Characterization of Streptomycin Resistance in Isolates of *Erwinia amylovora* in California

Helga Förster, Gayle C. McGhee, George W. Sundin, and James E. Adaskaveg

First and fourth authors: Department of Plant Pathology and Microbiology, University of California, Riverside 92521; and second and third authors: Department of Plant, Soil, and Microbial Sciences, Michigan State University, East Lansing 48824.
Accepted for publication 18 May 2015.

ABSTRACT

Förster, H., McGhee, G. C., Sundin, G. W., and Adaskaveg, J. E. 2015. Characterization of streptomycin resistance in isolates of *Erwinia amylovora* in California. *Phytopathology* 105:1302-1310.

In surveys from 2006 to 2014, streptomycin resistance in *Erwinia amylovora* from pear-growing areas in California declined from very high incidence in 2006 and 2007 to very low incidence in 2013 and 2014. The majority of resistant strains were designated as moderately resistant-low (MR-L), and were almost exclusively found in Sacramento County, whereas highly resistant (HR) strains were only recovered in Sutter-Yuba and San Joaquin counties. Resistance of HR strains was associated with a mutation in codon 43 of the chromosomal *rpsL* gene that results in a change from lysine to arginine, the same mutation that was originally reported for resistant strains from California in the mid-1970s. MR-L strains were found to harbor the *strA-strB* streptomycin resistance genes on transposon Tn5393a. This transposon lacks insertion sequence IS1133

that provides a promoter for efficient expression of *strA-strB*, resulting in lower minimum inhibitory concentrations of MR-L strains compared with those from other locations that harbor *strA-strB* on Tn5393::IS1133. In contrast to previously described plasmid-mediated resistance where Tn5393 is inserted in pEa34, or pEA29, Tn5393a in MR-L strains was located on plasmid pEU30. This plasmid was first described in *E. amylovora* from the western United States but was not associated with streptomycin resistance determinants previously. We hypothesize that Tn5393a was introduced into an *E. amylovora* strain carrying pEU30 and transposed into that plasmid. This hypothesis was supported by clustered regularly interspaced short palindromic repeat (CRISPR) sequence analysis that showed that two MR-L strains share the same CRISPR1 pattern as a streptomycin-sensitive strain. With current low resistance levels in California growing regions, streptomycin could be successfully used again, but applications per season should be limited and the antibiotic should be mixed and rotated with different modes of action.

Fire blight caused by *Erwinia amylovora* is one of the most destructive diseases of pome fruit crops worldwide. Few treatments are available for effective management. Copper products can be quite effective, but their use is currently mostly limited to early-season applications due to the risk of phytotoxicity to the developing fruit. Since the 1950s, management in the United States often relied on the use of the antibiotics streptomycin and later, in the 1970s, on the less effective oxytetracycline. The over-use of streptomycin resulted in the development of streptomycin resistance in the pathogen in some growing areas. Resistance was first detected in California in 1971 (Moller et al. 1972; Schroth et al. 1978) followed by Oregon and Washington in 1972 (Coyier and Covey 1975). Resistance is now widespread in the western United States (Loper et al. 1991) and in other pear and apple growing areas, including Michigan (McGhee et al. 2011) and British Columbia (Sholberg et al. 2001), and has also been reported from Missouri (Shaffer and Goodman, 1985), New York (Russo et al. 2008), as well as New Zealand (Thomson et al. 1993), Egypt (El-Goorani and El-Kasheir 1989), Israel (Manulis et al. 1998), Lebanon (Saad et al. 2000), and Mexico (Ponce de León Door et al. 2013).

The molecular mechanism of streptomycin resistance has been studied for several populations of *E. amylovora*. There are two basic mechanisms: (i) mutations in a chromosomal gene and (ii) plasmid/transposon-determined resistance (McManus et al. 2002). Chromosomal-determined resistance confers a high level

of insensitivity with minimum inhibitory concentration (MIC) values of 2,000 µg/ml (McManus et al. 2002). It is based on a point mutation in the *rpsL* gene that encodes ribosomal protein S12 on the 30S ribosomal subunit (Chiou and Jones 1995). This inhibits binding of streptomycin to the ribosome, and protein synthesis remains functional, in contrast to sensitive strains without this mutation. Chromosomal-determined resistance was considered to be the most common mechanism in the past (McManus et al. 2002) and was described for isolates from the western United States, including Oregon, Washington, California, and Idaho, as well as from Michigan, New Zealand (Chiou and Jones 1995), and Mexico (Ponce de León Door et al. 2013).

Plasmid/transposon-determined resistance confers a lower level of insensitivity with MIC values of 500 to 750 µg/ml (McManus et al. 2002; McGhee et al. 2011). This resistance mechanism was first discovered in *E. amylovora* isolates from Michigan in 1990 where it is now regarded as the more common type of resistance (Chiou and Jones 1991; McGhee et al. 2011). With plasmid/transposon-determined resistance, two linked genes, *strA-strB*, encoding phosphotransferases that modify streptomycin to a nontoxic form, reside on the 6.7-kb transposable element Tn5393 (McManus et al. 2002). In *E. amylovora*, this transposon was originally found to be integrated in pEa34, a 34-kb self-transmissible plasmid (Chiou and Jones 1991; McManus and Jones 1994). Subsequently, in a limited number of streptomycin-resistant isolates from an apple orchard in California, the *strA-strB* genes were found on plasmid pEa8.7 that is thought to be identical to the broad-host range plasmid RSF1010 (Palmer et al. 1997). In isolates from Michigan (McManus and Jones 1994; McGhee and Jones 2000) and later New York (Russo et al. 2008), Tn5393 was located on the nontransmissible plasmid pEA29 in at least two different insertion locations. Isolates with *strA-strB* integrated into pEA29 are currently the most common genotype among Michigan isolates with streptomycin

Corresponding author: J. E. Adaskaveg; E-mail address: jim.adaskaveg@ucr.edu

*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary figures are published online.

<http://dx.doi.org/10.1094/PHYTO-03-15-0078-R>
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resistance (McGhee et al. 2011). Thus, three types of plasmids have been described to harbor *strA-strB* in *E. amylovora*. Furthermore, in a limited number of Michigan isolates, Tn5393 with *strA-strB* was also found to be inserted in the bacterial chromosome (McManus and Jones 1994).

Although streptomycin resistance in *E. amylovora* was first described from California populations and was found at very high incidence at some locations, the extent of resistance in the state has not been assessed since the late 1970s (Schroth et al. 1978). Moller et al. (1981) indicated that by 1980, the incidence of resistance had dropped to approximately 5% at the location where resistance was originally found, and streptomycin was used again successfully for many years in California to manage fire blight. In 2006, however, pear growers in Sacramento County again started reporting fire blight control failures following streptomycin use. Thus, our first objective was to conduct multiple-year orchard surveys that were expanded to include other growing regions of the state to assess the extent and distribution of streptomycin resistance and to determine if streptomycin can remain part of an effective fire blight management program. A second objective of our investigations was to identify the mechanism of resistance in recent isolates of the pathogen in California and to determine if populations have undergone genetic changes similar to those observed in some other growing areas.

