STREPTOMYCIN RESISTANT *ERWINIA AMYLOVORA* IN NEW YORK AND IMPLICATIONS ON FIRE BLIGHT MANAGEMENT IN APPLE ORCHARDS

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by

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ABSTRACT

Erwinia amylovora is a bacterial phytopathogen that causes fire blight on several Rosaceous species, and is especially destructive on apple, leading to devastating losses in New York annually. In the eastern United States, the antibiotic streptomycin is the most effective and widely used chemical control for fire blight management. Recently, streptomycin resistant (SmR) *E. amylovora* was recovered from NY orchards, leading to concerns about the sustainability of antibiotic use. The goals of my dissertation were to explore the prevalence and diversity of SmR *E. amylovora* in NY, and to investigate practices that may contribute to the development and introduction of SmR *E. amylovora* in local orchards. In Chapter 1, the prevalence of SmR *E. amylovora* in NY orchards was investigated through state-wide surveys of orchards with fire blight outbreaks from 2011 to 2014. SmR *E. amylovora* was recovered from 19 commercial orchards in western NY, but was absent in eastern apple growing regions of the state. The next study, Chapter 2, focused on examining the diversity and possible origins of these isolates using CRISPR spacer arrays. CRISPR spacers arrays of NY SmR and streptomycin sensitive *E. amylovora* isolates were sequenced and compared to each other, as well as to previously described *E. amylovora* collected world-wide. Results implied that the presence of SmR *E. amylovora* in NY may be due to resistance development within NY orchards and also, to the introduction of resistant strains from the western US. In order to examine factors that may lead to the development of streptomycin resistance within NY orchards, the direct effects of antibiotic applications on the epiphytic bacterial community in the apple phyllosphere were studied (Chapter 3). In this study streptomycin and kasugamycin applications were applied in

increasing application numbers to apple trees and leaves were collected to examine the epiphytic bacterial community. Results suggested that these antibiotics may have differential effects on certain epiphytic bacteria, the overuse of streptomycin may increase overall SmR bacteria, and applications of kasugamycin may aid in the overall reduction of SmR bacteria. Furthermore, the sustainable use of these antibiotics may be achieved through rotation and minimal use, and the strict avoidance of antibiotic applications after bloom. Given that the introduction of SmR *E. amylovora* may occur through the movement of clonally propagated asymptomatic trees, the relationship between fire blight symptoms and the recovery of *E. amylovora* from asymptomatic budwood, used for tree propagation, was investigated (Chapter 4). Buds of apple trees were collected at defined distances from a center point tree with fire blight symptoms, and were tested for the presence of *E. amylovora*. Trees farther from a symptomatic tree may have lower incidences and lower populations of *E. amylovora*. However, *E. amylovora* was recovered from several distant asymptomatic trees, demonstrating that pathogen free trees cannot always be propagated from these materials and advanced pathogen screening practices are needed for propagation materials. Combined, these four studies provided new information on the status, characterization, and origins of SmR *E. amylovora* in New York.

BIOGRAPHICAL SKETCH

Kiersten A. Tancos completed her undergraduate degree in 2008 at St. John Fisher College under the direction of Dr. Maryann Herman, with whom she completed undergraduate research projects and gained an interest in teaching. In 2012, Kiersten was accepted into the graduate school at Cornell University to pursue a Ph.D. in Plant Pathology. Kiersten began her research with Dr. Kerik Cox in 2013 on the bacterial pathogen *Erwinia amylovora* and the status of streptomycin resistant *E. amylovora* in New York apple orchards. In addition to this research, Kiersten has also participated in teaching opportunities within the university and local schools, and outreach activities to convey scientific topics to diverse audiences within the community. These experiences motivated Kiersten to complete her degree with a focus on research, teaching, extension, and outreach.

DEDICATION

To my loving husband Matthew and my family for their unwavering support,

and to my advisors for their excellent guidance in all things

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INTRODUCTION

An overview of the apple industry in New York, and the impact of fire blight on grower operations

The New York apple industry is the second largest apple producer in the United States, yielding over 29.5 million bushels every year from approximately 700 commercial operations (New York Apple Growers Association 2015). These operations consist of over 55,000 acres, with the majority of acreage located along the southern shore of Lake Ontario, the Hudson Valley region, and the Lake Champlain Valley (New York State Department of Agriculture and Markets 2015). About 53% of New York apples are grown for the fresh market and 47% are used for processing, with top varieties such as McIntosh, Empire, Cortland, Red Delicious, Golden Delicious, Paula Red, Rome, Idared, and Gala. The production value of the New York apple crop was about $249.8 million in 2012, making apples the #1 fruit crop in the state (DiNapoli 2010; New York Apple Growers Association 2015). The New York apple industry's value is also reflected in over ten thousand jobs directly related to agricultural production and thousands of additional jobs indirectly related to apples through areas such as processing, distribution, crop protection, agricultural equipment, marketing, sales, and several other services (New York Apple Growers Association 2015).

Fire blight, a bacterial disease caused by *Erwinia amylovora,* poses a significant threat to New York's apple industry. This disease was first reported in New York in 1780 in the Hudson Valley region, with this region being the presumed origin of the pathogen. This pathogen has become increasingly challenging over the past two centuries (Bonn and van der Zwet 2002). In New York, fire blight was noted to be most destructive on pear and mildly problematic on apple

in the 19th and early 20th century (Folger and Thomson 1921). However, with the planting of highly susceptible varieties and rootstocks to meet market demands, coupled with production changes to promote higher yields per acre, fire blight has become a production challenge within the New York apple industry (Bonn and van der Zwet 2000; van der Zwet 2012).

Losses associated with fire blight are directly related to the advancement of disease in apple tissues and disease prevention strategies. Removal of branches, single trees, or whole orchards may be necessary to contain fire blight outbreaks resulting in substantial financial losses for growers. In recent years, several New York apple growers have experienced up to 50% tree loss in new plantings due to fire blight. These losses are projected to increase as older plantings, with fewer than 100 trees per acre, are converted to high-density plantings, with over 350 trees per acre to 3,000 trees per acre (Breth 2008, Robinson et al. 2013). Another significant factor that contributes to fire blight losses is the planting of susceptible apple varieties. A survey of New York orchards showed that over 57% of new plantings are planted to fire blight susceptible varieties (Ropel et al. 2006).

Fire blight symptoms, epidemiology, and environmental factors contributing to disease

Fire blight symptoms may be observed in several apple tissues. Three commonly observed stages of fire blight are blossom blight, shoot blight, and rootstock blight. Blossom blight includes the wilting and blackening of apple blossoms early in the growing season. Susceptible blossoms serve as avenues for bacterial entry and are usually infected by the fire blight bacterium *Erwinia amylovora* upon bloom (Thomson 1986). These affected blossoms may lose their petals, but the rest of the inflorescence remains affixed to the tree, which is characteristic of *E. amylovora* infection. The peduncle of the blossom may also produce ooze

droplets in a range of colors (light yellow, orange, and red) (Sundin 2014). Bacterial ooze consists of a polysaccharide matrix, which may contain extremely high populations of *E. amylovora* (Sundin 2014). This ooze serves as inoculum for further infection. Losses associated with blossom blight are the direct consequence of decreased fruit production due to blossom necrosis (van der Zwet et al. 2012; Bonn and van der Zwet 2000; Sundin et al. 2014).

Shoot blight is a result of *E. amylovora* infection in young green shoot tissue. Once infected, shoot tips may begin to wilt and lean to produce a shape similar to a shepherd's crook (Sundin 2014). The coloration of infected shoot material may range from orange-brown to dark and blackened in appearance. Ooze droplets may be present on infected shoots. Shoot blight may progress into limbs and result in death of branches, leading to direct loss of fruit bearing tissues (van der Zwet et al. 2012; Bonn and van der Zwet 2000; and Sundin et al. 2014).

Rootstock blight is usually indicative of advanced infection. Patches that are dark and appear water-soaked on rootstock bark are the first symptoms of rootstock blight. Internal tissues of the rootstock may appear to have dark red streaking (van der Zwet et al. 2012). Ooze production may occur and is a characteristic sign of fire blight infection. Rootstock blight severely impacts water and nutrient transport within the tree, and may result in girdling and death (Norelli et al. 2000). Tree removal due to fire blight is not only financially costly, but also costly in time, considering that replanted trees may take several years to produce a crop (van der Zwet et al. 2012; Bonn et al. 2000; and Sundin et al. 2014).

The life cycle of *Erwinia amylovora* includes stages of survival, infection, and dispersal in apple orchards. Survival of the bacterium over winter is essential in the temperate climate of the Northeastern United States. *E. amylovora* overwinters in cankers formed on limbs and trunks known as holdover cankers (Beer and Norelli 1977). In the spring these cankers may exude

bacterial ooze, which may be moved to blossoms via rain, wind, and insects. Once on blossoms, the bacteria multiply rapidly on the stigma and require moisture to move downward into the floral cup (Thomson 1986). Pollinating insects may serve as a vector for inoculum between blossoms. Infection of floral tissues constitutes primary fire blight infection (Schroth et al. 1974). From blossoms, bacterial ooze may spread to green tissues, such as shoots, to initiate secondary infection. *E. amylovora* enters shoots through natural openings, such as stomata, and through wounds. Once internalized, the bacteria have the potential to spread systemically throughout the tree leading to infection of branches, limbs, trunk, and rootstock tissues (Norelli et al. 2003). Cankers form on woody tissues and will house the bacterium over winter for the following season. Both cankers from the previous year and cankers formed in the current season expand and may serve as sources of initial inoculum (Sundin 2014). However, determinate cankers, with a demarcation around the infected tissue, are usually inactive, while indeterminate cankers, with no demarcation separating healthy tissue from infected tissue, produce infectious bacteria in the spring (Biggs 1994; Miller 1929).

Environmental conditions play a key role in the interactions between *Erwinia amylovora* and its apple host. Temperatures exceeding 65°F and high humidity are conducive to bacterial growth, production of bacterial ooze, and advancement of symptoms. The optimal range for fire blight infection is between 70°F and 81°F. Infection may occur at temperatures below 70°F, however progression of disease is slowed substantially (Miller 1929). High humidity is essential, with infection occurring rapidly at 80% relative humidity or higher (Brooks 1926). However, the exact temperature and humidity boundaries of bacterial growth and infection have not been studied extensively (van der Zwet et al. 2012). Precipitation and wind also contribute to fire blight severity. Rainfall in combination with high temperatures has been associated with fire

blight outbreaks and high epiphytic populations of *E. amylovora* (Ockey and Thomson 2006). Damage caused by rainstorms along with wind and hail also contributes to fire blight outbreaks. Occurrence of outbreaks on up to 80% of trees has been observed after severe hailstorms (van der Zwet et al. 1969).

Fire blight management in the northeastern United States

Apple growers in the Northeastern United States are reliant upon several tools to manage fire blight: disease forecasting, host resistance, cultural practices, biological controls, growth regulators, and chemical applications. Since blossom blight may lead to shoot and rootstock blight, it is important to begin fire blight management efforts early in the growing season. Disease forecasting models provide information on risk of blossom and shoot infection based on environmental factors such as precipitation, temperature, and relative humidity. NEWA, Network for Environment and Weather Applications, runs models for a variety of plant diseases (New York State Integrated Pest Management 2009, http://newa.cornell.edu). The Cougarblight model is used to predict infection risk of blossoms at bloom and shoot infection risk after trauma events. This model uses weather data collected by local weather stations across the Northeast United States, organized by NEWA, to advise growers when apple tissues are at risk for infection and then recommends when actions should be taken to prevent *E. amylovora* infection (Smith 1996).

Host resistance is another valuable tool when planting new orchards. Consideration of host resistance is fundamental to a proactive approach in controlling fire blight, but requires careful planning. Differential resistance to fire blight in apple cultivars has been known since the mid 19th century and has become a factor in the selection of apple varieties for regions facing

severe disease pressure (van der Zwet et al. 2012). Several studies beginning in the 1960's developed fire blight rating indices to place apple varieties into categories such as highly resistant, moderately resistant, susceptible, and very susceptible (Thompson et al. 1962; Mowry 1964). Currently, categorization of scion and rootstock susceptibility usually follows the USDA blight scoring system developed by van der Zwet (van der Zwet 1970).

While there is a history of emphasizing fruit quality and horticultural traits in breeding programs, the study of host resistance to fire blight is of international interest and progress has been made in identifying genes for resistance, mapping them on different linkage groups, and studying their genotype x environment interactions (Farkas et al. 2012; Horner et al. 2015; Peil et al. 2014).

Apple breeding at the New York State Agricultural Experiment Station in Geneva, NY has a long history. Factors such as fruit quality, yield, cold hardiness, and storage life have been emphasized in this program, however disease resistance is also a goal where possible. The Geneva program released cultivars Liberty and Freedom, which are highly resistant to fire blight (Lamb et al. 1979; Lamb et al. 1985). However, the development of scion varieties with desirable horticultural traits and multiple disease resistances, such as to apple scab and fire blight, is very challenging (van der Zwet et al. 2012).

J. N. Cummins and H. S. Aldwinckle undertook an alternate approach to combine horticultural traits and disease resistance with the initiation of the Geneva apple rootstock-breeding program in the late 1960's. This program has produced several fire blight resistant rootstocks over the past several decades (van der Zwet et al. 2012). Some of the most effective commercially available Geneva Apple rootstocks are G.11, G.16, G.65, G.41, G. 213, G.214,

G.814, G.935, G.222, G.202, G.969, G.30, G.210, and G.890 (Norelli et al. 2003; Fazio et al. 2016). These rootstocks maintain some of the dwarfing properties important to apple production systems while providing acceptable fire blight resistance to the rootstock (Norelli et al. 2000). There are additional commercially available rootstocks, such as Budagovsky 9**,** that have fire blight resistance (Russo 2008a). Along with traditional breeding, transgenic rootstock breeding has allowed researchers to investigate the possibly of fire blight resistance in susceptible Malling rootstocks such as M. 26, which are highly desirable for their horticulture traits (Aldwinckle and Norelli 2000; Wöhner et al. 2016).

Regardless of the scion variety or rootstock chosen for establishment of a new orchard, it is critical that all planting material be pathogen free. *Erwinia amylovora* may spread to new trees and established orchards when brought in on infected planting materials. The presence of *E. amylovora* in asymptomatic nursery propagating material, such as budwood, young trees, and shoots of established trees has been observed in several studies and described as latent infections (Keil and van der Zwet 1972; van der Zwet and Bell 1982; van der Zwet and Walter 1996; Hickey et al. 1999; McManus and Jones 1994a). Scouting for fire blight symptoms on planting material is crucial for disease management, however the presence of asymptomatic infection may result in disease development in orchards. Currently, there are no programs for the certification of *E. amylovora* free apple materials.

Despite measures taken to produce plant pathogen-free resistant apple varieties, active management of fire blight throughout the apple-growing season is crucial in New York, especially during bloom and after trauma events. There are several products that can be applied to blossoms in order to prevent blossom blight. Commonly used protectants include biological control bacteria, and bactericides such as copper and antibiotics. Biological control agents used

commonly for fire blight management include bacterial antagonists such as *Pseudomonas fluorescens* and *Pantoea agglomerans.* Commercial products such as BlightBan and Bloomtime biological containing these antagonistic bacteria have proven useful in reducing disease in the semi-arid climate of the Northwestern United States (Stockwell and Stack 2007; Pusey and Curry 2004). Copper is applied early in the season before bloom to reduce overwintering *E. amylovora*; however, copper is associated with undesirable fruit damage, such as russetting in many apple cultivars (van der Zwet and Beer 1991). In New York the most effective and widely used fire blight management product is the aminoglycoside antibiotic, streptomycin (Russo 2008b). Streptomycin is applied at bloom to protect blossoms from *E. amylovora* infection, providing more effective control than copper and biological products, with no fruit injury (van der Zwet 2012). Additional antibiotics used in fire blight management include oxytetracycline and kasugamycin. Oxytetracycline was used notably during severe fire blight outbreaks in Michigan in the 1990's when streptomycin resistant *E. amylovora* was being frequently reported, but it did not provide as effective control as streptomycin (Jones 1991). Kasugamycin has been used for fire blight management for a relatively short time period; however, this antibiotic has shown to provide as effective blossom blight control as streptomycin (McGhee and Sundin 2011). Kasugamycin was approved for use in New York on apple for blossom blight management at the beginning of the 2015 growing season, although streptomycin remains the standard due to its affordable cost and history of efficacy in New York orchards.

