Evaluation of Kasugamycin for Fire Blight Management, Effect on Nontarget Bacteria, and Assessment of Kasugamycin Resistance Potential in *Erwinia amylovora*

Gayle C. McGhee and George W. Sundin

Department of Plant Pathology, Michigan State University, East Lansing 48824.
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ABSTRACT

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The emergence and spread of streptomycin-resistant strains of *Erwinia amylovora* in Michigan has necessitated the evaluation of new compounds effective for fire blight control. The aminoglycoside antibiotic kasugamycin (Ks) targets the bacterial ribosome and is particularly active against *E. amylovora*. The efficacy of Ks formulated as Kasumin 2L for control of fire blight was evaluated in six experiments conducted over four field seasons in our experimental orchards in East Lansing, MI. Blossom blight control was statistically equivalent to the industry standard streptomycin in all experiments. *E. amylovora* populations remained constant on apple flower stigmas pretreated with Kasumin and were ≈ 100 -fold lower than on stigmas treated with water. Kasumin applied to apple trees in the field also resulted in a 100-fold reduced total culturable bacterial population compared with trees treated with water. We performed a prospective analysis of the potential for kasugamycin resistance (Ks^R) development in *E. amylovora* which focused on spontaneous resistance development and acquisition of a transferrable Ks^R gene. In replicated lab experiments, the development of spontaneous

resistance in *E. amylovora* to Ks at 250 or 500 ppm was not observed when cells were directly plated on medium containing high concentrations of the antibiotic. However, exposure to increasing concentrations of Ks in media (initial concentration 25 $\mu\text{g ml}^{-1}$) resulted in the selection of Ks resistance (at 150 $\mu\text{g ml}^{-1}$) in the *E. amylovora* strains Ea110, Ea273, and Ea1189. Analysis of mutants indicated that they harbored mutations in the kasugamycin target *ksgA* gene and that all mutants were impacted in relative fitness observable through a reduced growth rate in vitro and decreased virulence in immature pear fruit. The possible occurrence of a reservoir of Ks^R genes in orchard environments was also examined. Culturable gram-negative bacteria were surveyed from six experimental apple orchards that had received at least one Kasumin application. In total, 401 Ks^R isolates (42 different species) were recovered from apple flowers and leaves and orchard soil samples. Although we have not established the presence of a transferrable Ks^R gene in orchard bacteria, the frequency, number of species, and presence of Ks^R enterobacterial species in orchard samples suggests the possible role of nontarget bacteria in the future transfer of a Ks^R gene to *E. amylovora*. Our data confirm the importance of kasugamycin as an alternate antibiotic for fire blight management and lay the groundwork for the development and incorporation of resistance management strategies.

Fire blight, caused by the enterobacterial pathogen *Erwinia amylovora*, is a major disease problem and a limiting factor to apple and pear production in most regions of the world where these fruit are grown. On apple, blossom blight disease symptoms are typically initiated in the spring on flowers as overwintering inoculum from cankers is disseminated to the surface of stigmata by rain and insects (33). The pathogen grows as an epiphyte between the papillae on the stigma, and infection potential is most strongly influenced by flower age, temperature, and rainfall, which regulate bacterial population size (35,36,42,50,51). Rain events or heavy dew facilitate the movement of pathogen cells from the stigma to the hypanthium where infection occurs through the floral nectaries (57). Thus, the flower and, in particular, the stigma is a site of pathogen multiplication, and also a site in which pathogen populations can be targeted by management strategies because of their epiphytic surface association in this habitat. Blossom blight control is also critical because successful infection enables the pathogen to multiply and spread internally in trees (inaccessible to bactericides) and allows further amplification of pathogen populations supplying inoculum for subsequent shoot blight infections.

In the 1950s, the antibiotic streptomycin was first utilized for blossom blight control and proved highly efficacious (53). However, intensive use of this antibiotic resulted in the development of streptomycin resistance in *E. amylovora* populations, first in the western United States (10,23,40) and then in Michigan and in other countries, including Canada, Israel, and New Zealand (6,26,31,41,52). Alternatives to streptomycin include the antibiotic oxytetracycline (31,32), the biological control agent Serenade MAX, and antagonistic stigma-colonizing bacteria, including *Pseudomonas fluorescens* A506, *Pantoea agglomerans* C9-1, and *P. agglomerans* E325 (17,37,49). None of these alternate materials is as effective as streptomycin in controlling blossom blight.

In the absence of host resistance, successful bacterial disease control often requires large reductions in population size or effective cessation of bacterial growth. Antibiotics represent the main class of materials currently known with the potential to achieve these required results and, thus, provide the best potential for controlling plant-pathogenic bacteria. However, the availability of antibiotics for plant agriculture is limited because most antibiotics are used in clinical medicine to protect against or cure bacterial infections in humans and are not used in plant agriculture. This restriction is mainly due to the occurrence of transferrable antibiotic resistance in bacterial populations and the negative impact of antibiotic resistance on controlling human infections (11). In contrast, the antibiotics that can be used in

Corresponding author: G. W. Sundin; E-mail address: sundin@msu.edu

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animal agriculture, both therapeutically and for growth promotion, overlap widely with those used in clinical medicine (19). This overlap is thought to be responsible for antibiotic resistance problems in human pathogens arising from either the transfer of antibiotic-resistant human-pathogenic bacteria from agricultural animals to humans or the transfer of antibiotic resistance genes from an animal agricultural reservoir to the human bacterial community (43).

Although most known streptomycin-resistant (Sm^R) strains of *E. amylovora* are spontaneous mutants with a single nucleotide alteration in the chromosomal *rpsL* gene, the presence of the transmissible *strA-strB* resistance genes on the transposon Tn5393 is the predominant mechanism of streptomycin resistance in *E. amylovora* strains isolated in Michigan and New York (7,29,38). The Tn5393 transposon is widely distributed among other Sm^R plant pathogens, including *Pseudomonas syringae*, that were isolated from plant hosts treated with streptomycin for bacterial disease control (46,47). Tn5393 is also carried by nontarget phyllosphere bacteria isolated from orchards and ornamental tree nurseries where streptomycin was used for bacterial disease control (7,48).

It was originally hypothesized in the medical literature that antibiotic use initially selects for resistance in nontarget bacteria inhabiting niches in close proximity to that of the target pathogens (21). These nontarget bacteria are then thought to transfer the resistance determinants into the target pathogen. This same route of transfer was hypothesized to occur in the evolution of streptomycin resistance in *E. amylovora* because the Sm^R plasmid pEa34 encoding Tn5393 was thought to have been transferred from the orchard epiphyte *Pantoea agglomerans* into *E. amylovora* (7). Thus, any evaluations of new antibiotics for disease control must also include an examination of the effect of the antibiotic on nontarget bacteria and the potential for resistance development in both the pathogen and the nontarget populations.

Kasugamycin is an aminoglycoside antibiotic in the same class as streptomycin but with a different mode of action (9). Kasugamycin was originally developed as a fungicide to control rice blast caused by *Piricularia oryzae*, and has also been evaluated as a bactericide and examined for fire blight control in the 1980s (34). Further testing of kasugamycin on apple was suspended until recently due to problems with phytotoxicity. The negative impact of streptomycin resistance resulting in disease control failures, and the lack of suitable alternatives, has necessitated a new assessment of the potential applicability of kasugamycin for fire blight management. We hypothesized that Kasumin 2L (Arysta Lifescience, Cary, NC), a different formulation than that used in past studies, would be efficacious against the blossom blight phase of fire blight and would represent a viable long-term alternative to streptomycin in plant disease control because of a lack of clinical or animal agriculture uses of this antibiotic. However, the risk of resistance development needed to be evaluated, from both the standpoint of the potential for spontaneous resistance mutations and the possibility of resistance gene transfer from nontarget orchard bacteria. In this study, we evaluated the efficacy of Kasumin for fire blight control, assessed the potential for the selection of spontaneous mutants of *E. amylovora* with resistance to kasugamycin, analyzed the effect of Kasumin on nontarget orchard bacteria, and surveyed apple flowers, leaves, and orchard soil for bacterial isolates with reduced sensitivity to kasugamycin to estimate the risk potential for the development of kasugamycin resistance in *E. amylovora*.