MATERIALS AND METHODS

Isolation of *E. amylovora*, bacterial culturing, and verification of species identity. Pear samples with fire blight symptoms were obtained in the spring to early summer of 2006 to 2014 from orchards throughout California pear growing areas in El Dorado, Lake, Mendocino, Sacramento, San Joaquin, Solano, and Sutter-Yuba counties (Supplementary Fig. S1). Infected plant material (fruit, pedicels, and twig stems) was surface-disinfested for 1 min using sodium hypochlorite at 400 µg/ml, rinsed with sterile water, cut into small sections, and incubated in microcentrifuge tubes with 1 ml of sterile water for 15 to 30 min. Suspensions were streaked onto yeast extract-dextrose-CaCO₃ agar (YDC; Wilson et al. 1967). Single colonies were transferred and the identity of the isolates as *E. amylovora* was verified by colony morphology and by polymerase chain reaction (PCR) using primers specific for the ubiquitous *E. amylovora* plasmid pEA29 (Bereswill et al. 1992). *E. amylovora* strains RJ2 and RJ2- came from a previous study and were characterized to contain both plasmids pEA29 and pEU30 or to contain only plasmid pEU30, respectively (Foster et al. 2004). Additional reference strains from Michigan were used for in vitro streptomycin sensitivity tests including the moderately resistant (MR), hereafter referred to as moderately resistant-high (MR-H), strains NW1-1 and NW18-6 that were previously determined

to harbor *strA-strB* at different positions on pEA29 (McGhee and Sundin 2012) and the highly resistant (HR) strain S5 with a chromosomal mutation in the *rpsL* gene. Strains were maintained on YDC agar and stored in 20% glycerol at -80°C.

Determination of streptomycin sensitivity. In vitro sensitivity against streptomycin was evaluated using the spiral gradient dilution (SGD) method (Förster et al. 2004; Paton et al. 1990). For this, radial concentration gradients of 0.05 to 5 µg/ml or of 0.5 to 50 µg/ml were established in 15-cm nutrient agar (Difco Laboratories, Detroit, MI) plates by spirally plating an aqueous stock concentration of 1,000 or 10,000 µg/ml, respectively, of streptomycin (Sigma-Aldrich Inc., St. Louis, MO) using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood, MA). After streaking out suspensions of the test bacteria (10 µl of approximately 2 × 10⁸ cfu/ml, as determined by measurement of optical density at 600 nm) along the radial concentration gradient,

Fig. 1. Spiral gradient dilution plate demonstrating differences in streptomycin sensitivity of sensitive, moderately resistant-low (MR-L), moderately resistant-high (MR-H), and highly resistant (HR) strains of *Erwinia amylovora*. A concentration gradient from approximately 0.5 µg/ml (edge of plate) to 50 µg/ml (center of plate) streptomycin was established by applying a stock concentration of 10,000 µg/ml using a spiral plater. Eight strains are evaluated on a single plate with each strain duplicated in opposing streaks. Strains are as follows: 1 and 9, 529 (sensitive from California); 2 and 10, 538 (MR-L from California); 3 and 11, 547 (MR-L from California); 4 and 12, NW 1-1 (MR-H from Michigan); 5 and 13, NW 18-6 (MR-H from Michigan); 6 and 14, 552 (HR from California); and 7 and 15, 9-1 (HR from California) and S5 (HR from Michigan).

TABLE 1. Oligonucleotide primer pairs used in this study and their targets

Forward primer	Sequence (5'-3')	Reverse primer	Sequence (5'-3')	Annealing temperature (°C)	Target	Reference
AJ44	GAAGCAAAAGCTAAACAGGAGCT	AJ42	CGTGGCATGGAAATACTCCG	55	<i>E. amylovora</i>	Chiou and Jones 1995
strA-F	TGAATCGCATCTGACTGGTT	strA-R	AAGTTGCTGCCCATTTGA	58	<i>rpsL</i>	Palmer et al. 1997
strB-F	GGAAGTGCCTGGGCTACA	strB-R	GCTAGATCGCGTTGCTCCTCT	58	<i>strB</i>	Palmer et al. 1997
pEa34-F	CGTTTATCGGATGCCTTTGAGTC	pEa34-R	AGTCGTGATTTCCATTTTATTTC	54	pEa34	McGhee et al. 2011
tnpA-F	CATTCCGAGACGCAAACA	tnpA-R	CTATGTCGATCCGAGAACAGTT	58	Tn5393	Jones and Schnabel 2000
AJ507	GATCAAGCAGGTGCGACAGAC	pEU30R	TCTCAATGCTGGCAGGAATGTC	56	3' end of <i>strB</i> to into pEU30	McGhee et al. 2011; This study
pEU30F	ATTACGCGAGACTGCCTGGTTTC	pEU30R-2	ATTTCCCGCGAGCCTTTCATAGTG	61	pEU30	This study
tnpR-F	GGTCGCAAGGCGGACGAAA	strA5'out	AAGGCATGCCCGTCCACACG	59	<i>strA</i> to <i>tnpR</i> of Tn5393	This study

plates were incubated for 2 days at 25°C. Measurements were taken for MICs (growth inhibition by $\geq 95\%$) (Adaskaveg et al. 2011). Actual antimicrobial concentrations were obtained by entering the radial distances of inhibition (measured from the center of the plate) into the Spiral Gradient Endpoint computer program (Spiral Biotech, Inc.). Each isolate was evaluated in two independent experimental repeats with two replications per experiment. Strains with MIC values of ≤ 2.5 , >2.5 to 37, and >50 $\mu\text{g/ml}$ were designated sensitive, moderately resistant-low (MR-L), and HR to streptomycin, respectively. All HR isolates were streaked onto Luria-Bertani media (Difco) containing streptomycin at 2,000 $\mu\text{g/ml}$ for confirmation of high-level resistance.

PCR amplifications. Amplifications were performed in a total volume of 25 μl containing 1 μl of bacterial suspension (approximately 10^4 to 10^5 cfu), 2 μl of dNTPs (2.5 mM each dNTP), 2.5 μl of PCR buffer (New England Biolabs, Inc., Ipswich, MA), 0.25 μl of bovine serum albumin (10 mg/ml; Sigma-Aldrich Inc.), 1 μl of each forward and reverse primer (10 μM), and 0.25 μl of *Taq* DNA polymerase (New England Biolabs). Amplifications were performed in a PCT-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA) programmed as follows: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at selected annealing temperatures (Table 1), and 30 s (or 4 min for primers pEU30F and pEU30R-2) at 72°C, and a final extension at 72°C for 5 min. PCR products were visualized in 1.5% agarose gels in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer after ethidium bromide staining. As DNA size standards, the TrackIt 100 bp, TrackIt 1 kb, and the 1 kb Plus DNA ladders (Life Technologies, Thermo Fisher Scientific, Waltham, MA) were used.