The management of shoot blight later in the growing season includes the use of antibiotics and growth regulators. Antibiotics are used preventatively after trauma events, such as windstorms and hail, which create wounds for bacterial entry. Streptomycin is commonly applied after trauma events to provide effective shoot blight control in New York. Growth regulators are

also applied for indirect shoot blight control. Fire blight control is due to the manipulation of the host plant's growth rather than having a direct effect on *E. amylovora*. Prohexadione-calcium (brand name Apogee) is used to control vegetative growth in apple to maintain tree size. The reduction of new green tissue later in the growing season reduces the amount of highly susceptible tissue and therefore reduces risk of shoot blight (Aldwinckle et al. 2000). Recent evidence also suggests that prohexadione-calcium increases cell wall thickness in vegetative tissues, which may contribute to decreased infection of shoots (McGrath et al. 2009; Yoder et al 1999).

Once *E. amylovora* becomes established in an orchard, sanitation efforts are necessary to reduce inoculum levels for the following seasons. Removal of symptomatic tissues, such as strikes, holdover cankers, or even whole trees may help prevent the spread of *E. amylovora* during trauma events and during the following season (van der Zwet 2012). Blighted tissues are usually removed during winter tree pruning, however, it has been shown that removal of blighted tissues during the growing season is also effective at reducing subsequent fire blight symptoms (van der Zwet and Beer 1991; van der Zwet 2012).

Streptomycin resistance in *Erwinia amylovora*; the distribution of SmR *E. amylovora* in the United States and potential impacts on the sustainability of streptomycin use for fire blight management

Streptomycin has been employed for agricultural use since 1955 and is currently the most effective and widely used fire blight management tool in New York orchards (McManus et al. 2002; Agnello et al. 2016). Growers apply streptomycin before wetting events when temperatures exceed 60°F to protect blossoms from *E. amylovora* infection that can occur during

these high-risk periods. Streptomycin is also applied after trauma events, which create entry wounds on green tissues. The antibiotic serves to prevent infection, thus helping to prevent shoot blight. Because streptomycin serves as a protectant, it will not aid in the management of established blossom or shoot blight. Misuse of streptomycin by applying at inappropriate time points, applying at rates lower than the label recommendation, and overuse to treat established shoot blight infections are factors that may contribute to the development of resistance in *E. amylovora* (Agnello et al. 2016). Because streptomycin is the primary tool for fire blight management in New York, the sustainability of this control is of high importance to New York apple growers.

The sustainable use of streptomycin is dependent upon several factors, most importantly is the ability of this antibiotic to provide an adequate level of fire blight control. Development of streptomycin resistance in *E. amylovora* threatens the efficacy of streptomycin in controlling fire blight outbreaks. There are two known mechanisms of streptomycin resistance in *E. amylovora*; one mechanism is mutational while the other is of an acquired nature. The mutational mechanism of streptomycin resistance is due to a point mutation at codon 43 within the *rpsL* gene, which codes for the S12 ribosomal protein in *Erwinia amylovora* (Chiou and Jones 1995A). Streptomycin, in a non-mutant bacterial cell, binds the ribosome at the S12 protein to shut down translation of mRNA into protein, which results in cell death. The mutation at codon 43 results in an altered S12 protein that no longer allows the binding of streptomycin, making the antibiotic ineffective.

The second mechanism of resistance is due to the presence of the tandem genes *strA* and *strB*. The *strA/strB* genes code for aminoglycoside-modifying phototransferase enzymes. These enzymes modify streptomycin within the cell making the antibiotic ineffective (Chiou and Jones

1995A; Chiou and Jones 1995B). In *Erwinia amylovora* these genes reside on the transposable element Tn5393 found on the ubiquitous non-conjugative plasmid, pEA29. *StrA* and *strB*, and homologs of these genes, are often found in environmental bacteria conferring streptomycin resistance. *StrA/strB* are found in many common apple epiphytes, such as *Pseudomonas* species and *Pantoea agglomerans*, and reside on conjugative R plasmids, some within Tn5393 (Burr et al. 1988; Chiou and Jones 1993). It is theorized that *strA/strB* may have been acquired by *Erwinia amylovora* as a result of a horizontal gene transfer event from the closely related bacteria *Pantoea agglomerans* (Burr et al. 1988; Chiou and Jones 1993; van der Zwet et al. 2012).

Streptomycin resistant (SmR) *E. amylovora* was first isolated in California in 1971. SmR *E. amylovora* was found in Washington and Oregon in 1972, and since has spread eastward into several Midwest states (Miller and Schroth 1972; Coyier and Covey 1975; McManus and Jones 1994B). SmR *E. amylovora* was isolated in Southwest Michigan in 1991 and had spread rapidly to additional apple growing regions throughout the state by 2007. This rapid spread was likely due to the movement of infected planting material from nursery operations in Southwest Michigan (McGhee 2008).

The most recent report of SmR *E. amylovora* occurred in New York. In 2002 two isolates, termed NY17.1 and NY17.2, were recovered from a fire blight outbreak in a newly planted orchard in Wayne County. Eradication efforts immediately were deployed to contain the outbreak of SmR *E. amylovora* and prevent spread to additional orchards. However, several SmR *E. amylovora* isolates were recovered from orchards in the same region in 2011 as a result of a fire blight survey. Subsequent surveys in 2012, 2013, and 2014 also led to the isolation of SmR *E. amylovora*. The prevalence of SmR *E. amylovora* in New York orchards is explored in

Chapter 1. The goal of this study (Chapter 1) is to define the current prevalence of SmR *E. amylovora* in New York and inform growers of the presence of these bacteria so that they may employ fire blight management strategies that may contain or eradicate SmR *E. amylovora* in their orchards.

While SmR *E. amylovora* is continuing to spread in New York, the sources and movement of strains are poorly understood. Strain tracking of this bacterium is complicated by the lack of genetic diversity in the *E. amylovora* genome. A study comparing whole genomes of *E. amylovora* isolates from the United States and Europe showed over 99.99% genetic homogeneity, reflecting recent global spread and low rates of evolution within the species (Smits et al. 2010). Previous studies investigating diversity have shown limitations in distinguishing strains (McGhee and Sundin 2012; Mann et al. 2013).

CRISPRs (clustered regularly interspaced short palindromic repeats) are found in 48% of bacteria and have been used to explore diversity and strain tracking in several human pathogenic bacteria such as, *Escherichia coli*, *Yersinia pestis* and *Salmonella enterica* (Almendros et al. 2014; Barros et al. 2014; and Shariat et al. 2013). In 2012, McGhee showed that CRISPR spacer array sequence analysis in *E. amylovora* could allow a high level of differentiation among isolates on a regional scale, using isolates from apple growing regions across the United States and several additional countries, and furthermore may be used to explore parental strains of SmR *E. amylovora* in Michigan (McGhee and Sundin 2012). Because the presence of SmR *E. amylovora* in New York is a relatively new occurrence and has been the focus of widespread fire blight surveys, it is an ideal time to use CRISPR spacer array sequence analysis to investigate SmR isolates. The use of CRISPR spacer array sequence analysis to examine diversity and possible origins of SmR *E. amylovora* isolates in New York is investigated in Chapter 2. The

goals of this study (Chapter 2) were to investigate the possible origins of SmR *E. amylovora* in New York and examine the movement of SmR *E. amylovora* strains throughout the state. These results may also provide evidence for the future inspection of routes in which SmR *E. amylovora* may be introduced or spread.

Approaches to prevent or mitigate the spread of streptomycin resistance within New York and to additional apple growing regions

Managing streptomycin resistance in *Erwinia amylovora* has become a major component of fire blight disease management in New York. Streptomycin is still the most widely used and most effective management tool for fire blight, but the sustainability of this control is unclear due to the spread of SmR *E. amylovora*. With few alternatives, none as effective and economical as streptomycin, growers remain reliant on this antibiotic. The future use of streptomycin will depend upon the appropriate use of this antibiotic to prevent the occurrence of resistance development within individual orchards. Appropriate and responsible use of streptomycin includes using the full labeled rate and using the product only when it is advised, such as at bloom and after trauma events. Future streptomycin use may also depend on whether the application of antibiotics in agriculture remains legal in the United States (McManus et al. 2002).

Overuse of streptomycin throughout the growing season and over many years has been considered as a high-risk action that may lead to the development of streptomycin resistance in orchards (Moller et al. 1981; McManus et al. 2002). History of streptomycin use in individual orchards has been linked to the recovery of SmR *E. amylovora* isolates in Washington and Michigan (Loper et al. 1991; Yashiro and McManus 2012). However, the direct effects of streptomycin applications on SmR bacteria and epiphytic bacterial community structure are

largely unknown. With the recent isolations of SmR *E. amylovora* in New York orchards, it is crucial for growers to understand how overuse of streptomycin within a season could affect streptomycin resistance and non-target epiphytic bacteria. Chapter 3 discusses the effects of streptomycin and the newly available alternative antibiotic, kasugamycin, on target and non-target epiphytic bacteria in the apple phyllosphere. The goal of this research is to understanding how overuse of antibiotics affects bacterial communities and the development of antibiotic resistance in the field setting. The results of this study will provide evidence to support recommendations against the misuse of both streptomycin and the newly available kasugamycin.

Preventing the occurrence of streptomycin resistance in orchards is also dependent upon the use of clean planting materials free of SmR *E. amylovora,* which cannot be guaranteed due to a lack of certification programs. It is theorized that the original isolates discovered by Russo in 2002 were present in New York as a result of an introduction event from another apple growing region of the United States, possibly Michigan given the presence of the *strA/strB* gene pair commonly found in Midwestern SmR *E. amylovora* isolates (Russo et al. 2008). Clean planting material is currently characterized by a lack of fire blight symptoms, although it is known that young trees and budwood may harbor *E. amylovora* while remaining asymptomatic (Keil and van der Zwet 1972; McManus and Jones 1994a; Smith 2002). Asymptomatic fire blight infections complicate efforts to eradicate the disease from plantings.

The prompt removal of fire blight symptomatic tissues and trees from nursery stock is recommended to prevent the movement of *E. amylovora* to additional orchards (Agnello et al. 2016; Bonn and van der Zwet 2000: van der Zwet et al. 2012). However, it is difficult to say if surrounding trees remain free of the pathogen after symptomatic tissues are removed. The removal of these tissues, while important for orchard sanitation, can complicate the collection of

budwood for the propagation of new trees because areas of the orchard harboring *E. amylovora* are disguised after external symptomatic tissues are removed. The presence of *E. amylovora* in budwood has been established, but the position of symptomatic tissues, such as fire blight strikes, in relation to asymptomatic budwood has not been established. The relationship of fire blight strikes to the recovery of *E. amylovora* from asymptomatic buds is explored in Chapter 4. The goal of this research (Chapter 4) is to establish a relationship between fire blight strikes and presence of asymptomatic infected budwood. The results of this study will further inform the selection of budwood for tree propagation in relation to the presence of fire blight strikes in an orchard.

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CHAPTER 1

PREVALENCE OF STREPTOMYCIN RESISTANT *ERWINIA AMYLOVORA* IN NEW YORK APPLE ORCHARDS[1]

ABSTRACT

Resistance to streptomycin in *Erwinia amylovora* was first observed in the United States in the 1970's, but was not found in New York until 2002 when streptomycin resistant (SmR) *E. amylovora* was isolated from orchards in Wayne County. From 2011 to 2014, a total of 591 fire blight samples, representing shoot blight, blossom blight, and rootstock blight were collected from 80 apple orchards in New York. From these samples, 1,280 isolates of *E. amylovora* were obtained and assessed for streptomycin resistance. Thirty-four SmR *E. amylovora* isolates were obtained from 19 individual commercial orchards. The majority of the resistant isolates were collected from orchards in Wayne County, and the remaining were from other counties in western New York. Thirty-two of the 34 resistant isolates contained the streptomycin resistance gene pair *strA/strB* in the transposon Tn*5393* on the non-conjugative plasmid pEA29. This determinant of streptomycin resistance has only been found in SmR *E. amylovora* isolates from Michigan and the SmR *E. amylovora* isolates discovered in Wayne county New York in 2002. The remaining two isolates had the K43R mutation in the *rpsL* gene, which is commonly found

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in western US SmR isolates, that encodes ribosomal protein S12 where streptomycin binds. Currently our data indicate that SmR *E. amylovora* is isolated to counties in western New York and is concentrated in the county with the original outbreak. Since the resistance is primarily present on the non-conjugative plasmid, it is possible that SmR has been present in Wayne County since the introduction in 2002, and has spread within and out of Wayne County to additional commercial growers over the past decade. However, research is still needed to provide in-depth understanding of the origin and spread of the newly discovered SmR *E. amylovora* to reduce the spread of streptomycin resistance into other apple growing regions, and address the sustainability of streptomycin use for fire blight management in New York.

INTRODUCTION

Fire blight is a devastating disease of apple, pear, and many other Rosaceous hosts, caused by the bacterium *Erwinia amylovora. E. amylovora* overwinters in cankers formed during the apple growing season and initiates infection in the spring when the orchard temperatures warm and cankers begin to release ooze containing high numbers of bacteria (Beer and Norelli 1977; Vanneste 2000). The bacteria are then spread by insects, wind, and rain to flowers, which are highly susceptible to infection (De Wael et al. 1990). Flower stigmas provide a nutrient rich environment in which *E. amylovora* can colonize and multiply rapidly (Beer and Norelli 1977). Following rain or even dew, these bacteria are washed into the hypanthium and enter natural floral nectaries, which may result in blossom infection producing blackened and necrotic flower clusters (Rosen 1936; Sundin 2014). *E. amylovora* subsequently spreads internally throughout the vascular tissues resulting in systemic infections that may kill limbs, the trunk, and the

rootstock of the tree. Infection of susceptible rootstocks may result in death of the entire tree (van der Zwet 1969). The loss of entire trees and plantings has a tremendous impact on production. This is especially the case in moderately to highly susceptible apple cultivars, which are widely planted and in increasingly high demand (Bonn 2000). To complicate matters, modern planting systems in New York are typically composed of tightly spaced (>1000 trees/A) small vigorous trees of highly susceptible scion cultivars (Robinson et al. 2013; Robinson 2008a, b). Since high epiphytic populations of *E. amylovora* may lead to blossom blight, shoot blight, and potentially rootstock blight, protecting flowers from infection throughout bloom is essential for managing fire blight in orchards.