MATERIALS AND METHODS

Bacterial strains and growth media. A spontaneous rifampicin (Rif)-resistant mutant of *E. amylovora* Ea110 was used as pathogen inoculum in all field experiments. Strain Ea110 was isolated from a diseased apple tree in Michigan (31) and was

stored in 15% glycerol at -80°C prior to use. In laboratory experiments, we also used *E. amylovora* Ea273 and Ea1189; Ea273 was isolated in New York and the genome sequence of this strain has been determined (http://www.sanger.ac.uk/Projects/E_amylovora/), and Ea1189 was isolated in Germany and we routinely use this strain for genetic studies (58). Each of the strains used in the studies was sensitive to kasugamycin and streptomycin. *E. amylovora* strains were grown in Luria Bertani (LB) medium at 28°C and apple orchard bacteria were isolated on King's medium B (KB) (24) with cycloheximide added at $50\text{ }\mu\text{g ml}^{-1}$ to inhibit fungal growth. Antibiotics were added to media as necessary, including kasugamycin (100 , 250 , and $500\text{ }\mu\text{g ml}^{-1}$), Rif ($100\text{ }\mu\text{g ml}^{-1}$), and streptomycin ($100\text{ }\mu\text{g ml}^{-1}$).

Effect of Kasumin on *E. amylovora* populations on apple flower stigmas. Antibiotic treatments consisted of either a standard treatment (Agri-Mycin; 17% streptomycin sulfate; Nufarm Americas, Burr Ridge, IL) or a kasugamycin treatment (Kasumin; Arysta LifeSciences). The nonionic surfactant Regulaid (Kalo Inc., Overland Park, KS) was used in antibiotic applications to aid in surface area coverage and increase penetration of the antibiotics into flower tissue, and for increased retention of antibiotics to plant material.

One-year-old shoots of apple cv. Gala with 5 to 10 king flowers open were removed from trees maintained in an East Lansing, MI orchard and transported to the laboratory. Tissue ($\approx 2.5\text{ cm}$) was trimmed from the base of each shoot, and groups of three shoots were then placed in floral foam saturated with water in 1-liter plastic containers. Bacterial inoculum consisted of *E. amylovora* Ea110 which was cultured overnight in LB at 28°C . Bacterial cells were washed twice in sterile $0.5\times$ phosphate buffered saline (PBS) and resuspended to a final concentration of $1 \times 10^8\text{ CFU ml}^{-1}$ in $0.5\times\text{ PBS} + 0.3\%\text{ Tween }20$ as a surfactant. Either $2\text{ }\mu\text{l}$ of bacterial cells or $2\text{ }\mu\text{l}$ of water as a control were pipetted directly onto the stigmatic surfaces of the king bloom within each flower cluster. The shoots with inoculated flowers were then incubated in a growth chamber at 27°C for 24 h, after which $5\text{ }\mu\text{l}$ of Kasumin ($100\text{ }\mu\text{g ml}^{-1}$ solution) + $0.03\%\text{ Regulaid}$, $5\text{ }\mu\text{l}$ of Agrimycin ($100\text{ }\mu\text{g ml}^{-1}$ solution) + $0.03\%\text{ Regulaid}$, or $5\text{ }\mu\text{l}$ of water + $0.03\%\text{ Regulaid}$ were pipetted directly onto the inoculated stigmas.

Treatments were placed randomly from available king flowers on each shoot and multiple treatments were placed on each shoot. Following a second incubation in a growth chamber at 27°C for 24 h, individual stigmas were dissected from flowers, placed in 1 ml of $0.5\times\text{ PBS}$, and sonicated for 1 min in an ultrasonic bath (model 250T; VWR Scientific, Houston). The solution remaining following sonication was serially diluted and appropriate dilutions were spread on LB + Rif agar plates. Plates were incubated at 27°C for 48 h, after which the Ea110 colonies were enumerated. Two separate experiments were conducted. Statistical analyses of pooled $\log_{10}$ -transformed population data from both experiments were done using a one-way analysis of variance (ANOVA), and 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/mstac.htm>) for all statistical analyses of data from this study.

A second set of experiments was conducted in which the antibiotics were applied to stigmas prior to the bacterial inoculum. The apple shoots and bacterial inoculum were prepared as described above. In these experiments, apple flowers on the shoots were sprayed to runoff with a solution of either Kasumin at $100\text{ }\mu\text{g ml}^{-1} + 0.03\%\text{ Regulaid}$, Agri-Mycin at $100\text{ }\mu\text{g ml}^{-1} + 0.03\%\text{ Regulaid}$, or $0.03\%\text{ Regulaid}$ as a control. The shoots were allowed to dry for 30 min in a laminar flow hood, after which stigmas from king flowers were inoculated with $2\text{ }\mu\text{l}$ of cells of *E. amylovora* Ea110 ($1 \times 10^8\text{ CFU ml}^{-1}$). The bacterial inoculum was allowed to dry on stigmas for 10 min before a time 0 ($T = 0$) sample was taken. The five stigmas from each king flower were

then dissected out of each flower, placed in 1 ml of sterile 0.5× PBS, and sonicated for 1 min. The solution remaining following sonication was diluted and plated on LB + Rif agar. Time points for sampling were at T = 0, 6, and 24 h postinoculation. Five flowers per time point for each treatment were removed and a mean of stigma populations for each of the five stigmas harvested was used to generate population data. This experiment was conducted twice. Statistical analyses of pooled log₁₀-transformed population data from both experiments were done using a one-way ANOVA, and mean separation ($P < 0.05$) was accomplished using Fisher's protected least significant difference test.

Efficacy experiment plot design: inoculum preparation, pathogen inoculation, treatment applications, and disease assessment. Six field experiments designed to test the efficacy of antibiotics for blossom blight control were conducted over the course of 4 years in experimental orchards located in East Lansing, MI. The orchards consisted of blocks of ≈50 to 100 apple trees of either cv. Jonathan grafted on MM.106 rootstock or cv. Gala grafted on M.9 rootstock. Antibiotic treatments were applied to trees ≈24 h prior to or 24 h following inoculation, or both, with *E. amylovora* Ea110. Treatments were arranged in a randomized complete block design with five single-tree replicates per treatment. Disease incidence data from each individual experiment were arcsine-transformed and analyzed using ANOVA, and mean separation ($P < 0.05$) was accomplished using Fisher's protected least significant difference test.

Bacterial cultures of *E. amylovora* Ea110 cells for field inoculations were grown in LB broth at 27 to 28°C to late log-early stationary phase. Cell suspensions were adjusted turbidimetrically to the desired cell density (10^6 to 10^7 CFU ml⁻¹) in 0.5× sterile PBS and kept on ice during transfer to the field and prior to inoculations. Apple trees were inoculated by spraying flower clusters around the perimeter of each tree with the *E. amylovora* Ea110 cell suspension. An 11.4-liter pump mist sprayer (Solo, Newport News, VA) was used for the inoculations. Inoculation occurred at full bloom during the evening to ensure conditions favorable for bacterial survival.

All field experiments included a control treatment (nontreated, pathogen-inoculated trees), a standard treatment (Agri-Mycin + 0.03% Regulaid, pathogen-inoculated trees), and kasugamycin treatments (Kasumin + 0.03% Regulaid, pathogen-inoculated trees). Antibiotic treatments were applied with a handgun sprayer at 24.6 kg/cm² and 2,800 liters of water per hectare. Application timings were generally at full bloom. Following a sufficient duration to allow blossom blight infection to occur (usually ≈28 days), trees were rated for the incidence of blossom blight symptoms by counting the number of flower clusters with blossom blight strikes in a random sample of 210 flower clusters per tree. A cluster was considered infected if one or more flowers exhibited fire blight symptoms.

Experiment 1 was conducted in 2006 with antibiotic application timings on 3 and 5 May and pathogen inoculation on 4 May. Experiments 2 and 3 were conducted in 2007 in two separate blocks of apple cv. Jonathan trees with antibiotic application timings on 4 and 7 May and pathogen inoculation on 6 May. Experiment 4 was conducted in 2008 with antibiotic application timings on 8 and 12 May and pathogen inoculation on 10 May. Experiment 5 was conducted in 2008 with antibiotic application timings on 5 and 7 May and pathogen inoculation on 6 May. Experiment 6 was conducted in 2009 with pathogen inoculation on 7 May and antibiotic application on 8 May. Pathogen inoculum consisted of suspensions of *E. amylovora* Ea110 in water at a concentration of 10^7 CFU/ml (experiments 1, 4, 5, and 6) or 10^6 CFU/ml (experiments 2 and 3).