Genetic analysis of HR strains. The mechanism of high-level resistance to streptomycin was investigated by sequence analysis of the *rpsL* gene as described previously (Chiou and Jones 1995). Primers AJ44 and AJ42 (Table 1) were used to amplify the *rpsL* gene of sensitive strains (i.e., strains 531 and 2009-3-4), MR-L strains (i.e., strains 541 and 543), and HR strains (i.e., strains 549, 551, and 552). PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced at the Core Instrumentation Facility of the University of California Riverside Institute for Integrative Genome Biology. Nucleic acid and deduced amino acid sequences were compared for the strains included in this

analysis and with those published previously (Chiou and Jones 1995).

Genetic analysis of MR-L strains. Initially, the presence of streptomycin resistance genes *strA-strB*, transposon Tn5393, and plasmid pEa34 was evaluated using previously published primers and annealing temperatures (Table 1) for selected isolates of the three resistance phenotypes. The location of *strA-strB* on the transposon was verified for 26 strains by using primers tnpR-F (located in the resolvase gene *tnpR* of Tn5393) and strA5'out (located at the 5' region of *strA*) in PCR amplifications. PCR products were visualized in 1.5% agarose gels in 0.5 $\times$ TBE after ethidium bromide staining. Subsequently, the location of the transposon in the bacterial genome of representative MR-L strains (i.e., strains 538, 539, 540, 541, and 542) was determined by outward sequencing from the 3' end of *strB* using primer AJ507. When necessary, primer walking was used to complete sequencing. Sequencing was done at the Michigan State University Genome Technology Research. 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>).

Because Tn5393 with *strA-strB* appeared to be inserted in the previously described plasmid pEU30 (Foster et al. 2004), primers pEU30F (position 2805 to 2828 of pEU30) and pEU30R (position 3269 to 3292 of pEU30) (Table 1) were designed and used in amplifications to determine the presence of this plasmid in strains of *E. amylovora* with different levels of streptomycin sensitivity. Additionally, primers AJ507 and pEU30R that amplify a 600-bp region from the 3' end of *strB* into pEU30 were used for a larger sample of MR-L strains to verify the physical association of *strB* with pEU30. The presence of pEU30 and pEA29 was further verified by plasmid preparation of strains 529, 530, 531, 642, 650 (sensitive to streptomycin), 534, 536, 539, 541, 542 (MR-L), 550, 552, 9-1-2009, 645, 652 (HR), as well as strains RJ2 and RJ2- using a commercial kit (Pure Link, Quick Plasmid Miniprep kit; Invitrogen, Carlsbad, CA). An aliquot of 5 μl of plasmid preparation was digested with *KpnI* for 2 h at 37°C, and the digest was separated in a 0.8% agarose gel in 0.5 $\times$ TBE buffer stained with ethidium bromide.

TABLE 2. Summary of surveys for streptomycin resistance in *Erwinia amylovora* populations in California

County	Year	Number of orchards	Number of strains	% MR-L strains ^a	% orchards with MR-L strains	% HR strains ^b	% orchards with HR strains
El Dorado Lake	2010	3	10	0.0	0.0	0.0	0.0
	2008	11	30	0.0	0.0	0.0	0.0
	2009	7	7	0.0	0.0	0.0	0.0
	2012	5	10	0.0	0.0	0.0	0.0
	2013	21	44	0.0	0.0	0.0	0.0
Mendocino	2014	6	19	0.0	0.0	0.0	0.0
	2006	1	4	50.0	100.0	0.0	0.0
	2008	3	14	0.0	0.0	0.0	0.0
	2009	1	3	0.0	0.0	0.0	0.0
	2014	1	7	0.0	0.0	0.0	0.0
Sacramento	2006	14	67	76.1	85.7	0.0	0.0
	2007	17	172	66.9	94.1	0.0	0.0
	2008	7	57	5.3	42.9	0.0	0.0
	2009	21	132	33.3	61.9	0.0	0.0
	2010	10	56	46.4	80.0	0.0	0.0
	2011	9	39	33.3	44.4	0.0	0.0
	2013	13	105	6.7	15.4	0.0	0.0
	2014	18	129	2.3	5.6	0.0	0.0
San Joaquin	2008	1	5	0.0	0.0	100.0	100.0
Solano	2013	1	3	0.0	0.0	0.0	0.0
Sutter-Yuba	2006	2	30	0.0	0.0	0.0	0.0
	2007	2	12	0.0	0.0	100.0	100.0
	2008	1	3	0.0	0.0	0.0	0.0
	2009	7	24	0.0	0.0	45.8	42.9
	2012	3	11	0.0	0.0	72.7	66.7

^a MR-L = moderately resistant-low (minimum inhibitory concentration = 7.0 to 37.3 $\mu\text{g/ml}$).

^b HR = highly resistant (minimum inhibitory concentration = $\geq 2,000$ $\mu\text{g/ml}$).

RESULTS

Primers pEU30F and pEU30R-2 (Table 1) located at nucleotides 2805 and 3269, respectively, on plasmid pEU30 were used to amplify the entire transposon for sequencing. Strains possessing pEU30 and lacking the transposon yielded a 488-bp PCR product, whereas pEU30-positive strains harboring the transposon yielded a PCR product approximately 6 kb in length. PCR products were purified from the reaction mixes using the QIAquick PCR purification kit (Qiagen Sciences, Valencia, CA) prior to sequencing according to the manufacturer's instructions. Transposon sequences for each strain were assembled and compared among strains. Individual confirmation of sequence differences was made by visually examining chromatograms using Finch TV version 1.4.0 (Geospiza, Seattle, WA). Alignment and analysis was performed using the Lasergene (version 11.2.0) suite of programs (DNASTAR, Inc., Madison, WI).

Molecular diversity among strains of *E. amylovora* from California using clustered regularly interspaced short palindromic repeats (CRISPR) analysis. Oligonucleotide primers used to amplify the CRISPR 1 array sequences were designed using the *E. amylovora* Ea273 (ATCC 49946) genome sequence (GenBank accession number NC_013971) as previously described (McGhee and Sundin 2012) for eight strains with varying levels of streptomycin resistance. The cycling parameters were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, annealing at 58°C for 30 s, followed by 72°C for 4 min with a final extension for 7 min at 72°C. Amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced. CRISPR array spacer and repeat patterns were generated using the CRISPR recognition tool (CRT) Version 1.0 (<http://www.room220.com/crt>).