Blossom blight is typically managed by a combination of biological and chemical controls, and environmental monitoring. Biological controls inhibit *E. amylovora* through antibiosis or competitive inhibition, preventing the buildup of high populations levels on the stigma (Pal and Mc Spadden Gardener 2006; Johnson et al. 2009). While many biological controls are fairly effective in controlling blossom blight in semi-arid apple production regions in the western United States, they do not provide effective control of blossom blight in the humid temperate production regions of the midwestern and northeastern United States (Sundin et al. 2009). Aside from the biological controls, conventional chemical controls for fire blight include copper and antibiotics. Copper is only effective for control of blossom blight when applied prior to infection and can cause deleterious effects to the developing fruit and foliage (Jama and Lateur 2007; Montag et al. 2006). Hence, copper is usually not applied for blossom blight in orchards producing apples destined for the fresh market. Oxytetracycline can provide effective blossom blight control in semi-arid apple production regions such as those in the western United States (Stockwell et al. 2008), but the humid climate of the northeastern U.S. diminishes the

effectiveness of oxytetracycline's bacteriostatic mode of action (van der Zwet et al. 2012). The most effective means of chemical control of blossom blight in the midwestern and northeastern United States is the aminoglycoside antibiotic streptomycin (Russo et al. 2008; Cox et al. 2012; Sundin et al. 2009.). This is primarily due to streptomycin's bactericidal mode of action and the fact that there are no other products offering a similar cost-effectiveness (Russo et al. 2008; Sundin et al. 2009; and van der Zwet 2012). The aminoglycoside antibiotic kasugamycin was registered for use by the EPA in 2015 (EPA Reg. No. 66330-404) and can provide a similar level of control, but does not have the same cost-effectiveness as streptomycin (McGhee and Sundin 2011; Yoder et al. 2011).

Because of the aforementioned utility, streptomycin has been used widely in the United States for over 50 years providing effective and necessary control of *E. amylovora* outbreaks. However, reports of streptomycin resistance have raised concern about the sustainability of this antibiotic for fire blight management. The first reports of streptomycin resistance occurred in California in 1971 followed by Washington and Oregon in 1972 (Miller and Schroth 1972; Coyier and Covey 1975; McManus and Jones 1994). Since these discoveries, streptomycin resistant (SmR) *E. amylovora* has become established in apple growing regions along the western coast of the United States and in certain apple growing regions of Missouri and Michigan (McManus et al. 2002). SmR isolates of *E. amylovora* were not observed in New York until 2002 (Russo et al. 2008; Beer and Norelli 1976). In this instance two SmR isolates *of E. amylovora*, NY17.1 and NY17.2, were recovered from fire blight outbreaks in two adjacent orchards in Wayne County (Russo et al. 2008). Eradication efforts, which included prompt removal of trees, were put in place in order to contain and destroy SmR *E. amylovora* infected

plant materials and prevent spread of these bacteria to other orchards (Russo et al. 2008; Russo and Aldwinckle 2009).

Currently, there are two known determinants of streptomycin resistance in *E. amylovora*. The first is the presence of a point mutation at codon 43 in the *rpsL* gene, which codes for the S12 ribosomal protein (Chiou and Jones 1995a). This single base pair mutation causing a substitution of lysine for arginine (K43R) alters the binding site of streptomycin, which would otherwise bind to the S12 protein and inhibit translation of mRNA to proteins (McManus et al. 2002). In a sensitive strain of *E. amylovora*, streptomycin binds to the S12 protein on the ribosome to block protein synthesis, killing the bacterium. This mechanism confers resistance in *E. amylovora* at streptomycin concentrations up to 2500 μg/ml (Chiou and Jones 1995a). This mutation is persistent in populations, and is the most common mechanism found in streptomycin resistant *E. amylovora* in the western United States (Moller et al. 1981). Strains with this type of resistance are rarely found in apple orchards east of the Mississippi River (McManus et al. 2002).

A second determinant of streptomycin resistance is the gene pair *strA/strB*. These tandem genes code for aminoglycoside-modifying phosphotransferase enzymes that modify streptomycin making the antibiotic ineffective within the cell (Chiou and Jones 1995b). These genes are commonly found in many epiphytic bacteria, such as *Pseudomonas* spp., and reside on conjugative R plasmids (Burr et al. 1988). In *E. amylovora, strA/strB* has been found on plasmid RSF1010 (Palmer et al. 1997), and within the transposable element Tn*5393* on either a conjugative plasmid pEA34 or a nonconjugative plasmid pEA29 (McManus and Jones 1994; McGhee et al. 2011). The *strA*/*strB* gene pair in Tn*5393* on plasmid pEA29, the basis of

resistance for the isolates discovered in 2002 in New York is the most common determinant of resistance in Michigan (Russo et al. 2008; McManus and Jones 1994; McGhee et al. 2011).

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

METHODS AND MATERIALS

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

From 2011 to 2014, samples of fire blight were collected from outbreaks at nurseries and production orchards across the apple growing regions of New York State. Collection efforts took

place as a cooperative effort between the New York State Agricultural Experiment Station, Cornell cooperative extension, the Lake Ontario Fruit Program, New York State Integrated Pest Management, and Eastern New York Regional Fruit Program. Sample collection efforts were largely driven by grower and cooperative extension reported instances of fire blight outbreaks within individual orchards of relevance to production sustainability. Samples consisted of blighted blossom clusters, shoot blight of first and second year scion, and blighted rootstocks. When fewer than 10 symptomatic trees were present each tree was sampled. If more than 10 trees were sampled, 1 sample was collected for every 2 to 5 symptomatic trees. Usually (<3% of all samples) no more than 1 sample was taken per tree. Isolates representing multiple samples from the same tree were designated by a letter after the sample.

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

Identification of *Erwinia amylovora* colonies.

PCR amplification of a portion of plasmid pEA29 was used to confirm the identity of putative *E. amylovora* isolates with cratered colony appearance on CG. The pEA29 has shown to be a reliable marker for the identification *E. amylovora* because it is found ubiquitously in isolates collected worldwide; furthermore, laboratory strains cured of the plasmid show reduced virulence and were deemed unlikely to persist in nature (Chiou and Jones, 1993; Bereswill et al. 1992; McGhee and Jones 2000). However, it is possible that if naturally *E. amylovora* isolates lacking this plasmid exist in New York, these isolates may have been overlooked in this study. PCR amplification of pEA29 was conducted using primers previously developed by McManus and Jones (1995) (Table 1.1). PCR reactions were performed in 25 µl reaction volumes, and consisted of 12.3 µl H_2O, 5 µl 5X Green GoTaq Flexi Buffer (Promega Corp., Madison, WI), 1 µl each of forward and reverse primer (AJ75/AJ76), 0.5 µl 10 mM dNTP mix (Promega Corp.), 2.5 µl 25mM MgCl2 (Promega Corp.), 0.2 µl GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial suspension sample. Cycling parameters were 5 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, annealing at 52°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. PCR products were separated using gel electrophoresis with 1% agarose gels in 1x TAE buffer (44.5 mM Tris-borate, 1 mM EDTA, pH 8.0) at 90 volts for 60 minutes. The resulting amplified region was sequenced for a subset of colonies for confirmation of the presence of pEA29. PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research, Irvine, CA). Purified products were sequenced at the Cornell Biotechnical Resource center in Ithaca, NY using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems, Waltham, MA).

Streptomycin resistance screening of *E. amylovora* isolates.

Isolates positively identified as *E. amylovora* were assessed for streptomycin resistance in vitro. Initially, single colonies of the isolates were grown in 1 ml of Luria-Bertani (LB) broth (Bertani 1952) at 28°C for 24 hours. Following incubation, 100 µl of the solution was spread on LB media and allowed to dry. Autoclaved filter paper discs were soaked in 0, 100, and 2500 µg/ml streptomycin sulfate (Sigma-Aldrich, St. Louis, MO) and placed on the LB containing the isolate lawns. Plates were incubated at 28°C for 48 hours, and streptomycin resistance was evaluated by observing the presence or absence of a zone of inhibition around the streptomycin discs. The lack of a zone of inhibition at each concentration confirmed resistance at varying concentrations of streptomycin sulfate, and isolates with resistance phenotypes were re-tested to confirm resistance.

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

Identification of the *strA/strB* gene pair in streptomycin resistant *E. amylovora* isolates.

All streptomycin resistant isolates and the SmS isolate Ea273 (negative control) were tested for the presence of the *strA/strB* gene pair using primers previously developed by Russo et al. (2008) (Table 1.1). PCR reactions were performed in 25 µl reaction volumes, and consisted of

12.3 µl H_2O, 5 µl 5X Green GoTaq Flexi Buffer (Promega Corp.), 1 µl of forward and reverse primer (strA406-F/strA406-R for *strA* and strB403-F/strB403-R for *strB*), 0.5 µl 10 mM dNTP mix (Promega Corp.), 2.5 µl 25mM MgCl2 (Promega Corp.), 0.2 µl GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial suspension sample resulting in a 50 µl reaction. Cycling parameters were 5 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, annealing for 30 seconds (56°C for *strA* primers and 53°C for *strB*), and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. PCR products were visualized using gel electrophoresis on a 1% agarose gel. For a subset of isolates, PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research). Purified products were then sequenced at the Cornell Biotechnical Resource center in Ithaca, NY using an ABI 3730xl capillary sequencer (Applied Biosystems).

Determination of the Tn*5393* insertion site on pEA29 pair streptomycin resistant *E. amylovora* isolates.

All streptomycin resistant isolates with the *strA-strB* gene pair and two SmR isolates DM1 and MI5-1 (positive controls) (McGhee et al. 2011) were tested for the presence and insertion of Tn*5393* in pEA29 using primers Tn5393E and AJ1717 (pEA29 position 1,515) and AJ507 and AJ339 (pEA29 position 17,527) as described by McGhee et al. (2011). PCR reactions were performed in 25 µl reaction volumes, and consisted of 12.3 µl H_2O, 5 µl 5X Green GoTaq Flexi Buffer (Promega Corp.), 1 µl of forward and reverse primer, 0.5 µl 10 mM dNTP mix (Promega Corp.), 2.5 µl 25mM MgCl2 (Promega Corp.), 0.2 µl GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial suspension sample resulting in a 50 µl reaction. Cycling parameters were identical to those reported for the two sets of primer pairs in

McGhee et al. (2011). PCR products were visualized using gel electrophoresis on a 2% agarose gel. For a subset of isolates, PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research).

S12 ribosomal protein *rpsL* gene identification and sequencing in streptomycin resistant *E. amylovora* isolates.

Isolates confirmed to have a streptomycin resistance phenotype that would allow them to grow in the presence of 2500 µg/ml streptomycin sulfate were examined for the presence of mutations at codon 43 in the *rpsL* gene. Using primers previously developed by Russo et al. (2008) (Table 1.1), a portion of the *rpsL* gene containing codon 43 was amplified and sequenced. PCR reactions consisted of 24.6 µl H_2O, 5 ul 5X Green GoTaq Flexi Buffer (Promega Corp.), 2.0 µl each of forward and reverse primer (rpsL212-F/rpsL212-R), 1.0 µl 10 mM dNTP mix (Promega Corp.), 5.0 µl 25mM $MgCl_2$ (Promega Corp.), 0.4 µl GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 5.0 µl of bacterial suspension sample resulting in a 50 µl reaction. Cycling parameters were 5 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, annealing at 53°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research). Purified products were sequenced at the Cornell Biotechnical Resource center in Ithaca, NY using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems).

RESULTS

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

From 2011 to 2014, samples were collected from a total of 80 commercial orchards with fire blight outbreaks. The majority of the samples were from orchards in Wayne, Monroe, Niagara, Ontario, Orleans, and Tompkins counties in western New York. By comparison, only 19 of the 591 samples were from Albany, Clinton, Orange, Suffolk, and Ulster counties in eastern New York. Of the 591 samples collected, 97 were blighted blossoms, 415 were shoot blight of first or second year scions, and 74 were blighted rootstocks. In addition, there were 5 samples collected from the coleopteran *Xylosandrus germanus* or its galleries that appeared to be oozing with fire blight. Across all years, samples were predominantly from cultivars 'Gala', 'Ginger Gold', 'Honeycrisp', 'SnapDragon', and 'RubyFrost'. From the samples, 1,384 bacterial isolates were obtained and 1,280 were confirmed to be *E. amylovora*. Single colonies of isolates produced the characteristic cratered appearance on CG and when PCR was performed using primers AJ75 and AJ76, all isolates produced an 840 bp band, indicative of the presence of the ubiquitous, nonconjugative plasmid, pEA29.

Streptomycin resistance screening and pathogenicity of *E. amylovora* isolates.

Of the 1,280 isolates, 34 displayed a streptomycin resistant phenotype on CG in that a zone of inhibition failed to develop around filter papers discs with streptomycin concentrations of 100 µg/ml. For two of the 34 isolates, 306b and 189a, a zone of inhibition failed to develop around filter papers discs with streptomycin concentrations of 2500 µg/ml (Table 1.2). All 34 streptomycin resistant isolates of *E. amylovora* isolates and Ea273 were found to produce

necrotic lesions and bacterial ooze within 48 to 96 hours on immature pear fruits confirming pathogenicity (Table 1.2).

Of the 34 SmR isolates of *E. amylovora*, 20 were recovered from orchards in Wayne County and 5 were from orchards in Ontario County (Tables 1.2, 1.3). The remaining isolates were recovered from orchards in Monroe, Orleans, Tompkins, and Niagara counties in western New York (Figure. 1.1). These isolates were recovered from 19 individual orchards in which SmR *E. amylovora* was isolated from 4% to 100% of samples (Tables 1.2, 1.3). Isolates of SmR *E. amylovora* were recovered from 20 cultivars of apple with three or more SmR isolates recovered from 'Idared', 'RubyFrost', and 'McIntosh' apples. Nearly all of the isolates (28) were obtained from blighted shoots that developed in the current year's scion tissue (Table 1.2). Three isolates were obtained from blossom clusters, two from rootstock blight samples, and one was obtained from the coleopteran *Xylosandrus germanus.*

Identification of the *strA/strB* gene pair and Tn*5393* insertion site on pEA29 in streptomycin resistant *E. amylovora* isolates.

Amplification of the Tn*5393* region containing the *strA/strB* gene pair revealed the presence of a 406 bp and 403 bp band in 32 of the SmR isolates of *E. amylovora.* These bands were not present for SmS isolate Ea273 and the two streptomycin resistant isolates 306b and 189a, which grew in the presence of 2500 ug/ml streptomycin. Sequencing of these regions confirmed the identity of the 406bp and 403bp bands (GenBank Accession No. KT899306 and KT899307) to be the *strA* and *strB* genes previously described for streptomycin resistant isolates of *E. amylovora* (Russo et al. 2008). The Tn*5393* insertion site for all 32 isolates occurred at position 17,527 on pEA29 evidence by the presence of an expected 396 bp band (Figure 1.2).

S12 ribosomal protein *rpsL* gene identification and sequencing in streptomycin resistant *E. amylovora* isolates.

Amplification of the region containing codon 43 of the S12 ribosomal protein yielded a 212 bp band for all 34 SmR isolates of *E. amylovora* and Ea273, the SmS control. Sequencing of the 212 bp band confirmed the identity of the 212 bp region to be a portion of the *rpsL* gene of *E. amylovora*. Sequences of each resistant isolate were compared to the sequence of the sensitive isolate Ea273 (GenBank Accession No. KT899305). Two isolates, 306b (GenBank Accession No. KT899304) and 189a, were found to have a point mutation at codon 43 (Table 1.2). In both isolates, this mutation resulted in an amino acid change from lysine to arginine (K43R). The remaining 32 isolates and the SmS isolate Ea273 did not have a mutation present in this region.

DISCUSSION

A total of 34 SmR isolates of *E. amylovora* were found in 19 individual orchards across 6 counties in western New York (Tables 1.2, 1.3). Although 19 samples were collected from orchards in 5 counties in eastern New York, SmR *E. amylovora* was not found in the eastern apple production regions of the state. Given that the use of streptomycin for fire blight management practices is fairly consistent throughout the state (K. Cox, unpublished data), the absence of SmR in eastern New York may suggest that the emergence of streptomycin resistance in western New York after 2002 may be due to local transport of asymptomatic propagative materials or localized spread of isolates in regionalized storms over the last decade. However,

such patterns of emergence cannot be used as evidence or an explanation for the spread SmR *E. amylovora*.