Effect of Kasumin on nontarget bacteria inhabiting apple flowers and leaves. The effect of Kasumin on population size of nontarget bacteria on apple flowers and leaves was evaluated with a field experiment conducted in 2008 and 2009. Four single-tree

replicates of apple cv. Jonathan grafted on MM.106 rootstock were used for each treatment. All field experiments included a control treatment (water), a standard treatment (Agri-Mycin at 100 ppm), and a kasugamycin treatment (Kasumin at 100 ppm). All treatments were applied with a handgun sprayer at 24.6 kg/cm² and 2,800 liters of water per hectare. A single application was done at full bloom. Flower samples for bacterial population analysis were collected immediately prior to antibiotic application and 1 week after application. Leaf samples were collected 2 weeks after antibiotic application. Flower sample replicates consisted of 25 flowers from each of five trees (25 total flowers per replicated sample). Five samples were collected from each orchard. Leaf samples were taken similarly, except one leaf sample consisted of 25 leaves.

All samples were placed on ice in coolers for return to the laboratory and maintained at 4°C in the laboratory. Individual flower samples (25 flowers) were weighed and placed into 25 or 50 ml of sterile PBS and shaken vigorously by hand for 1 min. Appropriate serial dilutions from this suspension were spread onto KB medium amended with cycloheximide at 50 µg ml⁻¹ (KBc) for population analysis of total culturable bacteria. Individual leaf samples were cut into 2.5-cm strips, placed in a plastic bag, and shaken to mix. From each of the five leaf samples, 2 g was placed in 20 ml of chilled 0.5× PBS and sonicated for 7 min in an ultrasonic bath. Samples (0.1 ml) from appropriate serial dilutions of the sonicated solution were spread on KBc and on KBc also amended with kasugamycin at 100 µg ml⁻¹ (KBksc). All population data were log₁₀-transformed and data from both experiments were pooled prior to statistical analyses. An ANOVA was run, and mean separation ($P < 0.05$) was accomplished using Fisher's protected least significant difference test and the M-STAT-C 4.0 program. Data were analyzed for differences between sampling times within each treatment, and the effect of different treatments on population size was also assessed per each sampling time.

Isolation and identification of kasugamycin-resistant nontarget bacteria from apple orchards. We sampled flowers, leaves, and soil from five apple orchards in Michigan. Flower and soil samples were taken from two of the orchards prior to application of Kasumin to trees. Following application of Kasumin, leaf samples were taken from three orchards and soil samples were taken from all five orchards. All samples were chosen randomly from the orchards. Flower and leaf samples were collected and processed as described above. Soil samples consisted of five bulked soil cores (≈7.5 cm in depth) taken from under the dripline of trees; five samples were taken per orchard. From each of five soil cores composing a sample, 2 g was bulked and transferred into 20 ml of chilled 0.5× PBS and sonicated for 7 min in an ultrasonic bath (model 250T; VWR Scientific). Samples (0.1 ml) from appropriate serial dilutions of the sonicate were spread on KBc and KBksc. All incubations of culture plates were done at 28°C for 72 h following which bacterial colonies were counted.

After bacterial colonies were counted on plates, a sample of colonies was selected for analysis from both KBc and KBksc. The colonies selected from KBc were included to obtain a small representation of genera present on flowers and leaf surfaces because very few of these organisms were recovered on KBksc. Colony selection was done randomly; however, we also attempted to select a variety of colony morphologies. Bacteria were initially screened using the rapid gram-reaction test in 3% KOH to detect gram-negative colonies (39). In total, 588 presumptive gram-negative colonies were recovered for further analysis, purified by single-colony streaking, and stored in 15% glycerol at -70°C.

We sequenced a portion of the 16S rDNA for identification analysis (8). Genomic DNA preps were done on each isolate by boiling for 15 min in a lysis buffer (0.5 M KCl, 0.01 M Tris-HCl [pH 8.5], and 1% Tween20). The lysate was used as template in PCR reactions. Forward primer 63f (5'-CAG GCC TAA CAC

ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3') were used to amplify $\approx 1,300$ bp of the bacterial 16S rRNA gene as described by Marchesi et al. (27). Amplified products were purified using the QIAquick PCR Purification Kit (Qiagen; Valencia, CA) and sequenced. All sequencing was performed at the Michigan State University Department of Energy Plant Research Laboratory sequencing 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 for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/blast>).

Selection of spontaneous kasugamycin resistance and genetic basis of resistance. Two separate experiments were designed to determine whether *E. amylovora* could develop spontaneous kasugamycin resistance. In the first experiment, *E. amylovora* strains Ea110, Ea1189, and Ea273 were each grown overnight in LB broth, diluted 10-fold in 0.5 $\times$ PBS, and plated onto LB containing kasugamycin (LBks) at either 100, 250, or 500 $\mu\text{g ml}^{-1}$. In addition, cells were plated on LB containing streptomycin at 100 $\mu\text{g ml}^{-1}$ as a control. The plates were incubated for 48 h at 24°C in the dark prior to visual examination for the occurrence of resistant colonies. Plates were also reexamined after 120 h of incubation. This experiment was repeated three times for each strain.

In the second experiment, *E. amylovora* strains Ea110, Ea1189, and Ea273 were each grown overnight in LB broth, and then subcultured into fresh LB broth medium amended with kasugamycin at 25 $\mu\text{g ml}^{-1}$. If the strain was able to grow in the kasugamycin concentration it was exposed to, cells were subcultured to a progressively higher kasugamycin concentration every 24 h. If the cells were unable to grow, they were passaged in the same antibiotic concentration until they were able to acclimate to the antibiotic level. In this manner, *E. amylovora* strains were gradually exposed to levels of kasugamycin at 25, 50, 100, 125, 150, 175, and 200 $\mu\text{g ml}^{-1}$. This process was continued until the strain could grow and form colonies on LBks amended with kasugamycin at 150 $\mu\text{g ml}^{-1}$. All strains capable of growth on LBks were also assayed for resistance to streptomycin on LB containing streptomycin at 100 $\mu\text{g ml}^{-1}$.

The *ksgA* gene, a known target for kasugamycin in *Escherichia coli*, was isolated using PCR and sequenced for any *Erwinia amylovora* strain growing on LB containing kasugamycin at 150 $\mu\text{g ml}^{-1}$. Mutation of *ksgA* has been previously shown to be a genetic determinant of resistance (55) because the KsgA protein functions in the methylation of the ribosome. As described above, genomic DNA from kasugamycin-resistant (Ks^R) and wild-type *E. amylovora* was boiled for 15 min in lysis buffer (0.5 M KCL, 0.01 M Tris-HCl [pH 8.5], and 1% Tween 20). The lysate was used as template in PCR reactions. Forward primer KsgA-F (5'-ATCAGGGGCTTCCGGTGCTAAAT) and reverse primer KsgA-R (5'-AATATGCGGCTGTTCTCCACCAC) were developed from the *E. amylovora* genome (http://www.sanger.ac.uk/Projects/E_amylovora) to amplify a 1,232-bp fragment of the *E. amylovora* chromosome containing the *ksgA* gene. Amplified products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced at the Michigan State University Gene Technology Sequencing Facility using ABI dye-terminator chemistry and an ABI 3730 genetic analyzer (Applied Biosystems). Sequence analysis was conducted using the Lasergene suite of software.

Relative fitness of spontaneous Ks^R mutants of *E. amylovora*. Experiments were conducted to compare the virulence of newly evolved spontaneous Ks^R *E. amylovora* strains with their wild-type kasugamycin-susceptible (Ks^S) ancestral strains. Virulence assays using 10 replicate immature pear fruit per strain per experiment were performed essentially as described previously (5), except a 5- μl inoculum dose (1×10^8 CFU/ml) was used.

Following inoculation, pear fruit were incubated in a humidified chamber for 7 days at 28°C. In similar virulence experiments, bacterial population size was assessed over the first 3 days after inoculation using destructive sampling. Necrotic lesion size was measured each day beginning on day 2 after inoculation and continuing through day 7. Two replicate experiments were conducted for each strain. Statistical analyses of lesion size data pooled from two experiments were done using a one-way ANOVA; pooled data values for each day were analyzed separately. Mean separation ($P < 0.05$) was accomplished using Fisher's protected least significant difference test.