Determination of streptomycin sensitivity in *E. amylovora* from surveys of California pear orchards. A total of 993 strains of *E. amylovora* was isolated from diseased pear tissues collected from seven counties in California between 2006 and 2014. Sensitivity to streptomycin was determined using the SGD method. An example of an assay plate with sensitive, MR-L, and HR strains from California is shown in Figure 1. Growth of HR strains was not inhibited by streptomycin at 50 µg/ml (approximate concentration at the center of the plate), whereas sensitive and MR-L strains were inhibited at different radial distances. Additionally, this assay plate also included HR strain S5 from Michigan that carries a mutation in *rpsL*, and MR-H strains NW 1-1 and NW 18-6 from Michigan that carry *strA-strB* on Tn5393, with the transposon inserted at different locations in pEA29. These latter strains were also not inhibited by the highest streptomycin concentration on the plate.

MIC values for streptomycin-sensitive strains from California ranged from 0.2 to 2.5 µg/ml (mean = 0.8 µg/ml). The majority of the 264 MR-L strains were isolated from Sacramento County where most of the pears are grown in the state (Table 2). MIC values ranged from 7.0 to 37.3 µg/ml (mean = 20.6 µg/ml). The incidence of MR-L strains varied widely among years. In 2006 and 2007, 76.1 and 66.9% of the strains evaluated, respectively, had the MR-L phenotype. The incidence of orchards with at least some MR-L strains for these years was 85.7 and 94.1%, respectively. The incidence of MR-L was lower in 2008, 2013, and 2014. The 36 strains with high levels of resistance were isolated from five orchards in Sutter-Yuba County where they were detected at high incidence in 2007, 2009, and 2012, and from a single location in San Joaquin County in 2008 (Table 2). All of these strains were confirmed to grow on LB medium amended

TABLE 3. Isolates of *Erwinia amylovora* from pear used in analyses of streptomycin resistance mechanisms in this study

Sensitivity to streptomycin	Strain ID	Year collected	Country-orchard	MIC (µg/ml) ^a	Results of molecular tests					
					<i>rpsL</i> analysis ^b	Presence of <i>strA-strB</i> ^c	Presence of Tn5393a ^d	Presence of pEa34 ^e	Tn5393a on pEU30 ^f	Presence of pEU30 ^g
Sensitive	530	2008	Mendocino-1	1.1		–	–		–	–
	529	2007	Sacramento-1	1.0		–	–		–	–
	531	2009	Sacramento-2	0.5	–	–	–	–	–	–
	3-4	2009	Sacramento-3	0.5	–	–	–	–	–	–
	28-1	2009	Sacramento-4	0.5		–	–	–	–	–
	650	2012	Sutter-Yuba	1.2		–				
Moderately resistant-low	534	2007	Sacramento-5	20.4		+	+		+	+
	535	2007	Sacramento-6	21.0		+	+		+	+
	536	2007	Sacramento-8	31.6		+	+		+	+
	537	2007	Sacramento-1	28.4		+	+		+	+
	538	2008	Sacramento-9	11.5		+	+		+	+
	539	2008	Sacramento-10	14.8		+	+		+	+
	540	2009	Sacramento-2	16.2		+	+	–	+	+
	541	2009	Sacramento-11	12.6	–	+	+	–	+	+
	542	2009	Sacramento-12	15.7		+	+	–	+	+
	543	2009	Sacramento-13	11.5	–	+	+	–	+	+
	544	2011	Sacramento-2	25.1		+	+		+	+
	545	2011	Sacramento-2	27.5		+	+		+	+
	546	2011	Sacramento-14	32.2		+	+		+	+
	549	2007	Sutter-Yuba-1	>2,000	+	–	–	–	–	–
	550	2008	San Joaquin-1	>2,000		–	–		–	–
Highly resistant	551	2009	Sutter-Yuba-2	>2,000	+	–	–	–	–	+
	552	2009	Sutter-Yuba-3	>2,000	+	–	–	–	–	+
	7-1	2009	Sutter-Yuba-2	>2,000		–	–			+
	9-1	2009	Sutter-Yuba-1	>2,000		–	–			+
	645	2012	Sutter-Yuba-4	>2,000		–	–			+
	652	2012	Sutter-Yuba-5	>2,000		–	–			+

^a Minimum inhibitory concentration (MIC) to reduce growth by ≥95%.

^b Sequence analysis of the *rpsL* gene with codon change in position 43.

^c Presence of *strA-strB* was determined using primer pairs *strA-F/strA-R* and *strB-F/strB-R* (Palmer et al. 1997).

^d Presence of Tn5393a was determined using primers *tnpA-F* and *tnpA-R*.

^e Presence of pEa34 was determined using primers *pEa34-F* and *pEa34-R*.

^f Location of Tn5393a on pEU30 was confirmed using primers *AJ507* and *pEU30R* (McGhee et al. 2011).

^g Presence of pEU30 was determined using primers *pEU30F* and *pEU30R-2*.

with streptomycin at 2,000 µg/ml. Overall, high resistance was detected in six of the orchards surveyed.

Genetic analysis of HR strains. Sequence comparisons were done for amplified 452-bp regions of the *rpsL* gene from representative strains sensitive, MR-L, and HR to streptomycin. All sequences were identical except for a nucleotide substitution in the three evaluated HR strains in codon 43 from adenine to guanine that resulted in a codon change from lysine to arginine (Supplementary Fig. S2).

Genetic analysis of MR-L strains. Using previously designed amplification primers, homologous sequences of *strA*, *strB*, and of

transposon Tn5393 were detected in representative MR-L strains as indicated in Table 3, and also in all 10 MR-L strains collected in 2013 and 2014. Amplification products had expected lengths of 300 bp for *strA*, 302 bp for *strB*, and 300 bp for Tn5393 (Fig. 2A, panels 1, 2, and 3). No amplification products were detected using primers designed for plasmid pEa34. Amplification with primers spanning the 5' region of *strA* to *tnpR* of the putative Tn5393 yielded a product 488 bp in length (data not shown). This indicated the absence of insertion sequence IS1133 (IS1133 is found in Tn5393 and contains a promoter), and thus, the entire transposon