Although the fire blight collection taskforce made efforts to scout all of the major commercial production operations throughout the state, by no means to do our efforts represent a structured, systematic, sampling survey effort. Of the fire blight outbreaks that were observed by the survey team, they primarily were found on blocks planted to 'RubyFrost', 'Gala', 'Gingergold', 'SnapDragon', and 'Honeycrisp', but isolates of SmR *E. amylovora* were rarely found on these cultivars. It may be that cultivars 'RubyFrost', 'Gala', 'Gingergold', 'SnapDragon', and 'Honeycrisp' are rather susceptible to fire blight and growers with these cultivars should take additional care with fire blight management practices. It should be noted that all plantings of 'RubyFrost' and 'SnapDragon' were no more than 1 year old. Hence, apparent sensitivity of 'RubyFrost' and 'SnapDragon' may have been masked by the overly vigorous production in the establishment years.

Aside from cultivar trends, the majority of the SmR *E. amylovora* isolates were recovered from shoot blight samples. Shoot blight samples comprised the majority fire blight samples observed and collected by the survey team as blossom blight and rootstock blight were rarely observed by comparison. Shoot blight samples were all collected in late June through July (data not shown) when most of the streptomycin use had ceased. Shoot blight is typically initiated either by internal migration of *E. amylovora* to the growing shoot tips or by trauma to growing shoots, which become infected by *E. amylovora* spread from oozing cankers and infected flower clusters (van der Zwet et al. 2012). Hence, SmR *E. amylovora* isolates collected from shoot blight sample may not have emerged from local streptomycin control failures or streptomycin overuse during bloom. Alternatively, SmR *E. amylovora* found in shoot blight samples may have

resulted from the limited streptomycin applications often made to protect against fire blight during storms that occur within the period of shoot elongation. While data from the current study cannot prove that applications of streptomycin for shoot blight management lead to the development of streptomycin resistance, it cannot be discounted as several producers with SmR *E. amylovora* isolated from blighted shoots often reported using streptomycin after bloom for shoot blight management. The repeated use of streptomycin throughout the season would provide many selection events, which may result in the increase of SmR *E. amylovora*.

Of the SmR *E. amylovora* isolates recovered, a total of 32 contained the *strA/strB* gene pair located on the Tn*5393* transposon on pEA29. This *strA/strB* gene pair itself has been shown to confer a streptomycin resistance in several bacterial species commonly found on the surfaces of apples (Burr et al. 1988; Burr et al. 1993; Norelli et al. 1991). The *strA/strB* gene pair has been reported to confer resistance to streptomycin resistant isolates of *E. amylovora* from Michigan and California at concentrations 100 to 200 µg/ml in vitro (Russo et al. 2008; Palmer et al. 1997; Chiou and Jones 1991; Chiou and Jones 1993). In agreement with the previous reports, all 34 SmR isolates displayed resistance to streptomycin at a minimum of 100 µg/ml in vitro. This level of resistance is particularly relevant for the management of fire blight given that agricultural applications of streptomycin are applied at a target rate of 100 µg/ml (100 ppm). Hence, any grower operations with these isolates would not be able to fully manage blossom blight using applications of streptomycin would need to rely on kasugamycin, copper, or other biological controls.

Aside from the implications regarding the level of resistance, the presence of the *strA/strB* gene pair in the Tn*5393* transposon in *E. amylovora* may shed some light on the origin of streptomycin resistance. This gene pair transposon combination has only been found on

conjugative (pEA34) and non-conjugative (pEA29) plasmids in isolates from Michigan and New York (Chiou and Jones 1993; McManus and Jones 1994; Russo et al. 2008). The *strA/strB* gene pair has been shown to confer resistance in *E. amylovora* in California, but the gene pair was on plasmid RSF1010 (Palmer et al. 1997). In the current study, all 32 isolates had the *strA/strB* gene pair in Tn*5393* on pEA29. The isolates also belong to the lineage from Michigan with the 17,527-nucleotide position insertion site for Tn*5393* on pEA29 (McGhee et al. 2011). Since the gene pair transposon combination resides on a plasmid that has shown to be nontransmissable between strains of *E. amylovora*, the isolates from the current study must be physically dispersed to new locations (McGhee and Jones 2000). Hence, these 32 SmR *E. amylovora* isolates are likely to have been transported to or spread among locations in western New York instead of local emergence due to the ingress of resistance plasmids from local epiphytic bacterial species with streptomycin resistance. Local physical distribution of isolates would further explain the lack of SmR *E. amylovora* in apple operations in eastern New York. Indeed, half of the streptomycin resistant *E. amylovora* isolates with the *strA*/*strB* gene pair were obtained from orchards in Wayne County within 160 km of the location where the SmR *E. amylovora* isolates were discovered in 2002 (Russo et al. 2008). Planting material is rarely shared outside the neighboring production operations and statewide dispersal by storms is unlikely. The localization of SmR isolates in western New York raises questions about the success of the eradication efforts in 2002, and the possibility of additional acquisitions of trees from southwest Michigan in 2002 as described in Russo et al. (2008). Indeed, direct reports to the authors from producers in 2011 suggest that others may have participated in the acquisition of such trees in 2002.

Interestingly, isolates 436 and 439, collected from the same orchard, had a slightly larger band for the pEA29 PCR amplicon with primer AJ75 and AJ76 (data not shown). Upon

sequencing the product along with one of typical amplicon size, the pEA29 sequence revealed a variant, which was identical to isolate Ea356 (Genbank accession HF560643) isolated from Cotoneaster from Germany in 1979 (Zhao 2014). While the presence of the *strA/strB* gene pair in Tn*5393* on pEA29 is believed to have Michigan origin (McGhee et al. 2011; Russo et al. 2008), the presence of Tn*5393* on a sequence variant of pEA29 from Europe suggests the possibility of another transposition. Such a discovery warrants further investigation to better determine the origin and movement of these 32 isolates.

While the 32 SmR *E. amylovora* isolates from this study containing the *strA/strB* gene pair in the Tn*5393* transposon on pEA29 are consistent with a Michigan or western New York origin, the two isolates with the K43R *rpsL* mutation from this study may suggest additional introductions or spontaneous development of resistance within New York orchards. *E. amylovora* with the K43R *rpsL* mutation are most commonly found in California and Washington (Chiou and Jones 1991; Loper et al. 1991; Moller et al. 1981; Schroth et al. 1979;). While isolates with the K43R *rpsL* have been found in apple production operations in the eastern United States, they are rarely recovered (McManus et al. 2002). Apple production operations in western New York do order trees from nurseries in Washington State, but the trees from which isolates 306b and 189a were isolated were not obtained from the western United States and could represent one of these rare recoveries. The owners of the orchards from which these isolates were recovered reported fairly standard streptomycin use practices to the authors. Hence, the origin of isolates with the K43R *rpsL* mutation is still disconcerting, although it is possible that these isolates could have arisen from the mutation of native sensitive *E. amylovora* isolates due to selective pressure from overuse of streptomycin.

Given the possibility of multiple introductions and the presence of different resistant determinants, a more in-depth genetic analysis should be conducted to determine the origin of New York isolates and to investigate the movement of isolates throughout the state. Despite the information presented in this study, there is inherent difficulty in comparing these current isolates arising from the extremely limited diversity in the genome of *Erwinia amylovora* (Sebaihia et al. 2010; Smits et al. 2010). Traditional diversity studies involving techniques such as random amplified polymorphic DNAs, pulse field gel electrophoresis, variable number of tandem repeats analyses, and ribotyping have shown difficulties in distinguishing strains (Momol et al. 1997; Jock et al. 2002; Kim and Geider 1999; McGhee et al. 2012). To investigate movement of isolates on a local and regional scale a method that allows a high degree of differentiation must be employed. CRISPR (clustered regularly interspaced short palindromic repeats) spacer analysis has recently been of interest in exploring strain diversity and tracking in several human pathogenic bacteria including *Salmonella enterica*, *Escherichia coli* and *Yersinia pestis* (Shariat et al. 2013; Almendros et al. 2014; Barros et al. 2014). This method has also been used to explore diversity in *E. amylovora* and was hypothesized to allow differentiation of isolates on a regional scale (McGhee et al. 2012). We are currently constructing CRISPR spacer profiles for New York SmR and SmS *E. amylovora* isolates to investigate diversity and strain tracking.

In summary, streptomycin resistance is an emerging threat to the New York apple industry. A single isolated event in 2002 has become a greater issue over the past decade. Currently, SmR *E. amylovora* are present throughout three of the six major apple-growing counties of New York. While kasugamycin presents a viable antibiotic alternative to streptomycin for control of fire blight, it does not offer the same cost-effectiveness. Hence,

streptomycin is still widely used and resistance to streptomycin still poses a serious threat to the New York apple industry. To make matters worse, the most popular and highest value apple cultivars in the state are highly susceptible to *E. amylovora*. It is imperative that we continue to improve our understanding of this new threat in order to generate appropriate responses to mitigate the spread of streptomycin resistance in New York and prevent further spread of SmR *E. amylovora* to additional apple growing regions of the United States.

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TABLES

Table 1.1: Primers used in this study to confirm the identity of *Erwinia amylovora* isolates, and to determine the presence of the gene pair *strA/strB* and amplify the *rpsL* gene for sequencing.

Target[a]	Primer Desigation	Sequence (5'-3')[b]	Source
pEA29	AJ75	CGTATTCACGGCTTCGCAGAT	McManus et al. 1995
	AJ76	AACCGCCAGGATAGTCGCATA	
strA	strA406-F	TGACTGGTTGCCTGTCAGAG	Russo et al. 2008
	strA406-R	CGGTAAGAAGTCGGGATTGA	
strB	strB403-F	ATCGCTTTGCAGCTTTGTTT	Russo et al. 2008
	strB403-R	CGTTGCTCCTCTTCTCCATC	
rpsL	rpsL212-F	CGTACGCAAAGTTGCAAAAA	Russo et al. 2008
	rpsL212-R	GGATCAGGATCACGGAGTGT	

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

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

Table 1.2: Characteristics of streptomycin resistant (SmR) *Erwinia amylovora* isolates collected in from apple orchards in New York from 2011 to 2014

Year	Isolate	Orchard[a]	County	Cultivar[b]	Tissue[c]	SmR (%)[d]	pEA29[e]	Pathogenic[f]	*strA/strB*[g]	*rpsL*[h]
2011										
	161	W1	Wayne	'Idared'	Shoot	12	+	+	+	-
	162	W1	Wayne	'Rome'	Shoot	12	+	+	+	-
	173	W1	Wayne	'SnapDragon'	Shoot	12	+	+	+	-
	174	W1	Wayne	'RubyFrost'	Shoot	12	+	+	+	-
	175	W1	Wayne	'McIntosh'	Shoot	12	+	+	+	-
	176	W1	Wayne	'Red Delicious'	Shoot	12	+	+	+	-
	177	O1	Ontario	'Idared'	Shoot	50	+	+	+	-
	178	M1	Monroe	'Idared'	Shoot	33	+	+	+	-
	179	W2	Wayne	'Rhode Island Greening'	Shoot	100	+	+	+	-
2012										
	316	M2	Monroe	'RubyFrost'	Shoot	17	+	+	+	-
	301	N1	Niagara	'SweeTango'	Shoot	50	+	+	+	-
	230	O2	Ontario	'Idared'	Shoot	40	+	+	+	-

	313	O2	Ontario	‘Twenty ounce’	Rootstock	40	+	+	+	-
	306a	O3	Ontario	‘Lady’	Shoot	25	+	+	+	-
	306b	O3	Ontario	‘Lady’	Shoot	25	+	+	-	K43R
	249	Or1	Orleans	‘Aztec Fuji’	Blossom	15	+	+	+	-
	278	Or1	Orleans	‘Cameo’	Shoot	15	+	+	+	-
	254	W2	Wayne	‘M.26 RS’	Shoot	100	+	+	+	-
	292	W5	Wayne	‘Idared’	Shoot	20	+	+	+	-
	189	W4	Wayne	‘Gingergold’	Shoot	25	+	+	+	-
	189a	W4	Wayne	‘Gingergold’	Shoot	25	+	+	-	K43R
	189b	W4	Wayne	‘Gingergold’	Shoot	25	+	+	+	-
	2992d	W3	Wayne	‘RubyFrost’	Shoot	45	+	+	+	-
	3002d	W3	Wayne	‘Gala’	Shoot	45	+	+	+	-
	3002e	W3	Wayne	‘Gala’	Shoot	45	+	+	+	-
2013										
	465	M3	Monroe	‘Royal Court’	Blossom	4	+	+	+	-
	321	Or2	Orleans	M.9	Rootstock	17	+	+	+	-
	436	T1	Tompkins	‘McIntosh’	Blossom	22	+	+	+	-
	439	T1	Tompkins	‘McIntosh’	Shoot	22	+	+	+	-

330	W7	Wayne	‘Jonagold’	Shoot	8	+	+	+	-
333	W7	Wayne	‘Jonagold’	Shoot	8	+	+	+	-
345	W8	Wayne	‘Pink Lady’	Shoot	9	+	+	+	-
508	W6	Wayne	‘Idared’	Shoot	17	+	+	+	-
374	W9	Wayne	‘Macoun’	*Xylosandrus germanus*	5	+	+	+	-

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

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

c. The type of apple tissue from which the isolate was collected.

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

e. The presence or absence of the non-conjugative plasmid pEA29 ubiquitous in *E. amylovora*: (+) present or (-) absent

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

g. The presence or absence of the *strA/strB* gene pair responsible for conferring resistance to streptomycin: (+) ipresent or (-) absent

h. Presence of the K43R mutation in the *rpsL* gene: (K43R) present or (-) absent

Table 1.3: Summary of survey information for orchards where streptomycin resistant (SmR) *Erwinia amylovora* was detected.

Orchard[a]	Year	County, City	Total isolates[b]	SmR isolates[c]
W1	2011	Wayne, Huron	41	6
	2012		1	1
	2013		No fire blight	-
	2014		10	0
W2	2011	Wayne, Sodus	3	1
	2012-14		No fire blight	-
W3	2012	Wayne, Williamson	17	3
	2013-14		No fire blight	-
W4	2012	Wayne, Wolcott	4	3*
	2013		No fire blight	-
	2014		80	0
W5	2012	Wayne, Macedon	6	1
	2013		3	0
	2014		35	0
W6	2013	Wayne, Williamson	5	1
	2014		No fire blight	-
W7	2013	Wayne, Wolcott	23	2
	2014		No fire blight	-
W8	2013	Wayne, Sodus	21	1
	2014		No fire blight	-
W9	2013	Wayne, Sodus	24	1
	2014		13	0
O1	2011	Ontario, Phelps	1	1
	2012-14		No fire blight	-
O2	2012	Ontario, Phelps	7	2
	2013-14		No fire blight	-
O3	2012	Ontario, Geneva	7	2*
	2013-14		No fire blight	-
M1	2011	Monroe, Webster	2	1
	2012-14		No fire blight	-
M2	2012	Monroe, Brockport	6	1
	2013		13	0
	2014		No fire blight	-
M3	2013	Monroe, Hilton	30	1
	2014		10	0
N1	2012	Niagara, Appleton	2	1

	2013		No fire blight	-
	2014		30	0
Or1	2012	Orleans, Kendall	16	2
	2013		No fire blight	-
	2014		5	0
Or2	2013	Orleans, Medina	5	1
	2014		25	0
T1	2013	Tompkins, Lansing	7	2
	2014		No fire blight	-

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

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

c. Number of SmR *E. amylovora* isolates collected from symptomatic trees for each year of the survey. The presence of an asterisk indicates that one of the isolates has the K43R mutation in the *rpsL* gene.

