table 3). In total, 53 orchards from this region were sampled over the course of the survey, because some orchards were resampled in subsequent years. Prior to our surveys, Sm^R *E. amylovora* isolates were only recovered from orchards located within a relatively small contiguous area. This geographic “pocket” contained 12 orchards from which Sm^R *E. amylovora* were detected prior to 2003 (Fig. 2, filled triangles). Our sampling efforts were focused on orchards located on the borders of this geographic “pocket,” with a few additional orchards located within the pocket. Sm^R *E. amylovora* strains were newly discovered in nine orchards sampled in 2003 to 2007 (Fig. 2, filled circles). These results indicated a slight increase in the geographic range of orchards harboring Sm^R *E. amylovora*. An extensive sampling of orchards located outside of the pocket in 2003 to 2007 resulted in the recovery of only Sm^S *E. amylovora* isolates (Fig. 2, open squares). We only noted one orchard in this survey in which multiple samplings over time yielded variable results (e.g., we recovered only Sm^S *E. amylovora* in a particular sampling year and then recovered only Sm^R *E. amylovora* in a subsequent

sampling year). Our survey in 2009 revealed two new orchards harboring Sm^R *E. amylovora* (Fig. 2, denoted by arrow) and confirmed the detection of only Sm^S *E. amylovora* in eight resampled orchards (Fig. 2, open diamonds).

We sampled a total of 28 orchards in the Fruit Ridge apple growing area of Michigan in Kent County and adjacent Ionia, Montcalm, and Ottawa Counties and identified 21 orchard sites containing Sm^R *E. amylovora* and 7 orchards containing Sm^S *E. amylovora* (Table 3). The location of the first orchards found to contain Sm^R *E. amylovora* in 2003 and 2004 was in the southern area of the growing region. Resampling of two orchards located in this region that contained only Sm^S isolates in 2003 and 2004 revealed the presence of Sm^R *E. amylovora* in 2005 (Fig. 3, filled diamonds). However, there still remained additional orchards in the northern Fruit Ridge that contained only Sm^S isolates (Fig. 3, open squares). In 2006 to 2009, the survey was expanded, resulting in the detection of Sm^R *E. amylovora* in six orchards

TABLE 4. Genetic location of Tn5393 within pEA29 in *Erwinia amylovora* isolates differentiated by apple-growing region in Michigan

Region ^a	Sm ^{Rb}	No. of isolates with the indicated Tn5393 insertion in pEA29		pEa34 & pEA29 ^c
		Base pair 1,515	Base pair 17,527	
SW	88	45	43	0
FR	103	101	2	13
WC	19	0	19	0

^a Michigan regions: southwest (SW): Berrien, Cass, and Van Buren Counties; Fruit Ridge (FR): Ionia, Kent, Montcalm, Newaygo, and Ottawa Counties; and west central (WC): Oceana County.

^b Number of streptomycin-resistant (Sm^R) isolates.

^c Number of isolates with Tn5393 on pEa34 and pEA29.

Fig. 3. Locations of orchards in the Fruit Ridge growing area in the counties of Ionia, Kent, Montcalm, Newaygo, and Ottawa and also in Oceana County in west-central Michigan in which streptomycin-resistant (Sm^R) (●) or streptomycin-sensitive (Sm^S) (□) *Erwinia amylovora* strains were detected in 2003 to 2009. Orchards with a variable result (e.g., Sm^S strains in one year and Sm^R strains in a subsequent year) are designated with ♦.

sampled in Ionia, Kent, Montcalm, and Newaygo Counties (Fig. 3, filled circles).

Sm^R *E. amylovora* isolates were detected in 3 of 12 orchards located in west-central Michigan in Oceana County in 2006 (Fig. 3). Further sampling, including some resampled orchards in this county in 2007, resulted in the isolation of only Sm^S *E. amylovora* isolates (Table 3). In addition to the work with streptomycin, the 571 *E. amylovora* isolates recovered in 2003 to 2007 were screened on LBtc and all were determined to be sensitive to oxytetracycline (data not shown).