Fig. 2. A, Polymerase chain reaction (PCR) detection of **1**, *strA*, **2**, *strB*, and **3**, *tnpA* from Tn5393a. Amplification of the genes from sensitive (Sm-S), moderately resistant-low (MR-L), and highly resistant (HR) strains of *Erwinia amylovora* recovered from California pear orchards. Lane L, 100-bp marker; lane 1, 531 (Sm-S); lane 2, 3-4 (Sm-S); lane 3, 28-1 (Sm-S); lane 4, 540 (MR-L); lane 5, 541 (MR-L); lane 6, 543 (MR-L); lane 7, 542 (MR-L); lane 8, 544 (MR-L); lane 9, 549 (HR); lane 10, 551 (HR); and lane 11, 552 (HR). PCR fragments in each panel are designated by arrows with labels, i.e., (a), (b), and (c), that correspond to fragments drawn from genetic maps of pEU30::Tn5393a and pEU30 below. **B,** Genetic map of an approximately 5.6-kb region of plasmid pEU30 of *E. amylovora* spanning from the *repA* gene to *codB* including the insertion of the 5473-bp transposon Tn5393a at nucleotide 2839 as present in California MR-L strains and of pEU30 without the transposon as present in California streptomycin-sensitive and HR-L strains. Coding sequences from pEU30 are shaded in gray, and coding sequences from Tn5393a are in white. Tn5393a genes involved in transposition (*tnpA* and *tnpR*), and streptomycin resistance (*strA* and *strB*) are designated. The approximate location of DNA fragments amplified by PCR from pEU30::Tn5393a from *strA* (a), *strB* (b), *tnpA* (c) are shown as well as fragments demonstrating the insertion of Tn5393a in pEU30 (d) and detection of pEU30 without the transposon (e) are shown. **C,** Detection of pEU30 in *E. amylovora* strains isolated from California using primers pEU30F and pEU30R. Lane L, 1-kb ladder; lane 1, strain 530 (SM-S); lane 2, 531 (Sm-S); lane 3, 3-4 (Sm-S); lane 4, 535 (MR-L); lane 5, 536 (MR-L); lane 6, 537 (MR-L); lane 7, 551 (HR) lane 8, 552 (HR); lane 9, 550 (HR); and lane 10, 549 (HR). A 488-bp band was amplified from strains positive for pEU30 [lanes 7 and 8, fragment (e)]. When Tn5393a was inserted into pEU30, amplified bands were approximately 6 kb in size [lanes 4 to 6, fragment (d)].

was sequenced from five strains and confirmed as Tn5393a (i.e., transposon Tn5393 without IS1133; McManus et al. 2002; Sundin and Bender 1995).

The genomic location of Tn5393a was determined by sequencing outward from the transposon. Sequences adjacent to Tn5393a from five strains matched those of pEU30, indicating that the transposon was integrated into this plasmid (this plasmid occurs in some western United States populations of *E. amylovora* but was never reported to carry resistance determinants). Additionally, Tn5393a was found inserted in the same position at nucleotide 2839 within an intergenic region of pEU30 and in the same orientation for the five strains tested. A genetic map of pEU30 with Tn5393a inserted at position 2839 is shown in Figure 2B. In addition, we confirmed the presence of Tn5393a on pEU30 for 64 MR-L strains isolated from multiple locations over all collection years using a primer pair spanning the 3' end of *strB* in the transposon to pEU30 using primers AJ507 and pEU30 (data not shown).

Subsequently, the presence of pEU30 was determined for additional strains of *E. amylovora* with different levels of streptomycin sensitivity using primers pEU30F and pEU30R-2 specific for this plasmid (Fig. 2C). No amplification product was present in any strain that was sensitive to streptomycin. For MR-L strains, an amplicon of the expected size of approximately 6 kb was present, comprising plasmid and transposon sequences. For six of the eight HR strains tested, a fragment of 488 bp was amplified that comprised a plasmid portion without the inserted transposon. Two HR strains did not yield any band, indicating the absence of pEU30 (Fig. 2C).

The presence of pEU30 was further verified by restriction enzyme digestion of plasmid preparations. Digested plasmids from *E. amylovora* strains RJ2 (contains pEA29 and pEU30) and RJ2- (contains pEU30) were used as size standards. When digested with *Kpn*I, plasmids pEA29 and pEU30 yield five and three fragments, respectively, of lengths 1,005 (not visible in Figure 3), 2,759 (fragment g in Figure 3), 4,081 (fragment e), 8,710 (fragment c), and 11,630 bp (fragment b) for pEA29 and 3,501 (fragment f), 6,059 (fragment d), and 20,724 bp (fragment a) for pEU30. Compared with sensitive strains, three additional bands were present for MR-L strains and most of the HR strains examined. RJ2 and RJ2- patterns indicated that these bands corresponded to pEU30. In Figure 3, the largest fragment of pEU30 (i.e., fragment a) is approximately 4.9 kb larger for the three MR-L strains compared with the four HR strains due to the presence of Tn5393a in pEU30 in the three MR-L strains. In additional plasmid preparations, bands corresponding to pEU30 were absent in the HR strain 550 where absence of this plasmid was indicated before by PCR amplifications described above.

Molecular diversity among strains of *E. amylovora* from California using CRISPR analysis. The CRISPR locus is found in approximately 50% of bacterial species and consists of several genes and an associated region consisting of a small repeat sequence located between a series of spacer sequences. The spacers are characteristically 32 bp in length in *E. amylovora*; CRISPR spacers consist of sequences that were acquired from bacteriophage, plasmid, and other horizontally acquired DNA sequences (Sorek et al. 2013). The CRISPR system uses the spacer sequences as part of a surveillance system to identify invading sequences and target them for elimination (Bhaya et al. 2011). The order of the spacers is thought to represent a record of past encounters of the organism with potential invading sequences. The diversity and pattern of spacer arrays within individual bacterial strains has been used for population genetic and epidemiological strain-tracking experiments in human pathogens such as *Mycobacterium tuberculosis* (Shariat and Dudley 2014) and by us in *E. amylovora* (McGhee and Sundin 2012). Comparison of spacer array patterns among *E. amylovora* strains has revealed unique patterns enabling the differentiation of strains that were indistinguishable by previous typing methods and also revealed that array patterns are typically conserved in strains isolated from the same region (Rezzonico et al. 2011; McGhee and Sundin 2012).

We determined the sequence of the CRISPR1 array from eight *E. amylovora* strains in this study and identified a total of 97 spacers among them. These spacers were contained in arrays that were compared between strains, revealing patterns that were similar to *E. amylovora* strains previously isolated in the western United States (Fig. 4). The patterns observed in MR-L strains 16-1 and 32-21 were identical to that of the streptomycin-sensitive strain 1-1 (Fig. 4). The patterns observed in MR-L strains 15-3 and 17a, HR strains 8-1 and 66-2, and in the streptomycin-sensitive strain 19-6 were also closely related (Fig. 4).

DISCUSSION

The extent of streptomycin resistance in populations of *E. amylovora* from California pear orchards was reassessed for the first time after extensive surveys were done in the 1970s and in 1980 (Moller et al. 1972, 1981; Schroth et al. 1978) following the detection of resistance to this antibiotic. Our goals were to determine if streptomycin can remain part of an effective fire blight management program and to substantiate grower reports on the ineffectiveness of streptomycin applications in the field. Based on our investigations, treatment failures could be associated with a very high incidence of streptomycin resistance at some locations in some years.