FIGURES

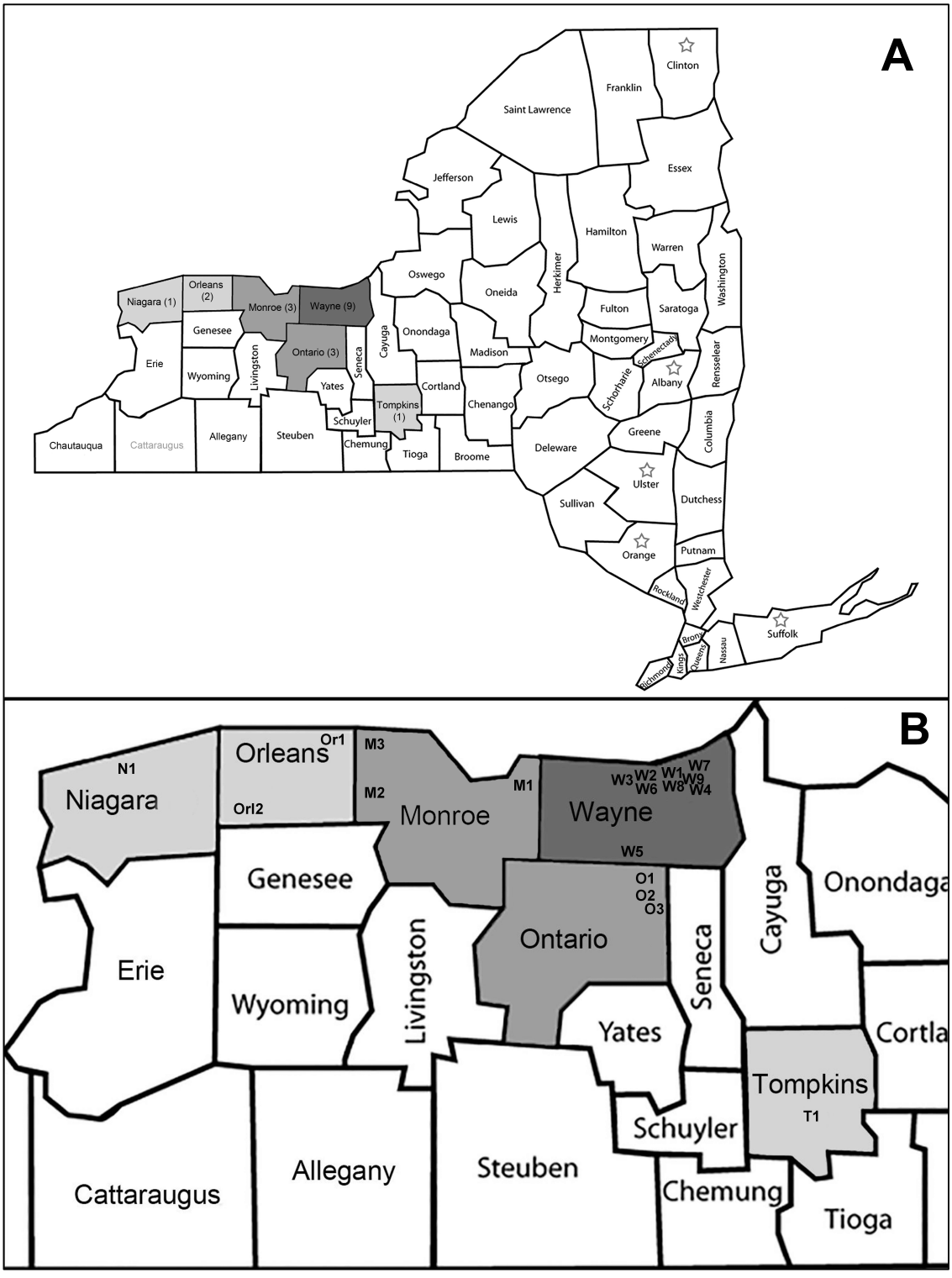
A
Clinton
Franklin
Saint Lawrence
Essex
Jefferson
Lewis
Hamilton
Warren
Washington
Herkimer
Oswego
Oneida
Fulton
Saratoga
Montgomery
Schenectady
Albany
Rensselear
Niagara (1)
Orleans (2)
Monroe (3)
Wayne (9)
Genesee
Erie
Wyoming
Livingston
Ontario (3)
Seneca
Cayuga
Onondaga
Madison
Yates
Cortland
Tompkins (1)
Schuyler
Chenango
Otsego
Schoharie
Chautauqua
Cattaraugus
Allegany
Steuben
Chemung
Tioga
Broome
Deleware
Greene
Columbia
Ulster
Sullivan
Dutchess
Orange
Putnam
Rockland
Westchester
Suffolk
Bronx
Kings
Queens
Nassau
Richmond
B
N1
Niagara
Orleans
Or1
Orl2
M3
M2
Monroe
M1
Wayne
W3
W2
W6
W1
W8
W7
W9
W4
W5
Genesee
Erie
Wyoming
Livingston
Ontario
O1
O2
O3
Seneca
Cayuga
Onondaga
Yates
Cortla
Tompkins
T1
Schuyler
Cattaraugus
Allegany
Steuben
Chemung
Tioga

Figure 1.1: New York State counties with orchards from which streptomycin resistant (SmR) isolates of *Erwinia amylovora* were identified in 2011 to 2014. (A) Counties with 4 or more, 3, or 1 to 2 orchards with SmR *E. amylovora* are shown in dark grey, medium grey, or light grey, respectively. Counties with star symbols had orchards with fire blight outbreaks and were surveyed, but did not have SmR *E. amylovora.* (B) Close up of the five counties displaying the approximate location of the orchard designated by a letter for the county and numerical identifier.

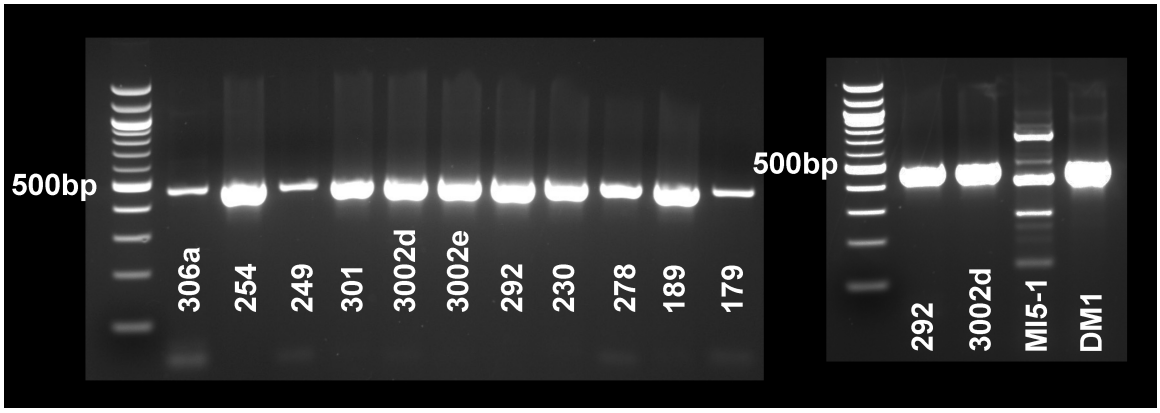

Figure 1.2: Amplification of the Tn*5393* insertion site in pEA29 at nucleotide position 17,527 in streptomycin resistant (SmR) *Erwinia amylovora* from western New York using primers AJ507 and AJ339 from McGhee et al. (2011). Shown are agarose gels with the expected the 397bp amplicon for the AJ507 and AJ339 primer pair spanning the 3' end of Tn5393 at nucleotide position 17,527 to nucleotide position 17,922 in pEA29. Both gels have Quick-Load 100-bp DNA Ladder (New England Bio-Labs) to denote amplicon size. Lanes in the gel on the left are loaded with amplicons SmR *E. amylovora* isolates collected from western NY in 2011 and 2012, while lanes in the right gel are loaded with amplicons SmR *E. amylovora* isolates collected from western NY in 2012 and the two SmR positive control isolates DM1 and MI5-1 (McGhee et al. 2011). Isolate MI5-1 has a Tn*5393* insertion at position 1,515 on pEA29, and does not make the expected amplicon for primers AJ507 and AJ339.

CHAPTER 2

EXPLORING DIVERSITY AND ORIGINS OF STREPTOMYCIN RESISTANT *ERWINIA AMYLOVORA* ISOLATES IN NEW YORK THROUGH CRISPR SPACER ARRAYS[2]

ABSTRACT

Streptomycin is the most effective and widely used chemical control in the eastern United States for blossom blight of apples caused by *Erwinia amylovora*, however resistance to this antibiotic has been a concern in New York since 2002. From 2011 to 2014, statewide collections of *E. amylovora* were conducted resulting in the isolation of SmR (streptomycin resistant) *E. amylovora* from several commercial orchards. Further genetic analysis of isolates was necessary to understand the origins and the diversity of these bacteria. CRISPR spacer sequencing was employed to explore the diversity and possible origins of New York SmR *E. amylovora* isolates. The spacer array CR1, CR2, and CR3 regions of twenty-seven SmR *E. amylovora* isolates and a seventy-six streptomycin sensitive (SmS) *E. amylovora* isolates were amplified and subsequently sequenced revealing nineteen distinct CRISPR spacer profiles for New York isolates. The majority of SmR *E. amylovora* isolates had the same CRISPR profile as SmR *E. amylovora*

[2] Reprinted from Tancos, K. A., and Cox, K. D. 2016. Exploring diversity and origins of streptomycin-resistant *Erwinia amylovora* isolates in New York through CRISPR spacer arrays. Plant Dis. 100:1307-1313.

isolates discovered in 2002. This may infer that eradication efforts in 2002 failed and the bacterial populations continued to spread throughout the state. Several CRISPR profiles for SmR *E. amylovora* were identical to SmS *E. amylovora* collected from the same orchards leading to the hypothesis that resistance may be developing within New York or that these isolates were imported together from other regions in the eastern US. Profiles not unique to New York were identical to many isolates from the mid-western, eastern, and western United States, implying that streptomycin resistance may be due to the introduction of SmR *E. amylovora* from other regions of the United States. The increased understanding as to how SmR *E. amylovora* isolates are introduced, evolve, or have become established afforded by CRISPR profiling has been useful for the management and restricting the movement of streptomycin resistance in New York.

INTRODUCTION

Erwinia amylovora, the causal agent of fire blight, is a destructive plant pathogen infecting several Rosaceous species throughout the world (Bonn and van der Zwet 2000). This bacterium is especially damaging in apple and pear production leading to devastating losses in the United States annually (Norelli et al. 2003). *E. amylovora* causes blossom blight shortly after bloom, which in turn, may lead to infection of shoots and rootstocks, termed shoot blight and rootstock blight (Vanneste 2000). Blighted tissues may become blackened, giving a burnt appearance for which the disease is named, fire blight (Sundin 2014). Because blossoms may serve as an entry point for bacteria and lead to infection of additional tissues, managing blossom blight is crucial for fire blight control (van der Zwet 1969).

Several cultural, biological, and chemical controls are recommended for fire blight management. Cultural practices include pruning of blighted and cankered limbs and avoiding late season planting (van der Zwet et al. 2012). Biological controls may include applications of antagonistic or niche competing bacteria on blossoms at bloom (Johnson et al. 2009). Chemical controls include the use of copper, growth regulators, and antibiotic products such as oxytetracycline and streptomycin (van der Zwet et al. 2012). Of these controls, the most effective in the Northeastern United States is streptomycin, an aminoglycoside antibiotic, which has been used to control fire blight in the United States since 1955 (Cox et al. 2012; McManus et al. 2002; Russo et al. 2008; Sundin and Ehret 2009).

The reliance of the apple industry on streptomycin has become a growing concern in recent years due to the development of streptomycin resistant *E. amylovora* in the United States, although the mechanisms behind this resistance are well known (Moller et al. 1981). There are two known mechanisms of streptomycin resistance known to occur in *E. amylovora*. The first is the presence of a point mutation at codon 43 in the *rpsL* gene, which codes for the S12 ribosomal protein. This point mutation confers a conformational change in the S12 protein, altering the binding site of streptomycin on the ribosome, which would normally inhibit translation of mRNA to protein within the bacterium (Chiou and Jones 1995b; McManus et al. 2002). The presence of this point mutation is most commonly found in streptomycin resistant (SmR) *E. amylovora* isolates from the western United States (Moller et al. 1981).

The second mechanism of resistance is the presence of streptomycin modifying enzymes within the bacterium. These aminoglycoside-modifying enzymes are coded for by the gene pair *strA/strB* (Chiou and Jones 1995a). These genes are commonly found in many epiphytic bacteria, such as *Pseudomonas sp.*, and reside on conjugative R plasmids (Burr et al. 1988, Burr et al.

1993). In *E. amylovora, strA/strB* has been found on the plasmid RSF1010 and also within the transposable element Tn*5393* on conjugative plasmids pEA34 and pEU30 or on a nonconjugative plasmid pEA29 (Chiou and Jones 1993, Förster et al. 2015; McManus and Jones 1994; McGhee and Sundin 2011, Palmer et al. 1997). The *strA-strB* gene pair in Tn*5393* on plasmid pEA29, the basis of resistance for the isolates discovered in 2002 in New York is the most common determinant of resistance in Michigan (Russo et al. 2008; McManus and Jones 1994; McGhee and Sundin 2011). The presence *strA-strB* gene pair in Tn*5393* on plasmid pEA29 has only been found in SmR *E. amylovora* isolates from Michigan and New York (Russo et al. 2008; McManus and Jones 1994; McGhee and Sundin 2011).

The first reports of streptomycin resistance in the United States occurred in California in 1972 and shortly afterwards in Washington (Coyier and Covey 1975; Miller and Schroth 1972). Currently resistance is found in several western and Midwestern states such as Missouri and Michigan (McManus and Jones 1994). SmR *E. amylovora* was first reported in New York in 2002 with the discovery of the two isolates NY17.1 and NY17.2 that were recovered from fire blight outbreaks in two adjacent orchards in Wayne County (Russo et al. 2008). This finding prompted immediate eradication efforts, the removal of plantings, in order to prevent the spread of SmR *E. amylovora* to additional orchards. Subsequent fire blight sampling from the affected orchards and other orchards in Wayne County in 2004 and 2006 did not lead to the identification of SmR *E. amylovora* isolates, which lead to the belief that eradication efforts were successful in containing the outbreak (Russo et al. 2008).

Following renewed concerns from New York growers regarding the performance of streptomycin for the management of fire blight in 2011, yearly statewide sampling from fire blight epidemics occurred to identify and characterize new SmR *E. amylovora* isolates. Surveys

of New York apple orchards with fire blight outbreaks from 2011 to 2015 led to the identification of SmR *E. amylovora* isolates from orchards in six counties in western New York. The majority of these SmR *E. amylovora* isolates were found in orchards in Wayne County with the remainder recovered from orchards in Monroe, Niagara, Orleans, Ontario, and Thompkins counties. The basis of streptomycin resistance in the majority of isolates was due to the presence of the *strA/strB* gene pair in transposon Tn*5393* on plasmid EA29, while only two had a point mutation at codon 43 of the *rpsL* gene (Tancos et al. 2015). The fact that the majority of isolates were found in Wayne County orchards near the site of 2002 outbreak, and had an identical resistance genotypes to the 2002 isolates, have caused a reconsideration of the extent of the 2002 subsequent eradication efforts. Moreover, the discovery of SmR *E. amylovora* isolates with the *rpsL* point mutation, commonly found in the western United States (Chiou and Jones 1995b), but previously undocumented in New York, have raised concerns about additional introduction events in later years (Russo et al. 2008; Tancos et al. 2015).