Genetic mechanisms of streptomycin resistance in *E. amylovora* isolates. Of 682 *E. amylovora* isolates analyzed, 261 were resistant to streptomycin and, in 9 of them (3.4% of the Sm^R isolates), resistance was conferred by a mutation in the chromosomal gene *rpsL*. Of the remaining 252 Sm^R isolates, 27 were not examined further. All of the remaining 225 Sm^R isolates that were examined were found to harbor Tn5393, based on a positive band generated from PCR reactions using primers (IS1133-F and IS1133-R) targeting IS1133 (data not shown).

The genomic location of Tn5393 was initially determined by sequencing outward from the transposon. We used the draft genome sequence of *E. amylovora* Ea273 (<http://www.sanger.ac.uk/>

Projects/E_amylovora/) and the complete sequence of pEA29 (19) to map the location of the Tn5393 insertions in 36 isolates recovered in 2003. Among the 36 isolates examined, only 2 unique insertions were identified at base pairs 1,515 and 17,527 of the plasmid (Fig. 1). Both of these insertions were in noncoding regions of the plasmid. From this point forward, we designed primer sets targeting the two insertion points (AJ507 and AJ339; AJ1717 and Tn5393E) which were used to screen isolates recovered in 2004 to 2009. In total, 210 isolates were screened for the location of Tn5393 on pEA29, and we determined that 146 contained an insertion of Tn5393 at base pair 1,515 and 64 contained an insertion at base pair 17,527. When we matched orchard location of isolates with the Tn5393 site of insertion data, we found that isolates with the base pair 1,515 insertion site were predominant among the Fruit Ridge isolates, and that 19 of the Sm^R isolates from Oceana County contained Tn5393 inserted at base pair 17,527. In contrast, isolates with either insertion site were present in the southwest Michigan samples (Table 4).

Comparative genetic analysis of *E. amylovora* strains. A comparative sequencing approach was used to determine whether we could genetically distinguish Sm^R *E. amylovora* strains from

TABLE 5. Comparative analysis of partial sequences of *groEL*, *msrA*, and a *parA* pseudogene from streptomycin-resistant and -sensitive (Sm^R and Sm^S, respectively) strains of *Erwinia amylovora*

Strain	Genomic location ^a	PCR ^b	No. of nucleotide differences from Ea273		
			<i>groEL</i> ^c	<i>msrA</i>	parA pseudogene ^d
Sm ^R , containing Tn5393					
CA11	pEa34	1	0	0	0
HS10	pEa34	1	0	0	0
GR5B1	pEa34 and pEA29, bp 1,515	...	0	0	0
R5F	pEa34 and pEA29, bp 1,515	...	0	0	0
CV1E	pEA29, bp 1,515	...	0	0	0
Gr4C-1	pEA29, bp 1,515	...	0	0	0
MI5-1	pEA29, bp 1,515	...	0	0	0
MA-1	pEA29, bp 1,515	...	0	0	0
MC-5	pEA29, bp 1,515	...	0	0	0
KR	pEA29, bp 1,515	...	0	0	Δ1 nt at 26,833
SCH2f	pEA29, bp 1,515	...	0	0	0
SWWC75M	pEA29, bp 17,527	...	0	0	0
DM1	pEA29, bp 17,527	1	0	0	0
RM5	pEA29, bp 17,527	...	0	0	0
SB1-9	pEA29, bp 17,527	...	0	0	0
W4	pEA29, bp 17,527	...	0	0	0
BBA-8	pEA29, bp 17,527	...	0	0	0
Sm ^{Se}					
EL01	...	1	0	0	0
K2	...	1	0	0	Δ1 nt at 26,833
RB07	...	1	0	0	0
MK1	...	1	0	0	0
GH9	...	1	0	0	0
DR5	...	1	0	0	Δ1 nt at 26,833
RRP12	...	1	0	0	0
Lyon14	...	1	0	0	0
RL3	...	1	0	0	0
DP11	...	1	0	0	0
Ea110	...	0	0	0	
MR1	...	4	3	6	36
CA1R	...	3	2 ^f	0	11 ^g
Sm ^R , mutational					
Ea88	Mutation in <i>rpsL</i>	3	2 ^f	0	11 ^g
S5	Mutation in <i>rpsL</i>	1	0	0	0

^a Genomic location of transposon Tn5393 carrying the streptomycin resistance genes *strA-strB*. In cases where the transposon was inserted into plasmid pEA29, location of the insertion (bp = base pair) is indicated. In strains R5F and GR5B1, copies of Tn5393 are located on both pEA29 and on pEa34.