In contrast to the central growing areas, the incidence of resistance in the northern regions of California (e.g., Lake and Mendocino counties) was very low with only two resistant strains detected in one orchard during the 9-year study. This possibly could be explained by a different fire blight spray management strategy where for many years, streptomycin was generally applied in a mixture with oxytetracycline. In surveys done between 1971 and 1980, streptomycin resistance was also only found in the central parts of the state, namely the Sacramento Valley (Moller et al. 1981). In the latter areas, temperature conditions during flowering are higher and more favorable for epiphytic growth and subsequent flower infection by the pathogen. This would lead to more intense selection for resistance because larger populations are exposed to the selection agent and additionally, more streptomycin is applied to manage fire blight. However, the authors of this and other studies also indicated that the occurrence of streptomycin

Fig. 3. *Kpn*I digestion of native plasmids from *Erwinia amylovora* strains: lane L, 1-kb ladder; lane 1, 642 (Sm-S); lane 2, 650 (Sm-S); lane 3, 529 (Sm-S); lane 4, 542 (MR-L); lane 5, 541 (MR-L); lane 6, 539 (MR-L); lane 7, 8-1 (HR); lane 8, 652 (HR); lane 9, 645 (HR); and lane 10, 9-1 (HR). Fragments a, d, and f originate from pEU30 and are 20,724, 6,059, and 3,501 bp, respectively, and fragments b, c, e, and g originate from pEA29 and are 11,630, 8,710, 4,081, and 2,759 bp, respectively. *Kpn*I fragment a in lanes 4 to 6 has shifted from 20,724 to 26,197 bp (denoted by arrow) indicating the insertion of Tn5393a in pEU30 in these strains. The smallest *Kpn*I fragment from pEA29 of 1,005 bp is not visible in this gel preparation.

resistance was often unrelated to streptomycin usage in the orchard, and resistance was sometimes detected with few or no streptomycin applications (Loper et al. 1991; Moller et al. 1972, 1981; Schroth et al. 1978).

In our studies, two resistance levels were differentiated among California strains of *E. amylovora*, moderate and high. MR-L strains comprised the majority of resistant strains and were almost exclusively found in Sacramento Co., whereas HR strains were only recovered in Sutter-Yuba and San Joaquin counties. Earlier surveys conducted in California and the Pacific Northwest only detected strains that grew at streptomycin concentrations of 100 µg/ml or higher (Coyier and Covey 1975; Loper et al. 1991; Moller et al. 1972, 1981; Schroth et al. 1978). This suggests that MR-L strains are of more recent origin and have possibly displaced HR strains.

The resistance mechanism of HR strains from our collections was associated with a mutation in codon 43 of the chromosomal *rpsL* gene that results in a codon change from lysine to arginine. The same codon change was originally reported for five resistant strains from California collected in the mid-1970s and for strains from Idaho, Michigan, Oregon, Washington, and New Zealand; whereas additional strains from Oregon showed a codon change to asparagine or threonine (Chiou and Jones 1995). Thus, the HR strains that we detected in our surveys likely had persisted from the original resistant populations in California that caused major disease outbreaks in the 1970s. This was also confirmed by CRISPR analyses of HR strains conducted in this study (Fig. 4) as HR strains had CRISPR array patterns characteristic of western U.S. strains. Their relative scarcity in our surveys may indicate a reduced fitness of HR strains. Supporting these findings, at the original discovery site of streptomycin resistance in California where more than 95% of the population was resistant and where streptomycin was no longer applied, the incidence of resistance declined gradually over the years, and was present at less than 5% after 10 years (Moller et al. 1981). In the past, mutational resistance has been considered highly stable and to persist in the environment in the absence of selection pressure (McManus et al. 2002). This conclusion, however, was based on surveys over a rather short time or on low detection incidences.

Strains of *E. amylovora* with intermediate resistance to streptomycin were also detected in early surveys at low incidence (Coyier and Covey 1975), but these strains were not further characterized. MR-L strains with MIC values of less than 50 µg/ml may also have been overlooked in some studies. For detecting resistance, media amended with streptomycin concentrations of 50 µg/ml or higher have often been used (Chiou and Jones 1991) because strains of *E. amylovora* with *strA/strB*-mediated resistance residing on pEa34 or pEA29 were characterized to grow at 100 µg/ml (Chiou and Jones 1991; McGhee et al. 2011), while those with *rpsL* mutations were able to grow at 2,000 µg/ml or higher (Chiou and Jones 1995). Strains from our samplings that were characterized as MR-L and harboring the *strA-strB* gene pair had MIC values of 7.0 to 37.3 µg/ml (mean = 20.6 µg/ml), and thus, were

Fig. 4. Clustered regularly interspaced short palindromic repeats (CRISPR) sequence analysis of the CRISPR1 spacer array pattern from seven *Erwinia amylovora* strains from California with streptomycin sensitive (Sm-S), moderately resistant-low (MR-L), or highly resistant (HR) phenotypes. Spacer sequences from strains are aligned vertically. Identical spacers (aligned within rows) are color coded the same and also numbered (numbering system from a previous study [McGhee and Sundin 2012]). White blocks indicate the absence of a particular spacer. The * symbol refers to a 31-bp sequence insert previously identified in 11 western U.S. strains from apple and pear (McGhee and Sundin 2012). Strains examined are as follows: 531(Sm-S), 542 (MR-L), 534 (MR-L), 552 (HR), 550 (HR), 536 (MR-L), and 529 (Sm-S). Pattern 12 from streptomycin-sensitive *E. amylovora* strain CA1R (isolated in California) was characterized in a previous study (McGhee and Sundin 2012).

inhibited by lower concentrations of streptomycin than previously characterized resistant strains of *E. amylovora*.

Molecular analysis of the transposon carrying *strA-strB* in MR-L strains from our surveys indicated that it lacked any insertion sequence (IS). In *E. amylovora* strains with plasmid-mediated *strA-strB* resistance described previously from other locations, IS1133 within Tn5393 possesses an outwardly-directed promoter that increases the expression of *strA-strB* (Chiou and Jones 1993; McManus et al. 2002; McGhee et al. 2011; Sundin and Bender 1995). The transposon in MR-L strains was therefore similarly organized as in *Pseudomonas syringae* pv. *syringae* where it was termed Tn5393a to differentiate it from Tn5393 that contains IS1133 (Sundin and Bender 1995). The absence of IS1133 in California MR-L strains provides a likely explanation for the lower levels of resistance that we determined. Streptomycin is usually applied at a field rate of 50 to 100 µg/ml in California. Nevertheless, we recovered high percentages of *E. amylovora* MR-L strains in several years associated with control failures in some cases. Streptomycin applications may be diluted to sublethal concentrations on plant surfaces once applied to the crop and the antibiotic can potentially break down in the environment. These factors, along with repeated usage and selection, can enhance the survival and increase of MR-L strains in California pear orchards.