To begin to investigate the origin of SmR *E. amylovora* it is necessary to explore the diversity of isolates collected in New York compared with isolates collected in other regions worldwide. However, examining diversity within this species has been challenging due to extremely limited diversity within the *E. amylovora* genome (Sebaihia et al. 2010; Smits et al. 2010). Studies investigating techniques such as random amplified polymorphic DNAs, pulse field gel electrophoresis, variable number of tandem repeats analyses, ribotyping and sequencing of housekeeping genes, such as *groEL*, have been used to explore diversity of *E. amylovora,* but have shown limitations in distinguishing strains (Jock et al. 2002; Kim and Geider 1999; McGhee and Sundin 2012; Momol et al. 1997). Another possibility for studying bacterial isolate diversity includes the use of clustered regularly interspaced short palindromic repeats

(CRISPRs). CRISPRs are DNA repeat regions found in about 48% of all bacteria, which are separated by spacers that share identity with laterally transferred DNA, such as that of bacteriophages, and are acquired in a temporal manner (Horvath et al. 2008). The polarity of spacer acquisition in a 3' to 5' manner produces a detailed account of foreign DNA elements the bacteria has come into contact with over time, and there by creating an inferred geographical record (Horvath et al. 2008; McGhee and Sundin 2012). The spacer content of CRISPR spacer arrays has recently been employed to explore strain diversity and to track human pathogenic bacteria such as *Escherichia coli*, *Yersinia pestis* and *Salmonella enterica* (Almendros et al. 2014; Barros et al. 2014; and Shariat et al. 2013). This method has provided a high degree of differentiation between isolates that was previously unattainable with many bacterial pathogens. CRISPRs have also been used successfully to study diversity of *E. amylovora* isolates (McGhee and Sundin 2012; Rezzonico et al. 2011). McGhee and Sundin (2012) examined the diversity of 85 *E. amylovora* isolates from fire blight affected regions worldwide by sequencing CRISPR spacer array regions, termed CR1, CR2, and CR3, which resulted in the differentiation of isolates on a regional scale. With such resolution, it would be possible to understand the emergence of SmR *E. amylovora* isolates within a region, the migration of isolates with specific antibiotic resistance determinants, and possibly strain tracking (Förster et al. 2015; McGhee and Sundin 2012; Rezzonico et al. 2011).

Given that streptomycin resistance in *Erwinia amylovora* is just beginning to emerge in apple orchards within New York, there is a unique opportunity to examine the possible origin of SmR *E. amylovora* isolates throughout the state. In this capacity the objective of this study was to investigate *Erwinia amylovora* isolate diversity with CRISPR spacer array analysis as a means to establish a baseline from which to investigate the possible origin and sources of SmR *E.*

amylovora, as well as explore potential for strain tracking of *E. amylovora* in future fire blight epidemics.

METHODS AND MATERIALS

Bacterial isolates

Twenty-seven SmR *E. amylovora* and 76 streptomycin sensitive (SmS) *E. amylovora* isolates collected from 61 commercial orchards, 16 of which containing SmR isolates, were used in this study. All isolates, with the exception of 2 isolates that contained the *rpsL* point mutation, contained the *strA/strB* determinant for streptomycin resistance, as described in Tancos et al. 2015. Isolates with the gene pair *strA/strB* had these genes occurring on the transposon Tn*5393* with the insertion site bp 17,527 on plasmid pEA29. All isolates were stored as single colonies cultures in a 15% glycerol Luria-Bertani (LB) broth (Bertani 1952) broth at -80°C before use. During experimentation, isolates were grown on Cross-Goodman media (Cross and Goodman 1973) at 28°C, and only single colonies with a cratered appearance on CG, characteristic of *E. amylovora* were used for CRISPR spacer array analysis.

DNA amplification and sequencing of CRISPR spacer array regions

Single colonies were placed into 200 µl of sterile H_2O and vortexed for 1 minute. The resulting mixture was used as a template for colony PCR amplification. PCR reactions, 50 µl in volume, contained 10 µl of 5X GoTaq Buffer (Promega Corp., Madison, WI), 3 µl of 25mM $MgCl_2$ (Promega Corp.), 1 µl of 0.2 mM dNTP mix (Promega Corp.), 2 µl of 10 mM forward primer, 2 µl of 10mM reverse primer, 2.5 U (0.2 µl) GoTaq DNA polymerase (Promega Corp.),

and 3 µl of template DNA. Universal primers for CR1, CR2, and CR3 were used to amplify these regions (Table 2.1). PCR cycling parameters, provided by McGhee and Sundin (2012), were 94°C for 5 minutes for initial denaturation followed by 40 cycles of 94°C for 30 seconds, annealing at 58°C for CR1 and CR2 or 55°C for CR3, and extension at 72°C for 4 minutes for CR1 and CR2 or 45 seconds for CR3. A final extension was performed at 72°C for 7 minutes.

Separation and visualization of PCR products was completed via gel electrophoresis using 1% agarose gels in 1x TAE buffer (44.5 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 90 volts for 60 minutes. PCR products were purified using a DNA Clean & Concentrator kit (Zymo Research, Irvine, CA). Amplified and purified PCR products were sequenced at the Cornell Biotechnical Resource Center in Ithaca, NY using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems, Waltham, MA). Primer walking, approximately 4 to 6 sequencing reactions, of large PCR products obtained for spacer arrays CR1 and CR2 was completed when necessary using primers developed in this study (Table 2.1).

Sequence analysis and CRISPR spacer array patterns

Sequencing data were assembled into complete CR1, CR2, and CR3 spacer array regions for each isolate using CLC Main Workbench (CLC Bio, Qiagen, Venlo, Netherlands). Spacer patterns were constructed by annotating each spacer based on a comprehensive spacer key for *E. amylovora* provided by McGhee and Sundin (2012) (Figure 2.1). Newly described spacers from this study were added to the existing key and designated a unique number. CRISPR spacer array profiles were constructed from the individual spacer array patterns of CR1, CR2, and CR3 spacer array regions (Table 2.2). Spacers and spacer patterns were compared between all isolates used in this study and Genbank sequencing data from previously described isolates collected from

locations across the United States. These comparisons were used to identify similarities or differences between isolates. Sequences of CR1, CR2, and CR3 CRISPR spacer array regions were submitted to the NCBI database and given a reference accession number. Accession numbers are listed in Table 2.3.

CRISPR spacer array profiles created for each isolate were recorded for each year of survey and linked to global positioning system (GPS) data using Google Maps (Google Inc., Mountain View, CA) for spatiotemporal considerations. CRISPR profiles of isolates were compared with other isolates collected at the same location if samples were collected from the same location over multiple years. Such location specific comparisons were limited by the development of fire blight from year to year as apple producers work to avoid fire blight management failures. CRISPR spacer array profiles of SmR *E. amylovora* were compared to those of SmS *E. amylovora* isolates at corresponding collection locations and within the same county in order to explore the possibility of resistance development within New York.

To observe genotypic differences among New York isolates from differing locations and with differing streptomycin sensitivity, cluster analysis was performed on CRISPR spacer array profiles for *E. amylovora* isolates. CRISPR spacer content for CR1, CR2, and CR3 was concatenated and converted to a binary data set based on the presence or absence of individual spacers. A distance matrix was first created using DISTANCE procedure of SAS (version 9.4; SAS Institute Inc. Cary, NC) using the Jaccard coefficient, and subsequently, the CLUSTER procedure of SAS (version 9.4; SAS Institute Inc.) was performed in conjunction with the TREE procedure of SAS (version 9.4; SAS Institute Inc.) to visualize the cluster analysis (Kuhfield and Kuo 2010).

RESULTS

CRISPR spacer array patterns and profiles

Twenty-seven SmR, including the SmR *E. amylovora* isolates collected in 2002, and 76 SmS *E. amylovora* isolates collected within 61 individual orchard sites in New York from 2011 to 2014 were evaluated for CRISPR spacer array content. Patterns for CRISPR spacer arrays CR1, CR2, and CR3 are diagrammed in Figure 2.1. Among the sequenced isolates there were 14 CR1 patterns, nine CR2 patterns, and one CR3 pattern. Of the 14 CR1 patterns and nine CR2 patterns, ten and four of these patterns had not been previously described, respectively.

CRISPR spacer array profiles created by combining the patterns of the CR1, CR2, and CR3 spacer array regions are listed for each isolate in Table 1. There were 19 distinct profiles for New York SmR and SmS *E. amylovora* isolates. Of these 19 profiles, only five profiles (4:27:38, 4:21:38, 5:27:38, 40:27:38, and 44:34:38) were shared between SmR and SmS *E. amylovora* isolates. Two profiles (15:34:38 and 41:23:38) were exclusive to SmR *E. amylovora* isolates. Isolates with the CRISPR profile 41:23:38 included the two isolates discovered in 2002 (Russo et al. 2008). The remaining twelve profiles (2:22:38, 4:56:38, 4:57:38, 4:58:38, 47:27:38, 50:27:38, 42:27:38, 5:55:38, 51:27:38, 43:27:38, 53:27:38, 52:27:38) were exclusive to SmS *E. amylovora* isolates (Table 2.2). Six SmR *E. amylovora* and 50 SmS *E. amylovora,* many from the same corresponding isolation sites, had the profile 4:27:38 (Table 2.2). This profile is also found in Michigan SmR and SmS *E. amylovora* isolates. 4:27:38 was the most common CRISPR profile found in New York *E. amylovora* isolates from a wide range of locations.

Cluster analysis revealed that CRISPR profiles clustered into three major groups accounting for about 68% of variance among genotypes (Figure 2.2). Group 1 contained isolates

with the profiles 15:34:38 and 44:34:38 from 3 orchards, which were either identical or similar to CRISPR sequences of SmR *E. amylovora* isolates from the western United States, respectively (McGhee and Sundin 2012). Isolates with the profile 15:34:38 were all SmR, while isolates with the profile 44:34:38 were both SmR and SmS. SmS *E. amylovora* isolates from these 3 orchards had profiles 4:27:38, 41:23:38, 42:27:38, and 5:27:38 found in group 3 (Figure 2.2). Group 2 contained isolates from 7 orchards with the CRISPR profiles 2:22:38, 4:56:38, 4:21:38, and 4:57:38, while group 3 contained isolates from 54 orchards with the CRISPR profiles 4:27:38, 4:58:38, 47:27:38, 50:27:38, 42:27:38, 5:27:38, 5:55:38, 51:27:38, 41:23:38, 40:27:38, 43:27:38, 53:27:38 and 52:27:38 (Figure 2.2).

The geographical location of SmR *E. amylovora* isolates with certain CRISPR spacer profiles was tracked over several years. The two isolates NY17.1 and NY17.2 from the original introduction of SmR *E. amylovora* in 2002 (Russo et al. 2008) had the profile 41:23:38. This profile was observed again in isolates collected from nine individual orchards, in four counties in 2012. SmR *E. amylovora* isolates with profile 4:27:38 were first observed in 2011 in one orchard and then observed again in 2013 at three orchards in three different counties. SmR *E. amylovora* isolates with the CRISPR profiles 5:27:38, 15:34:38, 4:21:38, 40:27:38, 44:34:38 were only isolated from single orchards in one year. While isolates with CRISPR profiles 4:21:38, 4:27:38, and 40:27:38 were isolated from 15 orchards with fire blight outbreaks in later years, all isolates collected from these orchards were streptomycin sensitive (data not shown).

DISCUSSION

CRISPR spacer array analysis has allowed New York SmR and SmS *E. amylovora* isolates to be grouped into 19 distinct CRISPR profiles clustered into 3 groups. In some cases, orchards had isolates from different groups, which could potentially indicate new introductions. The majority of SmR isolates shared the CRISPR profile 41:23:38 with the original SmR isolates NY17.1 and NY17.2 discovered in 2002 (Russo et al. 2008). Isolates with this profile were found within 7 individual orchards surrounding the 2002 orchard site across the Lake Ontario region. Based on the prevalence of SmR *E. amylovora* isolates with the CRISPR profile 41:23:38 and the lack of SmS *E. amylovora* isolates with this CRISPR profile it is possible that the neighboring orchards from which SmR *E. amylovora* isolates were first observed in 2002 is the primary source of the SmR *E. amylovora* found in many western New York orchards. It is possible that eradication efforts at this site were not successful in preventing the spread of these resistant strains to other orchards. Alternatively, nearby orchards may have also acquired plants from the same infected source and either effectively managed fire blight with cultural controls and growth regulators or did not report any fire blight management failures from 2002 to 2011. The absence of SmS *E. amylovora* with the profile 41:23:38 suggests that these isolates did not acquire resistance within New York, but rather SmR *E. amylovora* were introduced into the state. However, the lack of SmS isolates collected with this CRISPR profile may be limited by the number of infected trees at the locations from which to sample, seasonal variability in the development of fire blight due to unfavorable environmental conditions, and grower efforts to aggressively eradicate fire blight and SmR *E. amylovora* in later years. In this capacity, isolates of *E. amylovora* could not be isolated in later years from many of the sites where SmR *E.*

amylovora was discovered in 2011 to 2013. While cultivar information from each isolate was noted, there were no relationships between SmR *E. amylovora* isolates with certain CRISPR profiles and specific apple cultivars (data not shown). This is not surprising given the rarity of SmR *E. amylovora* in New York orchards and the infrequency of fire blight outbreaks from season to season.

The discovery of 6 additional CRISPR profiles (15:34:38, 44:34:38, 4:21:38, 4:27:38, 5:27:38, 40:27:38) in SmR *E. amylovora* isolates provides evidence that events other than the 2002 occurrence may have led to the development of streptomycin resistance in New York. The presence of isolates in New York with CRISPR profile 15:34:38, which is identical to those of western US isolates, possibly suggests an introduction event. The profile 15:34:38 is associated with isolates from the western United States with the *rpsL* mutation (McGhee and Sundin 2012). Two isolates in this study from two different commercial orchards were found to have the CRISPR profile 15:34:38 and a point mutation at codon 43 of the *rpsL* gene. The presence of this CRISPR profile within two geographically isolated orchards in Wayne and Ontario counties, and the lack of SmS *E. amylovora* isolates with a similar CRISPR profile suggest multiple introductions of SmR *E. amylovora* from western sources. An earlier introduction with subsequent dispersal is less likely given that the two orchards are further apart (> 8.6 Km) than the limit for local environmental dispersal (van der Zwet et al. 2012). Moreover, the two growers with isolate have never shared material or had reason to do so (K.D. Cox, personal communication).

While recent introduction events may explain the presence of SmR *E. amylovora* in some orchards, some CRISPR profiling data also suggests that resistance may be developing within the state. Fifty of 76 SmS *E. amylovora* isolates from 40 different orchards in 7 counties (Monroe,

Niagara, Ontario, Orange, Orleans, Oswego, and Wayne) had the CRISPR profile 4:27:38 (Table 2.2). Interestingly, this profile was also found in 6 SmR *E. amylovora* isolates within 4 orchards in 3 counties (Monroe, Orleans, and Wayne). Isolates of SmS and SmR *E. amylovora* with this profile were also recovered from apple orchards from Michigan (McGhee and Sundin 2012). The fact that *E. amylovora* isolates with the same CRISPR profile and both SmS and SmR phenotypes are present in the same orchard suggests that streptomycin resistance may have developed within the local populations. However, the fact that *E. amylovora* isolates with the same CRISPR profile and both SmS and SmR phenotypes are present in multiple orchards in different states would also suggest that they were introduced together from Michigan into New York or vice versa. *E. amylovora* isolates with CRISPR profiles 4:21:38, also found in Michigan (McGhee and Sundin 2012), and 44:34:38 with both SmR and SmS phenotypes were also recovered from the same orchards serving as evidence to further support the theory of resistance development within New York. Interestingly, the CRISPR profile 44:34:38 is similar to the profile 15:34:38 (Figure 2.2) and therefore similar to western United States isolates (McGhee and Sundin, 2012). This is concerning because it may suggest western United States CRISPR profiles may not be confined to the west coast and that New York is possibly the origin of these isolates. This would not be entirely surprising given that *E. amylovora* was first discovered in the Hudson Valley region of New York in 1794 (Denning 1794). It is possible that these seemingly introductions events could actually be reintroduction events into the state given the rarity of SmS *E. amylovora* isolates with CRISPR profiles similar to those of isolates collected in the western United States.