^b Polymerase chain reaction (PCR) ribotyping for some strains was previously reported (22).

^c Partial *groEL* gene was amplified from the *E. amylovora* chromosome using primers *groEL*-A and *groEL*-B. The PCR product was 772 bp in length.

^d The *parAB* pseudogene region was amplified from nucleotides (nt) 26,034 to 27,385 of plasmid pEA29 (GenBank accession no. NC_005706) using primers *gp*-F and *gp*-R. The region was 1,352 bp in length.

^e Sm^S = streptomycin susceptible.

^f Two nucleotide changes identified in the partial sequence of the *groEL* gene were identical in both CA1R and Ea88.

^g Eleven nucleotide changes found in the *E. amylovora* *parAB* pseudogene region in strains CA1R and Ea88 were identical and are located at nucleotides 26079, 26287, 26300, 26346, 26543, 26655, 26791, 26859, 27024, 27331, and 27176 on plasmid pEA29.

Sm^S strains or if we could identify an ancestral Sm^S genotype. If we could identify an ancestral Sm^S genotype, we could then examine characteristics related to its virulence and distribution which would allow us to make predictions on the future dissemination of the new Sm^R genotypes.

We sequenced a total of 2,660 bp from each of 33 *E. amylovora* strains. Of these, 11 were Sm^S strains isolated in Michigan during 1977 to 1995, and 18 were Sm^R strains isolated during 1991 to 2007, with Tn5393 located on pEa34, pEA29, or both plasmids (Table 5). Two Sm^R strains (isolated in Michigan and Washington) with *rpsL* chromosomal mutations and Sm^S *E. amylovora* strains from California and from *Rubus* spp. were included as controls. All sequences were compared with the draft genome sequence of *E. amylovora* Ea273. Among the Sm^R and Sm^S strains isolated in Michigan, we only detected two sequence differences. A deletion of 1 nucleotide was observed at position 26,833 of pEA29 in the *parA* pseudogene in the Sm^S strains DR5 and K2 and the Sm^R strain KR (Table 5). In contrast to the homogeneity observed among the Michigan apple strains, 45 nucleotide alterations, in total, were observed between MR1, the *Rubus* strain, and the others, and 13 nucleotide differences were observed between strains CA1R and Ea88, isolated in the western United States, and the strains isolated in Michigan (Table 5). Where the PCR ribotype of some of the strains was known from a previous study (22), ribotype 1 was shared among the Sm^R and Sm^S strains isolated in Michigan, whereas the *Rubus* strain MR1 and *E. amylovora* strains isolated in the western United States differed in ribotype (Table 5).

Comparative virulence assays. Virulence assays using immature pear fruit were conducted to determine whether there were any observable differences in virulence of Sm^R *E. amylovora* strains. If pEa34 was originally transferred into a lower virulence *E. amylovora* genetic background, for example, acquisition of streptomycin resistance may serve to reduce virulence in the Sm^R *E. amylovora* population. We detected very little difference in virulence among the Sm^S control strains Ea110 and EL01 and the Sm^R strains GR5B1 and W4 following replicated experiments using immature pear fruit (Fig. 4). Pear fruit infected with these strains exhibited typical increases in lesion size by 4 days after inoculation and large necrotic lesions with bacterial ooze production after 7 days (Fig. 4). In contrast, immature pear fruit inoculated with the Sm^R strains MI5-1, Ea88, and S5 exhibited either a lack of symptom development or a lesion that was significantly smaller than lesions initiated by strains Ea110, EL01, GR5B1, W4, and HS10 (Fig. 4). Strain HS10 was intermediate in virulence, with significant differences in lesion size from all of the other strains exhibited at days 6 and 7 following inoculation (Fig. 4).