Fitness experiments comparing bacterial populations of newly evolved Ks^R *E. amylovora* strains with their wild-type Ks^S ancestral strains were conducted separately from the virulence study using 20 replicate immature pear fruit per strain per experiment. Following inoculation as described above, five replicate pear fruit were destructively sampled per treatment for the day 0 time point prior to pear fruit being incubated in a humidified chamber for 4 days. Briefly, each day for days 0 to 3 after inoculation, five pear replicates were randomly selected for analysis. Using a no. 2 sterile cork borer calibrated to a depth of 1 cm, pear tissue plugs taken from the inoculation site were removed and placed individually into 450 μl of 0.5 $\times$ PBS in a 1.5-ml microfuge tube and ground to release bacteria from pear tissue. The homogenized pear tissue-PBS slurry was allowed to soak for 30 min to maximize bacterial release from tissue before appropriate serial dilutions were plated onto either LB (for wild-type controls) or LBks agar. This experiment was repeated twice per strain tested. In each experiment, the control was run concurrently with the mutant strain tested. Population data were $\log_{10}$ -transformed prior to analysis. Replicate data for each time point were pooled from both experiments, and populations means and standard error of the means were calculated.

The in vitro growth rate of the newly evolved Ks^R *E. amylovora* strains was also compared with that of their wild-type ancestor in minimal medium (MM) (28) and LB broth. All bacteria were grown overnight in LB broth, diluted 1:10 in 0.5 $\times$ PBS, and then diluted 1:100 in either MM or LB broth. Diluted bacterial suspension in media (2 ml) was placed in three replicate wells of a sterile 24-well microtiter plate (Evergreen Scientific, Los Angeles) and sealed with the Breathe-Easy gas permeable sealing membrane, regular strength (Diversified Biotech, Boston, MA). Sealed plates were then placed in a Tecan Safire plate reader (Tecan, Durham, NC). Optical readings were taken every 5 min using a wavelength of 600 nm, with 30 s of shaking at 2.5 min and prior to each read. Cultures were allowed to settle for 10 s after shaking and before optical density measurements were taken. Readings were taken for 24 h for LB cultures and 36 h for MM cultures. Media and well blanks were also included in each run.

Optical density at 600 nm (OD_{600}) values from growth curves were standardized by dividing by the initial OD_{600} (0 h) and then $\log_2$ transformed. The transformed values were plotted and the window of exponential growth was identified by linear regression. Lag time was calculated simply as the x -intercept of the linear equation (20).

Doubling time (g) was determined by the equation $g = 3.3[\log_{10}(N2) - \log_{10}(N1)]/t$, where $N1$ and $N2$ are the original nontransformed OD_{600} values corresponding to the window of exponential growth observed in the $\log_2$ transformation plot and t is the time interval between them (25). Values for both lag time and doubling time were compared by two-tailed paired t tests.

Differential sensitivity of bacteria to kasugamycin and Kasumin. During this work, we discovered that many bacterial isolates examined appeared to be more susceptible to the formulated product Kasumin than to kasugamycin, when either material was added to media to yield equivalent concentrations of kasugamycin. To further investigate this observation, a subset of

55 isolates both sensitive and exhibiting varying levels of kasugamycin resistance were selected from the 588 environmental isolates originally screened for kasugamycin resistance. We also examined the sensitivity of a collection of 18 *E. amylovora*, 1 *E. pyrifoliae*, and 1 *Erwinia* sp. strains which included *E. amylovora* Ea110, Ea273, and Ea1189 and the additional strains Al-1, Be-A8, Bcn20, CA11, Ea88, Ea322, EL01, IH3-1, LebA19, MA-1, MI5-1, MR1, OR25, S5, and UtRJ2 (12,26,29) and *E. pyrifoliae* Ep1/96 (26) and *Erwinia* sp. Ejp557 (28). Nine of the *E. amylovora* strains were resistant to streptomycin, either via a spontaneous mutation or through acquisition of Tn5393 (12,28,31). Bacterial growth was assessed on KB medium containing either Kasumin (containing 2% kasugamycin) or technical-grade kasugamycin (Sigma-Aldrich, St. Louis), with both at concentrations of 100, 250, and 500 $\mu\text{g ml}^{-1}$. In addition, we obtained the formulation blank (Kasumin formulation without kasugamycin) of Kasumin (KFB) from Dr. V. J. Spadafora (Arysta LifeScience), and equivalent volumes of the blank (relative to the volume of Kasumin to generate concentrations of 100, 250, and 500 $\mu\text{g ml}^{-1}$) were added to media as well. Growth was evaluated visually after 2 days of incubation at 28°C in the dark and again after 5 days for slower-growing bacteria. Cultures were considered negative for growth if, after 2 or 5 days, vigorous growth was observed on the unamended KB plate and no growth was observed on the test plate.

RESULTS

Effect of Kasumin on *E. amylovora* populations on apple flower stigmas in vitro. The effect of Kasumin or streptomycin on *E. amylovora* populations on apple stigmas was evaluated either with the antibiotics applied 24 h after *E. amylovora* inoculation or applied immediately prior to *E. amylovora* inoculation. In the first set of experiments, antibiotics were added 24 h after Ea110 inoculation and populations were determined after an additional 24 h of incubation. An examination of data pooled from two separate experiments shows that Ea110 populations on water-treated stigmas were almost 100-fold larger and significantly different than those on Kasumin-treated or streptomycin-treated stigmas (Fig. 1A). When antibiotics were applied immediately prior to *E. amylovora* inoculation, populations of Ea110 on apple stigmas remained steady after 6 h and increased ≈ 10 -fold after 24 h of incubation on streptomycin-treated stigmas (Fig. 1B). Although Ea110 populations on water-treated stigmas also were similar after 6 h, these populations increased ≈ 30 -fold after 24 h and were significantly larger than populations on stigmas treated with Kasumin (Fig. 1B).

Efficacy of Kasumin in blossom blight control. Incidence of fire blight disease, as measured by the occurrence of blossom blight symptoms in inoculated plots, was 35.6 to 72.5% infection in nontreated control trees in six experiments conducted between 2006 and 2009 (Table 1). Application of Kasumin to trees at least 24 h prior to and at least 24 h following inoculation with the virulent *E. amylovora* strain Ea110 resulted in high levels of disease control that were not significantly different from the standard Agri-Mycin treatment in all five experiments (Table 1). In three other experiments, Kasumin was not applied prior to but only applied following Ea110 inoculation. Similar results were observed in comparison with experimental treatments with two Kasumin applications, with disease control levels as high as 96% (Table 1). In one of the experiments comparing the efficacy of Kasumin with Agri-Mycin when both were applied after Ea110 inoculation, control with Agri-Mycin was significantly different than Kasumin (Table 1).

Effect of Kasumin on population size of nontarget bacteria inhabiting apple flowers and leaves. Bacterial populations increased >100 -fold on apple flowers that were sprayed with water whereas populations increased only slightly on flowers sprayed with Kasumin (Fig. 2). In contrast, populations also increased 100-fold on flowers sprayed with Agri-Mycin (Fig. 2). Postapplication bacterial populations on flowers were significantly larger in the water and Agri-Mycin treatments compared with the Kasumin treatment (Fig. 2). Leaf populations were not significantly different from all treatments. Within treatments, the application of water or Agri-Mycin resulted in a significant increase in bacterial populations on flowers, whereas the application of Kasumin did not result in any difference in bacterial population size on flowers (Fig. 2).

Isolation and identification of *Ks^R* bacteria from apple orchards. In total, between 2007 and 2009, 588 gram-negative bacteria were recovered from six experimental apple orchards in Michigan where Kasumin has been applied at least once. Isolates were initially chosen randomly from bacterial colonies growing on LB isolation plates from apple flower or leaf or orchard soil samples. Of these, 401 isolates (68.2%) were capable of growth on LBs at levels of 100 to 500 $\mu\text{g ml}^{-1}$ and were defined as resistant to kasugamycin (Table 2). A greater frequency of isolates from orchard soil (103 of 204; 50.5%) exhibited high-level resistance to kasugamycin with growth on LBs at 500 $\mu\text{g ml}^{-1}$ compared with flower and leaf isolates (71 of 384; 18.5%) (Table 2). Identification of bacteria was accomplished using sequence analysis of the conserved 16S rRNA gene and the identified organisms were differentiated as Enterobacteriaceae (closely related to *E. amylovora*) or other gram-negative bacteria. In total,

Fig. 1. Survival of *Erwinia amylovora* Ea110 on apple flower stigmas when **A**, the bacteria were inoculated 24 h prior to antibiotic application or **B**, antibiotics were applied prior to bacterial inoculation. Treatments are: Kasumin (gray bars), Agri-Mycin (white bars), and water (black bars). The presence of different letters above the bars at each isolation point indicates that the treatment means are significantly different ($P < 0.05$) according to Fisher's protected least significant difference test.

six *E. amylovora* isolates were also recovered during this orchard sampling experiment, and all were found to be sensitive to kasugamycin (Table 3).