The evidence from the present study suggesting multiple (re)introductions of SmR *E. amylovora* into New York may highlight the inadequacies of pathogen screening of plant

material imported into the state. Commercial orchards in New York rely heavily on trees and budwood from both local and out of state sources, and the majority of sources are located in regions with a history of SmR *E. amylovora.* Nurseries strive to maintain healthy plantings that are regularly scouted for diseased plants; however, it is known that *E. amylovora* may survive asymptomatically in young trees (Keil and van der Zwet 1972; McManus and Jones 1994; Smith 2002). Asymptomatic trees could serve as a reservoir for antibiotic resistant strains that produce disease in established orchards. Screening asymptomatic trees for *E. amylovora* (SmS or SmR) strains is not commonplace in nurseries, but may help mitigate the spread of these strains in later years.

The fear of local resistance development has largely influenced New York production guidelines outlining the use of streptomycin to manage fire blight (Agnello et al. 2015), and may have contributed to relatively few putative cases of local selection as presented in the current study. It is recommended that streptomycin should only be applied during bloom and after significant trauma events that put trees at high risk for infection in order to reduce selection pressure for resistant strains. Alternative controls, such as the biostatic antibiotic oxytetracycline, growth regulator prohexadione-calcium, and copper products, although they do not provide control comparable to streptomycin, are recommended in concert with streptomycin to manage fire blight and to mitigate resistance development within individual orchards (Cox et al. 2012; Sundin and Ehret 2009; Yoder et al. 2009). Kasugamycin is a highly effective antibiotic for fire blight management with no known resistance in *E. amylovora* in Michigan, where streptomycin resistance is a concern (McGhee and Sundin 2011; Sundin and Ehret 2011). However, this antibiotic has just become available for use in New York and is relatively expensive in comparison to streptomycin, making kasugamycin a less cost effective option for growers.

The streptomycin resistance development in populations of *E. amylovora* has become an increasingly important issue with New York apple growers over the past decade. Isolates of SmR *E. amylovora* have been found in orchards in all of the major apple-growing counties in western New York. CRISPR spacer array analysis was used to better understand isolate diversity and to infer possible isolate origins. Our results suggest introduction events or reintroductions may be primary source of SmR *E. amylovora* in New York. However, in cases where SmS and SmR isolates share identical CRISPR profiles, local selection for streptomycin resistance many have occurred. Were we able to apply this technique from 2002 to 2011 on local populations regardless of fire blight development, we would have a better understanding of the origin. In this capacity we are prepared to understand the origin of SmR *E. amylovora* in eastern New York, as there has been considerable surveying of the eastern New York apple orchards where streptomycin resistance is absent (Tancos et al. 2015). Continued CRISPR spacer array profiling of *E. amylovora* in commercial apple orchards will further elucidate factors that contribute to streptomycin resistance development within individual orchards and help mitigate the spread of resistance in surrounding regions.

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TABLES

Table 2.1: Primers used to amplify CRISPR spacer arrays CR1, CR2, and CR3

Spacer Array	Primer	Sequence (5'-3')	Source[a]
CR1	CR1-F1	CGC CGC CAC GCT GCC ATT T	McGhee and Sundin, 2012
	C1-R0	TCC AGC GCC TGT AAA GCG GC	McGhee and Sundin, 2012
	CR1RevRpt	CGG TTT ATC CCC GCT CAC GC	McGhee and Sundin, 2012
	s6F	CAA GCG ATC AAC CTG TTT TTC A	
	s13R	TCA GGT TTA TTA CGG GCG G	
	s14R	TAA CTA GCA GAC GAT CTT	
	s15F	TAA AGG AGC ATG CTT ATA CAA C	
	s24F	CCC TCG GGG AGG GCT TTG CGT T	
	s24R	TCT GAG TAA CAA CGC AAA GCC C	
	s25R	CCA ACG ATA CAT TCA ACG TAA C	
	s27R	AGC TCA CTG CGG ATT TTC GCG G	
	s68F	TAA ATG GTT GTC CGT TCT TGG C	
	s95R	CCG CCC ACA AAG AGG TCA CCA C	
	s332F	CAA CCA GTT TCG TTA GTT GTT T	
	s335F	CTG TAT CCA CTT CAC CCA CGC	
	s338F	ATG TCT TGT ATC CCG GCT CTG G	
	s342F	AGG GCT GTG GTT TAT CGT GAT G	
	s351R	GTT ATT AAC GCG GAA TCA GTC	
	s364F	GTG GGA TAC CCC TTT TAT GC	
	s368F	CCG GGC GAA TGC GGG GAT	
	s593R	GAA CAG GTA TCA GCG ATG A	
	s610R	GGT TTG CTA TGC GGC GAT TTT G	
	s616R	TTG ATT GCC ACA TCT GCG ATG G	
	s623R	CCT GAT GGT CGC CTT TGG TC	
CR2	Cr2-F1	GCG GCC AAC AGA TGC GGA AAG	McGhee and Sundin, 2012
	C2R-1	TGC GGG GAA CAC TCG ACA TCT AAT	McGhee and Sundin, 2012
	s37F	GAG ATG CAC TGG ATA TAC CGA C	
	s51R	ACC AGC GCC ACC ATC TGA CCG T	
	s58R	GCA ACA ATC GTT GCG TCA CCC T	
	s64F	CCG CAT CGG TCA GTA CTG CGC T	
	s68R	AGC CGT CTG CGC CAA GAA CGG	
	s105F	GCG GCG AAG AGA CCG GAG CAT G	
	s107F	CGG GCA TTA GCG GCT TTG AAA C	
	s152R	AAG GCG GAA AGT TGT CCT CTG G	
	s255F	TTG AGC ACG GTA ACC CTC GCC A	

CR3	CR3-F1	TTT TCG CCG GGT AAC AGG	McGhee and Sundin, 2012
	CR3-R1	ATG AGA AGC CCG TGA AGC AAA GTA	McGhee and Sundin, 2012

a. All primers were developed for this study unless noted from other sources.

Table 2.2: CRISPR profiles associated with streptomycin sensitive (SmS) and streptomycin resistant (SmR) *E. amylovora* isolates

			CRISPR Profile				
Orchard	**County**	**Town**	**CR1**	**CR2**	**CR3**	**# SmS Isolates**	**# SmR Isolates**
C1	Clinton	Peru	47	27	38	1	0
M1	Monroe	Hamlin	4	27	38	1	0
M2	Monroe	Brockport	4	27	38	2	0
			41	23	38	0	1
M3	Monroe	Hilton	4	21	38	1	0
			4	27	38	1	1
N1	Niagara	Appleton	4	27	38	1	0
			41	23	38	0	1
			53	27	38	1	0
N2	Niagara	Wilson	4	27	38	1	0
N3	Niagara	Gasport	4	27	38	1	0
N4	Niagara	Burt	4	57	38	1	0
N5	Niagara	Appleton	4	27	38	1	0
N6	Niagara	Ransonville	4	58	38	1	0
O1	Ontario	Geneva	53	27	38	1	0
O2	Ontario	Phelps	4	27	38	1	0
			41	23	38	0	2
O3	Ontario	Geneva	4	27	38	1	0
			15	34	38	0	1
			41	23	38	0	1
Ora1	Orange	Warwick	4	27	38	2	0
Orl1	Orleans	Kendall	4	27	38	1	0
			41	23	38	0	2
Orl2	Orleans	Medina	4	27	38	1	1
Orl3	Orleans	Knowlesville	4	27	38	1	0
Orl4	Orleans	Albion	4	27	38	1	0
Orl5	Orleans	Kendall	4	27	38	1	0
Orl6	Orleans	Albion	4	27	38	2	0

Orl7	Orleans	Albion	4	27	38	1	0
Orl8	Orleans	Knowlesville	4	27	38	1	0
Orl9	Orleans	Waterport	4	27	38	1	0
Orl10	Orleans	Knowlesville	5	55	38	1	0
Orl11	Orleans	Albion	4	27	38	1	0
			40	27	38	1	0
Orl12	Orleans	Albion	4	27	38	1	0
Orl13	Orleans	Waterport	4	27	38	1	0
Orl14	Orleans	Lyndonville	52	27	38	1	0
Os1	Oswego	Oswego	4	27	38	1	0
S1	Suffolk	Mattituck	2	22	38	1	0
S2	Suffolk	Wading River	4	21	38	1	0
S3	Suffolk	Calverton	4	21	38	1	0
T1	Tompkins	Lansing	44	34	38	1	2
U1	Ulster	Marlboro	2	22	38	2	0
W0	Wayne	Sodus	41	23	38	0	2
W1	Wayne	Huron	4	27	38	2	2
			41	23	38	0	1
W2	Wayne	Marion	4	27	38	1	0
W3	Wayne	Williamson	4	27	38	2	0
			41	23	38	0	3
W4	Wayne	Wolcott	4	27	38	1	0
			5	27	38	0	1
			15	34	38	0	1
			41	23	38	0	1
			42	27	38	1	0
W5	Wayne	Macedon	4	27	38	3	0
			41	23	38	0	1
W6	Wayne	Williamson	40	27	38	0	1
			43	27	38	1	0
W7	Wayne	Wolcott	4	27	38	2	1
W8	Wayne	Sodus	4	21	38	1	1
W9	Wayne	Williamson	50	27	38	1	0
W10	Wayne	Williamson	40	27	38	1	0
W11	Wayne	North Rose	51	27	38	2	0
W12	Wayne	Williamson	5	27	38	1	0
W13	Wayne	Sodus	4	27	38	2	0
W14	Wayne	Williamson	4	27	38	1	0
			40	27	38	1	0
W15	Wayne	North Rose	4	27	38	1	0
W16	Wayne	Williamson	4	27	38	1	0
W17	Wayne	Williamson	4	27	38	1	0

W18	Wayne	Sodus	4	27	38	1	0
W19	Wayne	Williamson	4	27	38	1	0
W20	Wayne	Wolcott	4	27	38	1	0
W21	Wayne	Alton	4	27	38	1	0
W22	Wayne	Wolcott	4	27	38	1	0
W23	Wayne	Sodus	4	27	38	1	0
W24	Wayne	Sodus	4	27	38	1	0
W25	Wayne	Williamson	40	27	38	1	0
Y1	Yates	Penn Yan	4	56	38	1	0

Table 2.3: Genbank accession numbers for sequences of CRISPR spacer regions CR1, CR2, and CR3 for isolates used in this study

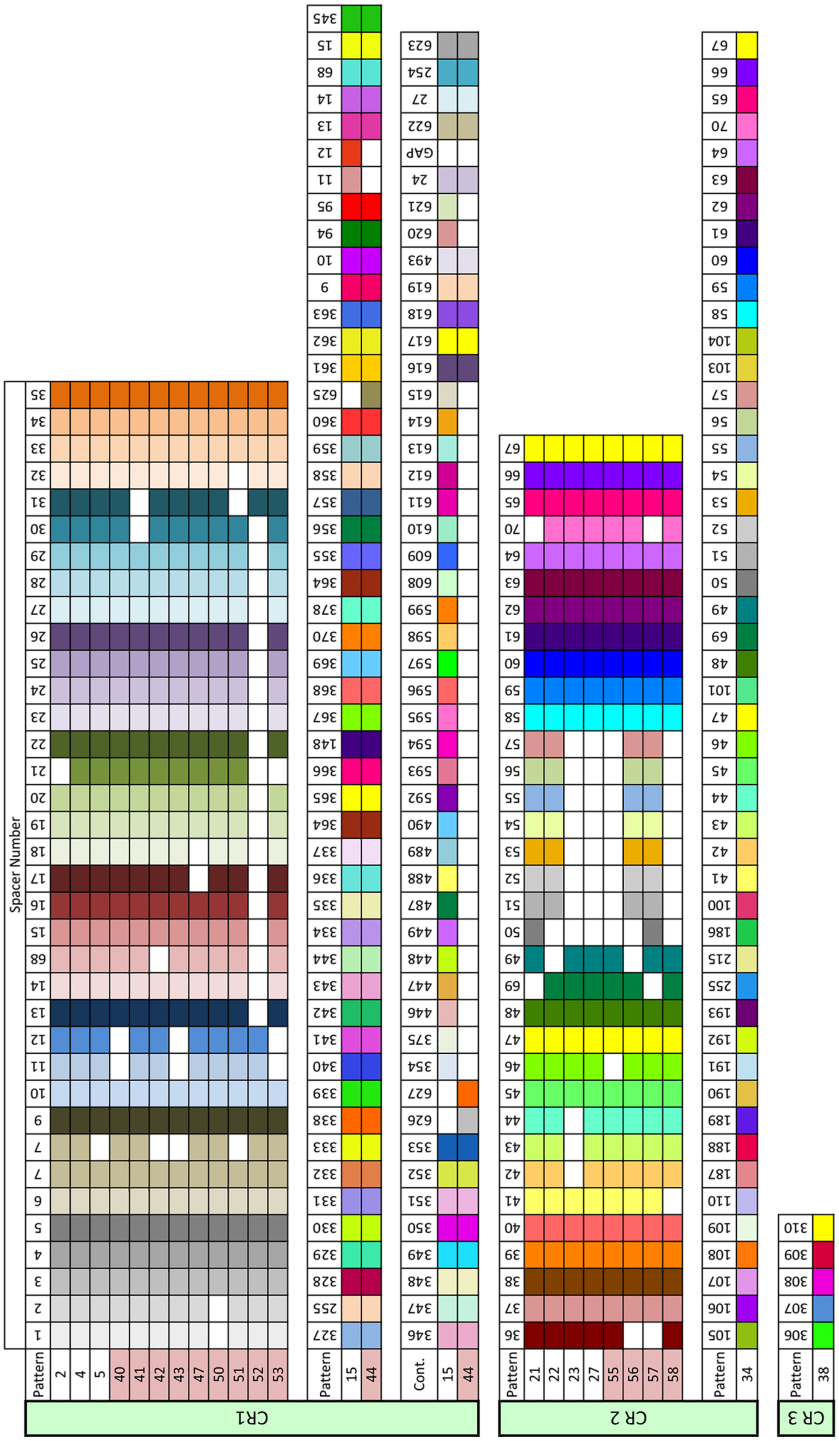
	Accession Number[a]		
Isolate	**CR1**	**CR2**	**CR3**
163.6	KR361332	KR361427	KR401000
173	KR361330	KR361425	KR400998
174	KR361331	KR361426	KR400999
182.2	KR361338	KR361433	KR401006
189	KR361388	KR361480	KR401056
189.1	KR361342	KR361437	KR401010
190	KR361413	KR361487	KR401081
214	KR361337	KR361432	KR401005
220	KR361389	KR361481	KR401057
222	KR361393	KR361482	KR401061
229	KR361345	KR361440	KR401013
230	KR361402	KR361517	KR401070
240	KR361340	KR361435	KR401008
245	KR361343	KR361438	KR401011
249	KR361405	KR361520	KR401073
251	KR361339	KR361434	KR401007
254	KR361407	KR361522	KR401075
265	KR361334	KR361429	KR401002
269	KR361419	KR361490	KR401087
272	KR361420	KR361491	KR401088
277	KR361347	KR361442	KR401015
278	KR361406	KR361521	KR401074
286	KR361335	KR361430	KR401003
291	KR361350	KR361445	KR401018
292	KR361412	KR361527	KR401080
301	KR361401	KR361516	KR401069