DISCUSSION

Our surveys and genetic analyses of isolates in 2003 to 2009 have demonstrated that two Sm^R genotypes of *E. amylovora* are spreading between orchards and between apple-growing regions in Michigan. In southwest Michigan prior to 2009, streptomycin resistance was only detected within orchards located in a geographic pocket near to where the first Sm^R strains were isolated 12 to 18 years previously (Fig. 2). Outside of this pocket of orchards, all *E. amylovora* isolates from adjacent orchards had remained sensitive to streptomycin in subsequent samplings. It is thought that movement of Sm^R strains from these orchards into orchards closer to Lake Michigan is probably slowed by the prevailing westerly winds off of the lake (A. L. Jones, *personal communication*). In 2009, we did detect Sm^R *E. amylovora* strains in two orchards located outside of the geographic pocket, indicating that strain movement between orchards continues to occur. However, the observation that streptomycin resistance is not universally present in all orchards in this region, despite the

widespread regular usage of streptomycin by growers for blossom blight control, also suggests that new Sm^R strains are not readily arising in the *E. amylovora* population.

The discovery of Sm^R *E. amylovora* in orchards in the Fruit Ridge area (Kent, Ionia, Montcalm, and Ottawa Counties) and in Oceana County indicates that resistance has spread across a region where apple orchards are noncontiguous. Similarly, Sm^R *E. amylovora* were detected in orchards for the first time in New York, and the resistance mechanism was identical to that found in the Michigan strains (33). The most likely mechanism facilitating initial spread of these Sm^R strains to new areas of Michigan and to New York is on contaminated nursery stock originating from southwest Michigan that was planted in the other regions. Analyses in later years of this study showing increasing numbers of orchards harboring resistant strains in the northern areas of the Fruit Ridge and into adjacent counties is indicative of rapid orchard-to-orchard spread within this region. Because the first detection of Sm^R *E. amylovora* in the Fruit Ridge growing area occurred in orchards on the west side of this region, spread of these resistant strains between orchards may have been increased by prevailing winds.

Genetic analyses revealed that 97% of the Sm^R *E. amylovora* strains isolated in Michigan carried the transposon Tn5393, which was inserted within the indigenous plasmid pEA29. Surprisingly, only two transposon-insertion sites were identified among all of the strains screened. We hypothesize that these two identified Tn5393-insertion sites within pEA29 have resulted from only two independent transposition events of Tn5393 into pEA29. Alternatively, it is possible that Tn5393 preferentially transposes into a specific target sequence similar to the well-studied transposon Tn7 (30). If this were true, the two insertion sites of

Fig. 4. Necrotic lesion size in immature pear fruit following inoculation with selected streptomycin-sensitive and streptomycin-resistant *Erwinia amylovora* strains. Bars are color-coded per strain as shown. This experiment was performed over 7 days, with measurements taken daily in days 3 to 7 following inoculation. In all, 10 immature pear fruit were used for each strain in each of two experiments. Results shown (data means and standard error) are combined from two separate experiments. Letters associated with data points indicate the means within day are not significantly different if given the same letter ($P < 0.05$).

Tn5393 into pEA29 identified in this study could have resulted from multiple, independent transposition events. There are five sequenced insertion sites for Tn5393 available currently, including the two sites on pEA29 identified in this study, an additional insertion of Tn5393 into the *thiO* gene of pEA29 identified in a previous study (19), a chromosomal insertion in *P. syringae* pv. *syringae* B728a (11), and an insertion in the plasmid pPSR1 of *P. syringae* pv. *syringae* A2 (43). None of these target sequences are similar (data not shown), which suggests that the transposition landing point for Tn5393 is not sequence target specific. Because pEA29 is a nonconjugative plasmid and is ubiquitous in *E. amylovora* (14), pEA29 plasmid variants containing Tn5393 are unlikely to be horizontally disseminated among *E. amylovora* strains within orchard populations. Thus, we believe that specific tracking of strains can be accomplished by tracking the occurrence of pEA29 plasmid variants with known Tn5393-insertion sites.

From a microbial ecology standpoint, the acquisition of an Sm^R determinant by *E. amylovora* has paralleled the acquisition of antibiotic resistance in clinical bacteria where resistance is typically initially selected in nontarget bacteria, a resistance determinant is then transferred to the bacterial pathogen, and, finally, further genetic rearrangements occur to stabilize the resistance determinant in the new bacterial host (10,15). Although an extensive analysis of nontarget bacteria in apple orchards harboring Tn5393 and pEa34 has not been performed, it is thought that the source of pEa34 was *Pantoea agglomerans*, a common orchard epiphyte which is an enteric bacterium closely related to *E. amylovora* (6). Over an ≈18-year period following the acquisition of pEa34, Tn5393 has presumably moved to pEA29 via transposition and, in most cases, pEa34 has subsequently been lost from the *E. amylovora* genome (Table 4). Loss of pEa34 would reduce the amount of ancillary DNA in the genome which would be expected to enhance ecological fitness. Likewise, the location of Tn5393 in noncoding regions on the ubiquitous plasmid pEA29 in *E. amylovora* suggests that the streptomycin resistance phenotype will be stable in the two well-distributed strain types we recovered. This is similar to previous observations in *Pseudomonas syringae*, in which plasmids encoding resistance to either streptomycin or copper were stable without selection and conferred a positive effect on the ecological fitness of the organism (39).