The most common bacterial species isolated from apple flowers and leaves in this study were *Pantoea agglomerans* (59.2% of total enterobacterial isolates), *Pseudomonas graminis*, *P. fluorescens*, and *Stenotrophomonas maltophilia* (Table 3). *Pseudomonas* spp. composed 42.9% of all bacteria isolated in this study. Analysis of the growth of the various isolates on LBks medium amended with kasugamycin at either 100, 250, or 500 µg ml⁻¹ revealed that 50.5% (52 of 103) of the enterobacterial isolates and 72.0% (349 of 485) of the other gram-negative isolates were resistant to kasugamycin. The most frequently isolated organism exhibiting high level Ks resistance was *S. maltophilia* while *P. fluorescens* and *Pseudomonas* spp. contained the most isolates with intermediate (250 µg ml⁻¹) resistance (Table 3). The predominance of any particular Ks^R genus varied by site and by year, indicating inherent variability in microbial communities based on geography and environmental factors (data not shown). Of potential concern were species such as *Pantoea agglomerans*, *Pseudomonas fluorescens*, and *P. syringae* that contained both Ks^S and Ks^R individuals (Table 3), indicating the possible acquisition of a kasugamycin resistance gene.

An assessment of the phenotype of sensitivity or resistance to kasugamycin combined with sensitivity or resistance to streptomycin was also done for the 588 identified gram-negative isolates. In all, 49.0% of isolates (288 of 588) were resistant to both antibiotics and 18.9% (111 of 588) were resistant to kasugamycin and sensitive to streptomycin (Table 4). In total, 21.8% of the bacterial isolates were sensitive to kasugamycin and resistant to streptomycin (Table 4). The Ks^R Sm^R multiresistance phenotype was present in isolates from flowers, leaves, and soil and found in all major bacterial groups identified in this study (Table 4).

Selection of spontaneous kasugamycin resistance in *E. amylovora* and genetic basis of resistance. In replicated experiments, *E. amylovora* was evaluated for the development of spontaneous resistance to kasugamycin. When wild-type *E. amylovora* strains Ea110, Ea1189, and Ea273 were grown overnight in broth culture and then directly plated onto LBks plates containing kasugamycin at either 100, 250, or 500 µg ml⁻¹, no resistant colonies were identified following 48 h of incubation. After 120 h, *E. amylovora* colonies did appear as pinpoint colonies. When these pinpoint colonies were subsequently transferred to new LBks plates with the identical level of kasugamycin, no colony growth was observed. This lack of growth on new plates indicated that the growth observed was the result of breakdown of kasugamycin in the media rather than spontaneously-resistant *E. amylovora* colonies.

Because Ks^R colonies were not recovered using a traditional method to select for spontaneous antibiotic resistance, we decided to also use an alternate approach. In this method, we initially seeded three replicate broth cultures of LBks containing kasugamycin at 25 µg ml⁻¹ with *E. amylovora* Ea110, Ea1189, or Ea273. After overnight incubation, a level of growth was observed in each of the broth tubes that was reduced compared with growth in unamended LB broth (data not shown). These cells were exposed

Fig. 2. Population size of total culturable bacteria isolated from apple flowers or leaves prior to and following application of Kasumin, Agri-Mycin, or water to trees. Population means (with standard error) consist of results from five individual tree replicates per treatment. The presence of different small letters above the bars indicates that the means within a treatment are significantly different ($P < 0.05$) according to Fisher's protected least significant difference test. The presence of different capital letters above the bars indicates that the means among treatments at a particular sampling time are significantly different ($P < 0.05$) according to Fisher's protected least significant difference test.

TABLE 1. Disease incidence of fire blight infection (blossom blight) in nontreated apple flowers or in apple flowers treated with antibiotics in six field experiments conducted between 2006 and 2009^w

Exp. cultivar ^x	Application timings ^y			Nontreated control		Kasumin		Agri-Mycin	
	25–50%	70–80%	100%	Infected (%) ^z		Infected (%) ^z	Control (%)	Infected (%) ^z	Control (%)
1, Gala	X	...	X	36.0 a		5.6 b	84	2.9 b	92
2, Jonathan	X	...	X	66.5 a		4.1 b	94	3.5 b	95
3, Jonathan	X	...	X	44.4 a		1.5 b	97	0.2 b	99
	...	...	X	44.4 a		3.3 b	93	nd	nd
4, Gala	...	X	X	35.6 a		5.0 b	86	1.2 b	97
5, Jonathan	...	X	X	44.3 a		2.0 b	96	0.6 b	99
	...	...	X	44.3 a		1.6 b	96	0.3 b	99
6, Jonathan	...	...	X	72.5 a		9.1 c	87	1.4 b	98

^w Means within a row followed by the same letter are not statistically different according to Fisher's protected least significant difference ($P \leq 0.05$); nd = not done.

^x Experiment and cultivar. Experiment 1 was conducted in 2006. Experiments 2 and 3 were conducted in 2007. Experiments 4 and 5 were conducted in 2008. Experiment 6 was conducted in 2009. Pathogen inoculum consisted of suspensions of *Erwinia amylovora* Ea110 in water at a concentration of 10⁷ CFU/ml (experiments 1, 4, 5, and 6) or 10⁶ CFU/ml (experiments 2 and 3).

^y Materials were applied to trees when the listed percentage of blossoms were open.

^z Mean values of proportion of infected flower clusters per tree. Disease ratings were conducted on 1 June 2006 (experiment 1), 30 May 2007 (experiments 2 and 3), 3 June 2008 (experiments 4 and 5), and 12 June 2009 (experiment 6).

to gradually increasing rates of kasugamycin over time in LBks broth and, by the conclusion of the 7-day experiment, each of the three *E. amylovora* strains were capable of forming colonies on LBks medium containing kasugamycin at 150 µg ml⁻¹. We then amplified and sequenced the *ksgA* target gene from each putative Ks^R mutant and identified sequence alterations in each strain compared with the wild-type *ksgA* sequence of strain Ea273

TABLE 2. Numerical distribution of gram-negative bacterial isolates recovered from apple flowers and leaves and apple orchard soil by kasugamycin resistance phenotype and year of isolation

Year, source	Total ^y	No. of isolates ^z			
		Sensitive	Low	Intermediate	High
2007					
Flower	6	0	3	0	3
Leaf	7	3	NT	2	2
Soil	123	60	NT	8	55
2008					
Flower	114	47	51	9	7
Leaf	38	0	38	0	0
Soil	NT	NT	NT	NT	NT
2009					
Flower	180	38	41	47	54
Leaf	39	29	2	3	5
Soil	81	10	NT	23	48

^y Total gram-negative isolates.

^z Number of isolates with each kasugamycin phenotype. Resistance was defined as low, intermediate, or high based on the growth of isolates on Luria-Bertani medium amended with kasugamycin at 100, 250, and 500 µg ml⁻¹, respectively; NT = not tested.

(available at http://www.sanger.ac.uk/Projects/E_amylovora/). Alignment of the entire *ksgA* sequence amplified from the original three strains unexposed to kasugamycin revealed 100% sequence identity (data not shown). Ks^R mutants Ea1189-2 and Ea273-1 and -2 exhibited identical point mutations at base pair 19 of *ksgA*, which would generate a premature stop codon at amino acid 7 of KsgA (Fig. 3). Mutants Ea273-3, Ea1189-1, and Ea1189-3 exhibited point mutations at base pair 49 and 139, respectively, resulting in mutations that changed the amino acid encoded (G17E and A47S). Ea110-3 had a deletion of a single nucleotide at base pair 126 and Ea110-1 had a 10-nucleotide deletion between base pairs 182 and 193. Both of these deletion events would cause frame shifts resulting in premature stop codons (Fig. 3).