In addition to the ubiquitous virulence plasmid pEA29, several other indigenous, cryptic plasmids have been described in *E. amylovora* and shown to have a limited distribution (Foster et al. 2004; Ismail et al. 2014; Llop et al. 2011). Of these, pEa34, pEA29, and pEa8.7 were shown to carry the functional *strA-strB* gene pair in selected populations. Plasmid pEU30 was first described in 2004, and in evaluations of a worldwide collection of *E. amylovora*, was only found in strains from apple and pear from the western United States, specifically, Oregon, Washington, and Utah (Foster et al. 2004). The function of this plasmid is not clear, but it does not appear to encode any virulence genes. In evaluating over 70 representative MR-L strains that we collected between 2006 and 2014 in California, Tn5393a and *strA-strB* were always associated with pEU30. We hypothesize that Tn5393a was introduced into an *E. amylovora* strain carrying pEU30 and transposed into that plasmid. This hypothesis is supported by our CRISPR results that show that two MR-L strains share the same

CRISPR1 pattern as a streptomycin-sensitive strain. Since the nt 2839 insertion point for Tn5393a was found in all cases, this introduction likely occurred only once. It should be noted that pEU30 encodes a full set of conjugation genes (Foster et al. 2004), and thus, the Tn5393a-encoding pEU30 plasmid could move by conjugation within the population.

The evolution of streptomycin resistance in *E. amylovora* has included both spontaneous mutation of the chromosomal *rpsL* target gene and several independent occurrences of acquisition of the *strA-strB* gene pair (Fig. 5). The *strA-strB* genes and transposon Tn5393 are widely distributed among gram-negative bacterial pathogens of humans, animals, and plants, and among environmental bacteria from many diverse habitats (Chiou and Jones 1993; Sundin and Bender 1996a). Thus, the acquisition of these genes by *E. amylovora* is not surprising, and the independent acquisition of variants of Tn5393 by *E. amylovora* in California and in Michigan indicates that *E. amylovora* strains have participated in genetic exchanges with other bacteria in orchard environments. The *strA-strB* genes have been acquired on two different variants of transposon Tn5393, and in *E. amylovora* populations in California and Michigan, these transposons have ultimately inserted into the indigenous plasmids pEU30 and pEA29, respectively. The evolution of antibiotic resistance involving acquired resistance genes in bacterial human pathogens typically involves stabilization of the acquired genes in their hosts after initial acquisition through movement of the genes into genetic elements that do not have a negative effect on host fitness (Levy 1986). For example, in streptomycin-resistant *E. amylovora* strains from Michigan, the initial acquisition of Tn5393 on the nonnative plasmid pEa34 was followed by movement of Tn5393 onto the native ubiquitous plasmid pEA29 (McGhee et al. 2011). Similarly in a streptomycin-resistant population of *P. syringae* from Oklahoma, Tn5393 has moved to several indigenous plasmids (Sundin and Bender 1996b). Except for *E. amylovora* populations in Washington and Oregon, the HR chromosomal mutation phenotype is only present at a low incidence in other regions in the United States where streptomycin resistance has been observed (Fig. 5). This observation suggests the possibility that streptomycin resistance conferred by Tn5393 represents a phenotype of enhanced ecological fitness compared with the HR phenotype in *E. amylovora*.

Although the incidence of MR-L strains was high in the first few years of our surveys, prevalence of these strains fluctuated widely and has declined to very low levels in 2013 and 2014. In addition, the frequency of HR strains was low in our surveys, correlating with previous results (Moller et al. 1981) indicating a decline of these strains in the population. Thus, both types of resistance appear to lack persistence under California conditions. Although we hypothesize that the presence of Tn5393a on pEU30 in California *E. amylovora* strains has reduced a negative fitness effect of the transposon in these strains, there remains a possibility that the pEU30::Tn5393a plasmid is unstable. This potential instability leading to reversion to streptomycin sensitivity, and the possibility that streptomycin can still inactivate MR-L strains when present on flower surfaces at levels higher than 50 µg/ml, could cause the reductions in incidence that we have observed. With current low resistance levels present in California growing regions, high labeled rates of streptomycin could be successfully used again in conventional pome fruit production, but the number of applications per season should be limited to one or two and the antibiotic should be mixed and rotated with different modes of action such as copper, oxytetracycline, or the newly registered kasugamycin (Adaskaveg et al. 2011; McGhee and Sundin 2011).

Streptomycin resistance in *Erwinia amylovora*

HIGH RESISTANCE – *rpsL*-mediated

MODERATE RESISTANCE – *strA-strB*-mediated

1. *strA-strB* resistance genes on transposon

2. *strA-strB* resistance genes not on transposon

Fig. 5. Evolution of streptomycin resistance in *Erwinia amylovora* through chromosomal mutation to high resistance or via acquisition of the *strA-strB* gene pair conferring differing levels of resistance based on their presence within Tn5393a or Tn5393. Further evolution of *strA-strB*-mediated resistance involves localization of resistance determinants on the indigenous plasmids pEU30, pEa34, pEA29, or pEa8.7, or integration in the bacterial chromosome.

ACKNOWLEDGMENTS

We thank University of California Cooperative Extension farm advisors C. Ingles and R. Elkins for assisting in sampling of pear orchards and K. Nguyen and M. Vilchez for helping with in vitro sensitivity assays. We

also thank the California Pear Board, the Michigan Apple Committee, and Michigan Agricultural Experiment Station for financially supporting this research.