The acquisition of a functional Tn5393 element capable of successfully expressing the *strA-strB* genes in enteric bacteria was a multistep process. Although Tn5393 is readily detected in various orchard bacteria from orchard environments where streptomycin has been used, the most common form of this transposon does not contain the insertion sequence IS1133 and, thus, the *strA-strB* genes are not expressed in *E. amylovora* (40,41). It was only after the fortuitous acquisition of IS1133 that the *strA-strB* genes could be expressed in *E. amylovora* and related orchard enteric bacteria such as *Pantoea agglomerans*. Our genetic analyses of the current Sm^R *E. amylovora* population, coupled with the observation of only one other single strain (which we did not reisolate in the present study) with a separate insertion point of Tn5393 in pEA29 from a previous study (19), imply that very few acquisitions of Tn5393 have occurred and that *E. amylovora* is not a ready partner for gene transfer events in nature.

A central question remains concerning why the Tn5393 streptomycin resistance mechanism predominates in *E. amylovora* in Michigan while spontaneous Sm^R mutants predominate in other locations. Although our research has not directly addressed this question, we can speculate that the relatively low level of genetic diversity in *E. amylovora* populations (26,31) possibly contributed to the ease of selection and dispersal of one or a few Sm^R genotypes in response to streptomycin selection. Thus, the initial acquisition of pEa34 harboring Tn5393 by *E. amylovora* in Michigan may have simply predated selection of spontaneous

Sm^R mutants, enabling an adaptive “selective sweep” resulting in population takeover by Tn5393-harboring strains under streptomycin selection pressure in orchards. In addition, a recent study indicated that some mutations at codon 43 of the *rpsL* gene resulting in spontaneous streptomycin resistance in *E. carotovora* subsp. *carotovora* were pleiotropic, resulting in reduced exoenzyme production and reduced virulence (1). Thus, potential pleiotropic effects of *rpsL* mutations may also have limited the selection of these mutants in Michigan. The current location of Tn5393 on a nonconjugative plasmid suggests that the Sm^R *E. amylovora* population in Michigan behaves in an ecological sense similar to a population of Sm^R mutants because the resistance genes are nontransferable and expansion of the Sm^R population occurs by dispersal and not through additional gene transfer events.

Finally, our comparative sequencing analysis of Sm^R *E. amylovora* strains in Michigan apple orchards suggests that the phenotype of streptomycin resistance has likely served to further reduce the genetic diversity of the pathogen population. However, the heterogeneity within the sequences chosen for comparison was relatively low, even when comparing strains from different geographic regions (maximum difference of 0.5%) or when comparing *E. amylovora* strains isolated from apple with a strain isolated from *Rubus* spp. (maximum difference of 1.7%). Thus, further sequence analysis is needed for a more robust confirmation of the clonal nature of these strains. We hypothesized that, if streptomycin resistance was acquired by low virulence strains, the overall virulence level of the population would suffer. However, the results of virulence studies conducted suggest that this is not the case (Fig. 4). We did identify one recent isolate (MI5-1) and two other isolates with low virulence in this experiment. Naturally occurring strains of *E. amylovora* with low virulence are known (5), although the epidemiological significance of these strains is not currently known.

In summary, our results indicate that Sm^R strains of *E. amylovora* are spreading between orchards in Michigan, and that new Sm^R strains are apparently not arising. Other antibiotics have become available for fire blight management, including oxytetracycline and kasugamycin (21,22,24). These additional antibiotics, along with existing biological control agents, provide an effective and varied arsenal for management of blossom blight on the highly susceptible cultivars that are currently widely grown.

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