Evaluation of virulence of spontaneous Ks^R *E. amylovora* strains. The virulence of eight spontaneous Ks^R *E. amylovora* mutants with alterations in the *ksgA* gene was evaluated against their corresponding parental strain using an immature pear assay. Wild-type (Ks^S) strain Ea110 caused significantly larger zones of necrosis than *ksgA* mutant daughter strains Ea110-1 and Ea110-3 at days 5, 6, and 7 after inoculation (Fig. 4A). Wild-type strain Ea273 also exhibited increased virulence compared with *ksgA* mutant daughter strains Ea273-1, Ea273-2, and Ea273-3 at days 5 and 7 following inoculation (Fig. 4A). Wild-type strain Ea1189 exhibited increased virulence compared with *ksgA* mutant daughter strains Ea1189-1, Ea1189-2, and Ea1189-3, with significantly larger zones of necrosis observed at day 7 after inoculation (Fig. 4A). We also evaluated bacterial fitness during pathogenesis by measuring cell growth in immature pear tissue during the first 3 days after inoculation. During this time period, the population

TABLE 3. Identification of gram-negative bacteria isolated from flower, leaf, and soil samples from apple orchards where Kasumin was applied, and quantification of number of isolates by kasugamycin sensitivity level^w

Bacterial genus, species ^x	Total ^y	No. of isolates capable of growth on medium amended with kasugamycin (µg ml ⁻¹) ^z							
		Flower and leaf				Soil			
		S	100	250	500	S	100	250	500
Enterobacteriaceae									
<i>Dickeya dadantii</i>	3	2	0	1	0	0	0	0	0
<i>Enterobacter</i> sp.	14	1	0	1	0	10	0	0	2
<i>Erwinia amylovora</i>	6	5	0	0	0	1	0	0	0
<i>E. persicina</i>	4	0	4	0	0	0	0	0	0
<i>Klebsiella</i> sp.	1	0	0	0	0	1	0	0	0
<i>Pantoea agglomerans</i>	61	24	31	0	2	2	0	2	0
<i>Pantoea</i> sp.	7	1	2	0	0	2	0	2	0
<i>Serratia</i> sp.	4	0	0	0	0	1	0	0	3
<i>Yersinia aldovae</i>	3	1	2	0	0	0	0	0	0
Total	103	34	39	2	2	17	0	4	5
Other bacteria									
<i>Chryseobacterium</i> sp.	22	0	0	1	3	0	0	0	18
<i>Pseudomonas aeruginosa</i>	6	1	2	1	1	0	0	0	1
<i>P. fluorescens</i>	46	2	5	25	10	0	0	1	3
<i>P. geniculata</i>	12	0	1	0	11	0	0	0	0
<i>P. graminis</i>	58	20	35	0	2	0	0	1	0
<i>P. putida</i>	14	0	9	1	2	0	0	2	0
<i>P. syringae</i>	26	23	2	0	1	0	0	0	0
<i>Pseudomonas</i> spp.	90	31	17	15	7	5	0	7	8
<i>Sphingomonas</i> sp.	16	0	6	4	1	2	0	1	2
<i>Stenotrophomonas maltophilia</i>	57	1	0	2	22	2	0	3	27
<i>Stenotrophomonas</i> spp.	19	0	5	1	3	1	0	0	9
<i>Xanthomonas campestris</i>	6	0	0	3	2	0	0	0	1
Miscellaneous	113	5	14	6	4	43	0	12	29
Total	485	83	96	59	69	53	0	27	98

^w Bacterial isolations were done in 2007, 2008, and 2009.

^x Isolate identification was done by sequence analysis of the 16S rDNA gene and BLAST comparison with the GenBank database as described in the Materials and Methods. *Pseudomonas* spp. includes *Pseudomonas cedrina*, *P. gingeri*, *P. lutea*, *P. rhodesiae*, *P. poea*, *P. veronii*, *P. viridiflava*, and *Pseudomonas* sp. *Stenotrophomonas* spp. includes *Stenotrophomonas chelatiphaga*, *S. rhizophila*, and *Stenotrophomonas* sp. Miscellaneous bacterial species includes *Acinetobacter* sp., *Aeromonas* sp., *Agrobacterium tumefaciens*, *Burkholderia* sp., *Caulobacter* sp., *Delftia lacustris*, *Duganella* sp., *Ensifer* sp., *Flavobacterium* sp., *Pedobacter* sp., *Psychrobacter* sp., *Rahnella aquatilis*, *Rhizobium* sp., *Variovorax ginsengisoli*, and *V. paradoxus*.

^y Total number of isolates.

^z Number of isolates capable of growth on Luria-Bertani medium amended with kasugamycin; S = sensitive.

size of wild-type strains appeared to be mostly similar to that of the mutant strains (Fig. 4B), suggesting that any difference in growth that might correspond with differences in virulence would have been observed later during the infection process.

In vitro relative fitness was also evaluated by comparing growth rate and lag time of wild-type and mutant strains in both rich medium (LB) and MM. Growth curves of each of these strains and their corresponding *ksgA* mutant daughter strains in LB is shown (Fig. 5; data not shown for MM experiments). The mean lag time of strain Ea110 was 3.6 h, which was significantly shorter than that of the corresponding mutant strains Ea110-1 (4.1 h, $P = 0.01$) and Ea110-3 (4.8 h, $P = 0.0002$). The mean doubling time of Ea110 was 1.8 h, which was significantly faster than that of Ea110-1 (2.4 h, $P = 0.01$) and Ea110-3 (2.5 h, $P = 0.01$) (Fig. 5). The mean lag time of Ea1189 of 3.5 h was significantly shorter than that of two mutants (Ea1189-1, 4.2 h, $P = 0.03$ and Ea1189-2, 4.7 h, $P = 0.05$) and not significantly different from Ea1189-3 (4.4 h, $P = 0.14$). In addition, the mean doubling time of Ea1189 of 2.1 h was not significantly different from that of any of the corresponding mutant strains (Ea1189-1, 2.1 h, $P = 0.9$; Ea1189-2, 2.5 h, $P = 0.14$; and Ea1189-3, 2.7 h, $P = 0.11$). The mean lag time of Ea273 was 3.2 h, which was significantly shorter than that of the corresponding mutant strains (Ea273-1, 5.4 h, $P = 0.002$; Ea273-2, 5.0 h, $P = 0.001$; and Ea273-3, 4.1 h, $P = 0.01$). Likewise, the mean doubling time of Ea273 of 1.6 h was also significantly reduced compared with the mutant strains (Ea273-1, 2.4 h, $P = 0.005$; Ea273-2, 2.4 h, $P = 0.008$; and Ea273-3, 2.0 h, $P = 0.05$).

Differential sensitivity of bacteria to kasugamycin and Kasumin. The isolate test panel for comparative sensitivity analysis was weighted in favor of the predominant species observed in the distribution of environmental isolates recovered, and in the selection of bacteria commonly isolated from aerial plant tissue. We also tested a collection of 20 strains of *E. amylovora*, *E. pyrifoliae*, and *Erwinia* sp. representing a wide geographic distribution. All 55 environmental isolates from the test panel grew on KB and KB + kasugamycin (100 µg ml⁻¹) but only 16 isolates could grow on KB + Kasumin with Kasumin included at the same rate of 100 µg ml⁻¹ (Table 5). Because of the disparity in growth on the kasugamycin and Kasumin-amended media, we also examined isolate growth on KB containing the equivalent amount of the KFB. Only 36 of 55 isolates from the test panel grew on KB amended with the KFB, indicating that the KFB had some antimicrobial activity (Table 5). When we added a corresponding amount of the formulation blank to KB medium containing technical-grade kasugamycin at 100 µg ml⁻¹, the number of isolates capable of growth was reduced from 55 to 36. We also found that, whereas 11 of the *Stenotrophomonas* spp. examined grew on KBs + blank medium, only 3 of these isolates grew on KB + KFB without antibiotic added (Table 5). All of the *E. amylovora*, *E. pyrifoliae*, and *Erwinia* sp. strains examined could

grow on KFB but none of these strains could grow on KB amended with kasugamycin or Kasumin (Table 5). In addition, the 18 *E. amylovora* strains tested included nine Sm^R strains; none of these strains could grow on KBs.