LITERATURE CITED

- Adaskaveg, J. E., Förster, H., and Wade, M. L. 2011. Effectiveness of kasugamycin against *Erwinia amylovora* and its potential use for managing fire blight of pear. *Plant Dis.* 95:448-454.
- Bereswill, S., Pahl, A., Bellemann, P., Zeller, W., and Geider, K. 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. *Appl. Environ. Microbiol.* 58:3522-3536.
- Bhaya, D., Davison, M., and Barrangou, R. 2011. CRISPR-Cas systems in Bacteria and Archaea: Versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* 45:273-297.
- Chiou, C.-S., and Jones, A. L. 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. *Phytopathology* 81:710-714.
- Chiou, C.-S., and Jones, A. L. 1993. Nucleotide sequence analysis of a transposon (Tn5393) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. *J. Bacteriol.* 175:732-740.
- Chiou, C.-S., and Jones, A. L. 1995. Molecular analysis of high-level streptomycin resistance in *Erwinia amylovora*. *Phytopathology* 85:324-328.
- 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.
- El-Goorani, M. A., and El-Kasheir, H. M. 1989. Distribution of streptomycin resistant strains of *Erwinia amylovora* and control of fire blight disease in Egypt. *J. Phytopathol.* 124:137-142.
- Förster, H., Kanetis, L., and Adaskaveg, J. E. 2004. Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus-fungicide interactions. *Phytopathology* 94:163-170.
- Foster, G. C., McGhee, G. C., Jones, A. L., and Sundin, G. W. 2004. Nucleotide sequences, genetic organization, and distribution of pEU30 and pEL60 from *Erwinia amylovora*. *Appl. Environ. Microbiol.* 70:7539-7544.
- Ismail, E., Blom, J., Bultreys, A., Ivanovic, M., Obradovic, A., van Doorn, J., Bergsma-Vlami, M., Maes, M., Willems, A., Duffy, B., Stockwell, V. O., Smits, T. H. M., and Pulawska, J. 2014. A novel plasmid pEA68 of *Erwinia amylovora* and the description of a new family of plasmids. *Arch. Microbiol.* 196:891-899.
- Jones, A. L., and Schnabel, E. L. 2000. The development of streptomycin-resistant strains of *Erwinia amylovora*. Pages 235-251 in: *Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora*. J. L. Vanneste, ed. CABI Publishing, Wallingford, UK.
- Levy, S. B. 1986. Ecology of antibiotic resistance determinants. *Banbury Rep.* 24:17-30.
- Llop, P., Cabrefiga, J., Smits, T. H. M., Dreö, T., Barbe, S., Pulawska, J., Bultreys, A., Blom, J., Duffy, B., Montesinos, E., and Lopez, M. M. 2011. *Erwinia amylovora* novel plasmid pEI70: complete sequence, biogeography, and role in aggressiveness in the fire blight phytopathogen. *PLoS One* 6:e28651.
- 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 in *Erwinia amylovora* isolated from pear orchards in Washington State. *Plant Dis.* 75:287-290.
- Manulis, S., Zutra, D., Kleitman, F., Dror, O., David, I., Zilberstaine, M., and Shabi, E. 1998. Distribution of streptomycin-resistant strains of *Erwinia amylovora* in Israel and occurrence of blossom blight in the autumn. *Phytoparasitica* 26:223-230.
- McGhee, G. C., Guasco, J., Bellomo, L. M., Blumer-Schuette, S. E., Shane, W. W., Irish-Brown, A., and Sundin, G. W. 2011. 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. *Phytopathology* 101:182-191.
- McGhee, G. C., and Jones, A. L. 2000. Complete nucleotide sequence of ubiquitous plasmid pEA29 from *Erwinia amylovora* strain Ea88: Gene organization and intraspecies variation. *Appl. Environ. Microbiol.* 66: 4897-4907.
- McGhee, G. C., and Sundin, G. W. 2011. Evaluation of kasugamycin for fire blight management, effect on nontarget bacteria, and assessment of kasugamycin resistance potential in *Erwinia amylovora*. *Phytopathology* 101: 192-204.
- McGhee, G. C., and Sundin, G. W. 2012. *Erwinia amylovora* CRISPR elements provide new tools for evaluating strain diversity and for microbial source tracking. *PLoS One* 7:e41706.
- McManus, P. S., and Jones, A. L. 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. *Phytopathology* 84:627-633.
- 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.
- Moller, W. J., Beutel, J. A., Reil, W. O., and Zoller, B. G. 1972. Fireblight resistance to streptomycin in California. *Phytopathology* 62:779.
- Moller, W. J., Schroth, M. N., and Thomson, S. V. 1981. The scenario of fire blight and streptomycin resistance. *Plant Dis.* 65:563-568.
- Palmer, E. L., Teviotdale, B. L., and Jones, A. L. 1997. A relative of the broad-host-range plasmid RSF1010 detected in *Erwinia amylovora*. *Appl. Environ. Microbiol.* 63:4604-4607.
- Paton, J. H., Holt, H. A., and Bywater, M. J. 1990. Measurement of MICs of antibacterial agents by spiral gradient endpoint compared with conventional dilution method. *Int. J. Exp. Clin. Chemother.* 3:31-33.
- Ponce de León Door, A., Romo Chacón, A., and Acosta Muñoz, C. 2013. Detection of streptomycin resistance in *Erwinia amylovora* strains isolated from apple orchards in Chihuahua, Mexico. *Eur. J. Plant Pathol.* 137: 223-229.
- Rezzonico, F., Smits, T., and Duffy, B. 2011. Diversity, evolution and functionality of CRISPR regions in the fire blight pathogen *Erwinia amylovora*. *Appl. Environ. Microbiol.* 77:3819-3829.
- 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.
- Saad, A. T., Hanna, L., and Choueiri, E. 2000. Evaluation of streptomycin and oxytetracycline resistance of *Erwinia amylovora* populations in Lebanon. (Abstr.) *Phytopathology* 90(suppl.):S68.
- Schroth, M. N., Thomson, S. V., and Moller, W. J. 1978. Streptomycin resistance in *Erwinia amylovora*. *Phytopathology* 69:565-568.
- Shaffer, W. H., and Goodman, R. N. 1985. Appearance of streptomycin-resistant *Erwinia amylovora* in Missouri apple orchards. (Abstr.) *Phytopathology* 75:1281.
- Shariat, N., and Dudley, E. G. 2014. CRISPRs: Molecular signatures used for pathogen subtyping. *Appl. Environ. Microbiol.* 80:430-439.
- Sholberg, P. L., Bedford, K. E., Haag, P., and Randall, 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.
- Sorek, R., Lawrence, C. M., and Wiedenheft, B. 2013. CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annu. Rev. Biochem.* 82: 237-266.
- Sundin, G. W., and Bender, C. L. 1995. Expression of the *strA-strB* streptomycin resistance genes in *Pseudomonas syringae* and *Xanthomonas campestris* and characterization of IS6100 in *X. campestris*. *Appl. Environ. Microbiol.* 61:2891-2897.
- Sundin, G. W., and Bender, C. L. 1996a. Dissemination of the *strA-strB* streptomycin resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol. Ecol.* 5:133-143.
- Sundin, G. W., and Bender, C. L. 1996b. Molecular analysis of closely-related copper- and streptomycin-resistance plasmids from *Pseudomonas syringae* pv. *syringae*. *Plasmid* 35:98-107.
- Thomson, S. V., Gouk, S. C., Vanneste, J. L., Hale, C. N., and Clark, R. G. 1993. The presence of streptomycin resistant strains of *Erwinia amylovora* in New Zealand. *Acta Hort.* 338:223-230.
- Wilson, E. E., Zeitoun, F. M., and Fredrickson, D. L. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. *Phytopathology* 57:618-621.