DISCUSSION

Our field experiments indicated that Kasumin significantly reduced blossom infection by *E. amylovora* when applied both before and after pathogen inoculation and when applied only after pathogen inoculation. The efficacy of Kasumin was not significantly different from that of Agri-Mycin, which was the standard material used for blossom blight control prior to the development of streptomycin resistance. The effect of Kasumin on reduction of *E. amylovora* populations on apple flower stigmas was also similar to that of streptomycin. In field studies conducted by other researchers, the efficacy of Kasumin for blossom blight control was also equal to that of Agri-Mycin (1,18). Taken together, all of these results indicate that Kasumin represents a viable alternative antibiotic, in particular for use for fire blight management in orchards harboring Sm^R strains of *E. amylovora*.

The long-term utility of kasugamycin for fire blight control is dependent upon the development of resistance in the pathogen population. Loss of kasugamycin efficacy to resistance in *E. amylovora* is predicted to occur through either of two mechanisms. The first possibility would involve the development and selection of spontaneous Ks^R mutants. In our laboratory studies, direct exposure of three different *E. amylovora* strains to high concentrations of kasugamycin did not result in the recovery of spontaneous Ks^R colonies. However, exposure of *E. amylovora* initially to low concentrations of kasugamycin followed by progressive increases in concentration of kasugamycin from 25 to 200 µg ml⁻¹ did result in the recovery of strains that could grow in

Fig. 3. Graphic representation of the *ksgA* gene from *Erwinia amylovora* indicating the location of mutations in eight spontaneous kasugamycin-resistant mutants of *E. amylovora* strains Ea110, Ea273, and Ea1189 and the corresponding amino acid change in the translated protein or frameshift.

TABLE 4. Assessment of sensitivity or resistance to both kasugamycin and streptomycin in gram-negative bacteria isolated from flower, leaf, and soil samples from apple orchards where Kasumin was applied

Bacterial group (family or genus)	Total no. of isolates	No. of isolates with each kasugamycin and streptomycin resistance phenotype ^z			
		Ks ^S Sm ^S	Ks ^S Sm ^R	Ks ^R Sm ^S	Ks ^R Sm ^R
Flower and leaf					
Enterobacteriaceae	77	16	17	4	40
<i>Pseudomonas</i> spp.	224	13	68	14	129
<i>Stenotrophomonas</i> spp.	34	1	0	6	27
Other gram-negative bacteria	49	1	4	16	28
Soil					
Enterobacteriaceae	26	4	13	4	5
<i>Pseudomonas</i> spp.	28	1	4	17	6
<i>Stenotrophomonas</i> spp.	42	3	0	21	18
Other gram-negative bacteria	108	23	22	28	35

^z Ks^S and Sm^S = sensitive and Ks^R and Sm^R = resistant to kasugamycin and streptomycin, respectively.

Fig. 4. Virulence assays in immature pear fruit performed with wild-type and eight spontaneous kasugamycin-resistant mutants of *Erwinia amylovora* strains Ea110, Ea273, and Ea1189. **A**, Necrotic lesion size (with standard error) in immature pear following inoculation with *E. amylovora* strains. Data pooled from two experiments are shown. The presence of different small letters above the bars indicates that the means within a treatment are significantly different ($P < 0.05$) according to Fisher's protected least significant difference test. These experiments were performed over 7 days with measurements taken at days 2, 3, 4, 5, 6, and 7. In all, 10 immature pear fruit were used for each strain in each experiment. Representative pear fruit inoculated with wild-type and mutant strains from day 7 are shown in the inset. The key indicates the color of the bars representing data from a particular strain. **B**, Population size of wild-type and mutant strains (and standard error) immediately following inoculation and over a 3-day period following inoculation into immature pear fruit.

the presence of kasugamycin at a concentration of at least 150 $\mu\text{g ml}^{-1}$. Furthermore, these bacteria exhibited heritable alterations in the *ksgA* gene that were presumably associated with decreased sensitivity. It is possible that populations of *E. amylovora* could be exposed to relatively lower concentrations of kasugamycin in orchards due to either inadequate spray coverage or environmental degradation of kasugamycin. However, the stability of the *ksgA* mutations and the relative fitness of spontaneous Ks^R strains would also play a role in the maintenance of such strains in orchard populations. Our results with the spontaneous Ks^R mutants selected in this study indicated that these strains were reduced in virulence in an immature pear assay. Although this result suggests that spontaneous Ks^R mutants would be reduced in fitness, these mutants would have to be further evaluated because it is known that spontaneous antibiotic-resistant bacterial mutants can evolve compensatory mutations that alleviate fitness defects (3).

A second potential method for the development of resistance to kasugamycin in *E. amylovora* orchard populations would be through the acquisition of a kasugamycin resistance gene by horizontal DNA transfer. Although there are currently no known transferrable kasugamycin resistance genes in bacteria, it is likely that such genes exist in nature because kasugamycin is a natural product of the bacterium *Streptomyces kasugaensis* in soil environments (16), suggesting that kasugamycin selection would theoretically be present in soil microbial communities. We isolated a wide range of Ks^R gram-negative bacteria from apple flowers and leaves and orchard soil in this study. Two ecological factors that could affect the transfer of a kasugamycin resistance gene, if present in the orchard microbial community, into *E. amylovora* would be the location of the gene in an organism that could attain relatively large population sizes in the apple flower and leaf habitat and the possible presence of a transferrable gene in an organism relatively closely related to *E. amylovora*. Of the Ks^R bacterial species and genera identified in this study, most were known residents of the plant phyllosphere or known colonists of apple such as *Pantoea agglomerans*, *Pseudomonas graminis*, *P. syringae*, and *Stenotrophomonas* spp. (4,13,15,17). Thus, it would be expected that, if these organisms carried a mobile kasugamycin resistance gene, they could reach sufficient population size on apple tissue to effect gene transfer to *E. amylovora*, potentially via conjugation.

We also determined that 72.4% of the Ks^R bacteria recovered (288 of 398 isolates) were also resistant to streptomycin. The occurrence of the $\text{Ks}^R \text{Sm}^R$ phenotype in Enterobacteriaceae isolates and *Pseudomonas* spp. is of concern because these organisms could potentially be involved in the dissemination of resistance genes to *E. amylovora*. The high percentage of Sm^R bacteria recovered was not surprising because streptomycin has been applied yearly in the orchard where we sampled for fire blight control for at least 25 years. It has been shown in studies involving different crop hosts that the use of streptomycin as a bactericide for plant disease control selects for streptomycin resistance in nontarget commensal bacteria that inhabit either plant surfaces or the surrounding soil (7,48). The isolation of a number of bacteria with resistance to both kasugamycin and streptomycin may be indicative of the selection of kasugamycin resistance in strains already harboring streptomycin resistance or may be due to the presence of bacteria that are intrinsically resistant to both of these aminoglycoside antibiotics. Because we found that spontaneous Ks^R *E. amylovora* strains were sensitive to streptomycin and that spontaneous Sm^R *E. amylovora* strains were sensitive to kasugamycin, there is no concern for cross resistance among singly resistant Ks^R or Sm^R spontaneous mutants. This will become important if Kasumin is utilized as a resistance management tool in situations where the *E. amylovora* background population is still sensitive to streptomycin.

In addition, we isolated a number of enterobacterial species from apple flowers and leaves; of these, *Pantoea agglomerans*

was the most prevalent. This is significant because *P. agglomerans* was thought to be the source of the streptomycin-resistance plasmid pEa34, which was acquired by *E. amylovora* in Michigan (7). Transferrable streptomycin resistance in *E. amylovora* and other plant pathogens, including *Pseudomonas syringae*, is conferred by the linked *strA-strB* genes which are encoded within

Fig. 5. Growth curves of wild-type and eight spontaneous kasugamycin-resistant mutants of *Erwinia amylovora* A, Ea110; B, Ea1189; and C, Ea273 in Luria-Bertani (LB) broth. Strains indicated with dashes (e.g., Ea110-1) are the corresponding spontaneous kasugamycin-resistant mutant strains. Diluted bacterial suspension (2 ml) in media was inoculated into three replicate wells of a 24-well microtiter plate and sealed. Optical density (OD) readings (600 nm) were taken every 5 min using a Tecan Safire plate reader for 24 h. The microtiter plates were shaken for 30 s at 2.5 min between readings and just prior to each optical read. Bacteria were allowed to settle for 10 s prior to reading. Representative curves for each organism were chosen and graphed as a function of absorbance over time in hours. The experiment was replicated twice in both LB and minimal media (data not shown).

the transposable element Tn5393 (7,44,45). The *strA-strB* genes and the Tn5393 element are also present in nontarget bacteria isolated from the same orchard and nursery environments as the pathogens (7,48), suggesting the likelihood that nontarget bacteria served as the donors of streptomycin resistance to the plant pathogens.

We must stress that, although large numbers of Ks^R organisms from diverse species were isolated in this study, it is currently unknown if any of these Ks^R nontarget bacteria isolated harbor a transferable kasugamycin resistance gene. However, it is clear that the possibility already exists that bacteria harboring a transferable kasugamycin resistance gene could be readily selected in orchards where Kasumin is applied for fire blight disease management. It is also possible that some of the Ks^R nontarget bacteria that were isolated are intrinsically resistant to kasugamycin through mechanisms such as reduced antibiotic uptake or enhanced efflux. For example, it is known that *Stenotrophomonas maltophilia* exhibits intrinsic resistance to aminoglycoside antibiotics such as kasugamycin (22), and a number of isolates of this organism exhibiting high-level kasugamycin resistance were recovered in this study. Thus, a critical next step for this research is to determine whether any transferable kasugamycin resistance genes are present in the bacterial communities of Kasumin-sprayed orchards.

Antibiotic resistance is a global problem with severe consequences in clinical medicine (2). From a scientific perspective, the emergence of antibiotic resistance in clinical bacteria is explained by several broad concepts: (i) most antibiotics are produced naturally by environmental bacteria, and resistance genes originally evolved long ago in response to natural antibiotic selection; (ii) antibiotic resistance genes are typically associated with mobile genetic elements; (iii) in clinical situations, mobile antibiotic resistance genes are usually acquired first by nontarget commensal organisms and then transferred to pathogens; (iv) the combination of the large size of bacterial populations, selection pressure imposed by large-scale human use of antibiotics, and transferable resistance genes has resulted in the current situation, in which similar antibiotic resistance genes are widely disseminated among a variety of unrelated bacterial pathogens (11).

Because antibiotic resistance genes are so easily transferred among bacteria, even among unrelated species, there are concerns in the medical community regarding the potential for the transfer of resistance genes selected in agricultural habitats to human pathogens. These concerns are valid in the animal agriculture arena, with an example being the selection of clinically important

vancomycin-resistant enterococci initially in chickens given the antibiotic avoparcin as a feed additive for growth promotion (56). The current use of the antibiotic gentamicin for bacterial disease control on plants in Mexico and other Central American countries has heightened these concerns, in particular, because of the use of gentamicin in clinical medicine. However, although gentamicin resistance is a problem in clinical medicine, gentamicin resistance genes are also already commonly found in habitats associated with or impacted by sewage effluent or farm animals (14). Thus, the possibility for transfer of gentamicin resistance from environmental sources to human-associated bacteria already exists and the number of these currently existing environmental sources would be expected to be orders of magnitude greater than the number of sources from plant agriculture.

It should also be noted that current antibiotic use in plant agriculture in the United States (streptomycin and oxytetracycline) represents <0.5% of the total agricultural antibiotic use (32,54). The availability of rapid sequencing technologies has aided in the identification of specific resistance genes and alleles in plant-pathogenic bacteria and in the determination of possible relationships with similar genes found in clinical bacteria. For example, the *strA-strB* streptomycin resistance genes found in plant-pathogenic bacteria are also widely distributed in clinical pathogens (47); however, the alleles of the genes were different, suggesting different routes of acquisition (44,45). Thus, the detection of similar antibiotic resistance genes in plant-associated bacteria and clinical pathogens does not necessarily signify direct horizontal transfer events between organisms from these disparate habitats.

Kasugamycin is an attractive antibiotic for the management of bacterial plant pathogens because there are no uses of this antibiotic in human medicine. In addition, there are no known kasugamycin resistance genes currently, although spontaneous Ks^R mutants of *E. amylovora* were selected in this study and have been genetically characterized in *Escherichia coli* (55). Our observations on the lack of sensitivity to kasugamycin of many nontarget bacteria inhabiting apple orchards could also reduce the risk potential for resistance development in *E. amylovora*. Because many bacteria capable of growth in the presence of kasugamycin at 100 µg ml⁻¹ were isolated from orchards, it is possible that applications of Kasumin for blossom blight control at 100 µg ml⁻¹ will not affect these organisms. This would be advantageous if these organisms were intrinsically resistant to kasugamycin. Thus, organisms with intrinsic resistance to kasugamycin would survive spray applications but would not contribute to the selective pressure to obtain and disseminate a potential

TABLE 5. Differential growth of gram-negative bacterial isolates on King's medium B (KB) amended with technical-grade kasugamycin (KBks) or Kasumin at 100 µg ml⁻¹, the Kasumin formulation blank (KFB), or kasugamycin plus KFB

Bacterial species ^y	No. ^z	No. of isolates growing on media				
		KB	KB + KFB	KB + KBks (100 µg ml ⁻¹)	KB + Kasumin (100 µg ml ⁻¹)	KBks (100 µg ml ⁻¹) + KFB
<i>Chryseobacterium</i> sp.	1	1	0	1	0	1
<i>Dickeya dadanti</i>	1	1	1	1	0	0
<i>Enterobacter</i> sp.	2	2	1	2	1	2
<i>Erwinia amylovora</i>	18	18	18	0	0	0
<i>E. persicina</i>	4	4	0	4	0	4
<i>E. pyrifoliae</i>	1	1	1	0	0	0
<i>Erwinia</i> sp.	1	1	1	0	0	0
<i>Pantoea agglomerans</i>	20	20	20	20	4	9
<i>Pseudomonas</i> sp.	2	2	1	2	1	1
<i>Pseudomonas aeruginosa</i>	4	4	2	4	2	2
<i>P. fluorescens</i>	6	6	6	6	3	4
<i>P. syringae</i>	2	2	2	2	0	0
<i>Stenotrophomonas</i> spp.	13	13	3	13	5	11

^y Bacterial species tested, except for *Erwinia amylovora*, *E. pyrifoliae*, and *Erwinia* sp., were isolated from experimental Michigan apple orchards that were previously treated at least once with Kasumin. The *E. amylovora*, *E. pyrifoliae*, and *Erwinia* sp. strains tested represented a collection of strains isolated from the United States, Asia, Europe, and the Middle East.

^z Number of strains tested.

kasugamycin resistance gene. With less selection pressure for resistance development in nontarget organisms, the potential for introduction of transferrable kasugamycin resistance genes is expected to be low.

The observation that some bacteria were more sensitive to the formulated product Kasumin than to kasugamycin was also interesting and may slow the potential for resistance development in certain bacterial species. The Kasumin formulated product consists of kasugamycin and additional proprietary components (V. J. Spadafora, *personal communication*). It is possible that these additional ingredients may stabilize the kasugamycin antibiotic in solution or may impact bacterial cell membranes, which could increase the permeability of the antibiotic. However, in some cases, the formulation blank itself had bactericidal activity, which could result via induction of membrane leakage. The increased sensitivity of most of the *Pantoea agglomerans* strains to Kasumin compared with kasugamycin could also be significant in that a *P. agglomerans* strain was implicated in the transfer of the Sm^R transposon Tn5393 into *Erwinia amylovora* (7).

In summary, we have determined that Kasumin, a formulated bactericide with the antibiotic kasugamycin as active ingredient, is highly efficacious for controlling the blossom blight phase of fire blight and represents a new alternative antibiotic for the management of *E. amylovora*. The lack of medical uses of kasugamycin significantly increases the attractiveness of this antibiotic for plant agricultural use. We determined that exposure of *E. amylovora* to low concentrations of kasugamycin heightened the risk of spontaneous resistance development. Thus, it will be critical that full label rates of Kasumin are consistently utilized for control and that spray methodologies such as alternate-row spraying that can reduce the amount of available product on trees should be avoided. We are currently examining a variety of orchard bacterial isolates for the possible presence of transferrable kasugamycin resistance genes. If any genes are identified, we can assess their distribution and potential for horizontal transfer among nontarget organisms and into *E. amylovora*.

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