307	KR361346	KR361441	KR401014
310	KR361336	KR361431	KR401004
313	KR361403	KR361518	KR401071
316	KR361400	KR361515	KR401068
317	KR361344	KR361439	KR401012
321	KR361352	KR361447	KR401020
330	KR361353	KR361448	KR401021
332	KR361375	KR361470	KR401043
333	KR361354	KR361449	KR401022
345	KR361325	KR361499	KR400993
347	KR361326	KR361500	KR400994
362	KR361356	KR361451	KR401024
390	KR361369	KR361464	KR401037
407	KR361385	KR361504	KR401053
411	KR361390	KR361507	KR401058
414	KR361361	KR361456	KR401029
415	KR361355	KR361450	KR401023
431	KR361370	KR361465	KR401038
436	KR361415	KR361510	KR401083
437	KR361417	KR361512	KR401084
439	KR361416	KR361511	KR401085
445	KR361367	KR361462	KR401035
450	KR361366	KR361461	KR401034
464	KR361374	KR361469	KR401042
465	KR361351	KR361446	KR401019
487	KR361358	KR361453	KR401026
499	KR361360	KR361455	KR401028
508	KR361394	KR361483	KR401062
509	KR361414	KR361488	KR401082
517	KR361421	KR361492	KR401089
525	KR361368	KR361463	KR401036
526	KR361372	KR361467	KR401040
530	KR361362	KR361457	KR401030
535	KR361357	KR361452	KR401025
545	KR361364	KR361459	KR401032
559	KR361359	KR361454	KR401027
564	KR361363	KR361458	KR401031
570	KR361386	KR361505	KR401054
741	KR361396	KR361485	KR401064
189a	KR361392	KR361509	KR401060
189b	KR361411	KR361526	KR401079
192A	KR361333	KR361428	KR401001
206A	KR361341	KR361436	KR401009

225A	KR361418	KR361489	KR401086
299-1b	KR361348	KR361443	KR401016
299-2d	KR361408	KR361523	KR401076
300-1a	KR361349	KR361444	KR401017
300-2d	KR361409	KR361524	KR401077
300-2e	KR361410	KR361525	KR401078
305-1	KR361322	KR361496	KR400990
306a	KR361404	KR361519	KR401072
306b	KR361391	KR361508	KR401059
328-1	KR361365	KR361460	KR401033
360-3	KR361371	KR361466	KR401039
557-1	KR361373	KR361468	KR401041
587a	KR361376	KR361471	KR401044
588a	KR361382	KR361477	KR401050
595c	KR361323	KR361497	KR400991
596a	KR361324	KR361498	KR400992
602a	KR361380	KR361475	KR401048
604a	KR361381	KR361476	KR401049
605a	KR361378	KR361473	KR401046
615a	KR361329	KR361503	KR400997
624a	KR361328	KR361502	KR400996
629a	KR361395	KR361484	KR401063
632a	KR361327	KR361501	KR400995
634e	KR361384	KR361479	KR401052
640a	KR361423	KR361494	KR401091
643a	KR361397	KR361486	KR401065
644a	KR361424	KR361495	KR401092
650a	KR361387	KR361506	KR401055
661a	KR361383	KR361478	KR401051
677a	KR361379	KR361474	KR401047
688a	KR361422	KR361493	KR401090
703b	KR361377	KR361472	KR401045
NY17.1	KR361398	KR361513	KR401066
NY17.2	KR361399	KR361514	KR401067

a. Genbank reference accession numbers for CR1, CR2, and CR3 spacer arrays, respectively.

FIGURES


CR1
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Spacer Number
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CR 2
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CR 3
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Figure 2.1: Spacer content of CRISPR arrays CR1, CR2 and CR3 for all *Erwinia amylovora* isolates used in this study. Spacers are represented by boxes and given a number in the top row. Unique numbering is given to spacers that differ from other spacer sequences by at least 5 nucleotides. Spacers are arranged in patterns, which are given a pattern number in the left column. Pattern numbers that are shaded are newly discovered in this study, while white boxes represent patterns previously described by McGhee and Sundin (2012). GAP denotes an area of the spacer array sequence that did not contain direct repeats or spacers.

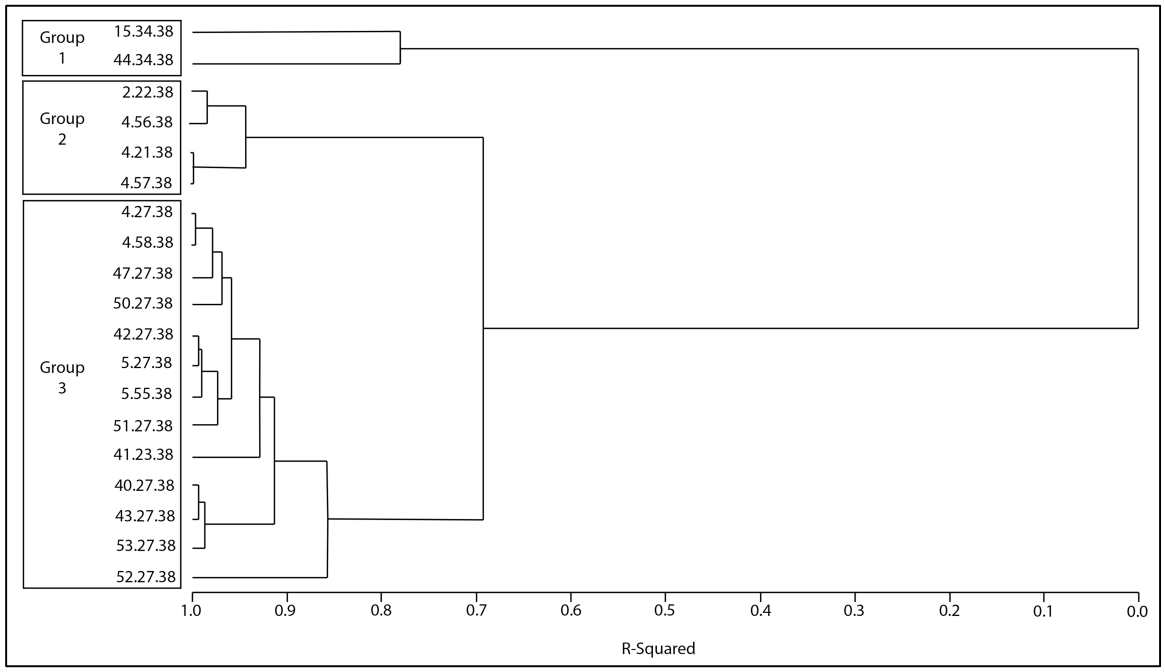

Figure 2.2: Distance matrix constructed based on cluster analysis of concatenated spacers for CRISPR arrays CR1, CR2, and CR3 for all isolates used in this study. Distances are represented as R^2 values on the x-axis. CRISPR profiles were placed into 3 groups based divergence accounting for 68% of variability among profiles.

CHAPTER 3

EFFECTS OF CONSECUTIVE STREPTOMYCIN AND KASUGAMYCIN APPLICATIONS ON EPIPHYTIC BACTERIA IN THE APPLE PHYLLOSPHERE[3]

ABSTRACT

Antibiotic applications are essential for fire blight management the eastern United States. Recently, streptomycin resistant *Erwinia amylovora* strains were found in New York. There are growing concerns that streptomycin resistance may develop from post bloom applications of streptomycin in local orchards. Our goal was to investigate the impacts of increasing applications of streptomycin and kasugamycin on bacterial epiphyte community composition and antibiotic resistance in the phyllosphere of 'Idared' apple plantings in 2014 and 2015. Rinsate samples from leaves treated with zero, three, five, and ten applications of streptomycin and kasugamycin were collected to isolate, enumerate, and identify epiphytic bacterial species. The majority of isolated epiphytic bacteria were identified as *Pantoea agglomerans* and fluorescent *Pseudomonas* spp., while *Erwinia amylovora* was rarely found. Overall, post-bloom streptomycin use did not result in an increased recovery of streptomycin resistant *E. amylovora*. However, the recovery frequencies of other streptomycin resistant epiphytes (*P. agglomerans* and *Pseudomonas* spp.) did increase with increasing application numbers of streptomycin.

[3] Tancos, K. A., and Cox, K. D. Effects of consecutive streptomycin and kasugamycin applications on epiphytic bacteria in the apple phyllosphere. Plant Disease. *Submitted.*

Increasing applications of kasugamycin reduced the overall number and percentage of streptomycin resistant epiphytes in the phyllosphere, which implies that kasugamycin may be particularly effective against SmR bacteria.

INTRODUCTION

Erwinia amylovora is a phytopathogenic bacterium that causes fire blight, a disease of rosaceous plant species that is especially devastating in apple and pear, which leads to substantial production losses in the United States annually (Vanneste 2000). During bloom *E. amylovora* infects blossoms causing blossom blight, which often leads to subsequent infection of shoots and rootstocks (Vanneste 2000). These blighted tissues typically become blackened and may exude bacterial ooze ranging from yellow to red in color (Sundin 2014). Economic losses are due to the loss of blossoms and subsequent fruit, fruit bearing shoots, and often whole trees. This disease can be especially severe in plantings of moderately to highly susceptible apple cultivars, which are in high commercial demand and are widely planted across the major apple growing regions of the United States (van der Zwet et al. 2012). In 2008, it was estimated that approximately 50% of new plantings in New York orchards were planted to susceptible varieties at densities of over 750 trees/ha (Breth 2008).

Fire blight management in the eastern United States relies heavily upon the use of antibiotics in susceptible apple orchards, particularly the aminoglycoside antibiotics streptomycin and kasugamycin, which are applied at bloom to protect blossoms from anticipated *Erwinia amylovora* infection (McGhee and Sundin 2011; Sundin 2014). Streptomycin has been used for fire blight management in the eastern United States for over 50 years (McManus et al.

2002), while use of kasugamycin is relatively new with approval for use in New York apple orchards beginning in 2015 (EPA registration number 66330-404) (McGhee and Sundin 2011). Currently, there are no other viable management alternatives that provide an acceptable level of blossom blight control in the temperate production conditions of the eastern United States (Sundin et al. 2009) making the use of antibiotics imperative for fire blight management. Because streptomycin has a long history of effective use in the northeast and is the most economically sound option, it remains the most widely used management tool (van der Zwet et al. 2012).

The emergence of streptomycin resistant (SmR) *E. amylovora* in the United States is of great concern due to widespread reliance on this antibiotic for fire blight control (Moller et al. 1981). SmR *E. amylovora* was first isolated in the western United States in California in 1972, and in Washington shortly afterward (Miller and Schroth 1972; Coyier and Covey 1975). Currently, SmR *E. amylovora* is found in several western and mid-western states, such Missouri and Michigan (McManus and Jones 1994). SmR *E. amylovora* was first isolated in New York in 2002 where SmR isolates were detected in two adjacent orchard sites. Orchard surveys, made from 2011 to 2014, led to the discovery of several strains of SmR *E. amylovora* from individual apple orchards in several western New York counties (Tancos et al. 2015).

In the northeastern United States, the most common resistance mechanism is the presence of the tandem gene pair *strA/strB*, while resistance caused by a point mutation in the *rpsL* gene is relatively rare (Chiou and Jones 1995a, 1995b; Tancos et al. 2015). The gene pair *strA/strB* codes for streptomycin modifying enzymes (Chiou and Jones 1995b) and, in *E. amylovora,* is found on the transposon Tn*5393* on the ubiquitous nonconjugative plasmid pEA29 (Chiou and Jones 1993; McGhee et al. 2011; McManus and Jones 1994). Several epiphytic bacteria found in

the apple phyllosphere, such as *Pseudomonas* species and *Pantoea agglomerans*, are known to carry the *strA/strB* gene pair on R plasmids (Burr 1988, 1993). It is theorized that epiphytes such as *Pantoea agglomerans*, which commonly carries *strA/strB* on the transposon Tn*5393* on the plasmid pEA34, may be responsible for the transfer of these streptomycin resistance genes to the closely related *E. amylovora* (Chiou and Jones 1991; McGhee et al. 2011). In order for such an event to occur, selective pressure to maintain populations of SmR *P. agglomerans* and the presence of epiphytic *E. amylovora* populations in an orchard is necessary.

The development of SmR *E. amylovora* has been correlated with streptomycin overuse after bloom to control the shoot blight phase of the disease, although evidence for this is mostly anecdotal. In this context, applications of streptomycin are often made to non-bearing trees in the nursery or high value plantings during early establishment to protect against late season shoot blight infections. The correlation between historical streptomycin use in apple and pear orchards and the recovery of SmR *E. amylovora* has been studied (Loper et al. 1991; Yashiro and McManus 2012). However, aside from these studies, the development of resistance following direct application of streptomycin remains largely unexplored, and it is unknown whether SmR *E. amylovora* could emerge following excessive post-bloom streptomycin applications. If this practice did result in the selection of SmR *E. amylovora* isolates, it would be due to mutation within local *E. amylovora* populations or due to the acquisition of horizontally transferred streptomycin resistance genes from other environmental bacteria. To bridge the knowledge gap concerning antibiotic application patterns and resistance development, the presence of streptomycin resistance in epiphytic bacterial populations following post-bloom applications of streptomycin and kasugamycin should be investigated. We hypothesize that increasing the number of streptomycin applications should lead to an increased recovery of streptomycin

resistant epiphytic bacteria and perhaps the recovery of SmR *E. amylovora*. By comparison, increased applications of kasugamycin should not preferentially select for streptomycin resistant epiphytic bacteria and perhaps lead to the recovery of kasugamycin resistant epiphytic bacteria.

METHODS AND MATERIALS

Antibiotic resistance selection experiments in apple orchards.

In 2014 and 2015, antibiotic resistance selection experiments were performed on two plantings of 'Idared' apples on B.9 rootstocks in Geneva NY, which were 15 (orchard 1) and 7 (orchard 2) years old, respectively. A completely randomized design was used for all experiments with four replicate plots per antibiotic treatment schedule, each plot consisting of a single data tree surrounded by single buffer trees within and across rows to minimize interplot interference. Resistance selection experiments focusing on streptomycin applications were conducted in both orchards in both years, while those focusing on kasugamycin applications took place only in orchard 1 and in both orchards in 2015. Antibiotic application schedules consisted of weekly applications of streptomycin or kasugamycin for either zero, 3, 5, or 10 weeks. These applications were made using the commercial products Agri-Mycin 17 (Nufarm, Morrisville, NC) and Kasumin 2L (Arysta LifeScience, Cary, NC) at labeled rates of 24 oz/A (1680 g/Ha) and 64 fl oz/A (73.1 ml/Ha), respectively. Applications were made at using a Solo 451 gas-powered mist blower calibrated to deliver approximately 935 L/Ha, which is a standard volume for high density apple plantings in the northeastern United States. In both years, antibiotic applications began in orchard 1 at 80% bloom (late May); in orchard 2 they began post-bloom

during terminal elongation (early August), representing late season applications to manage shoot blight in non-bearing plantings.

Collection, enumeration, and morphological identification of common bacterial epiphytes.

Upon completion of antibiotic application schedules, 50 healthy and fully expanded leaves were collected randomly from each replicate plot. Leaves were grouped into batches of five for a total of 10 batches per replicate plot. Each of the five-leaf batches were placed in 10 ml of 20% glycerol 1X PBS buffer in a sealed Ziploc Bag (SC Johnson, Racine, WI) and sonicated in a Bransonic ultrasonic cleaner (Fisher Scientific, Westminster, MD) for 5 minutes. Following sonication, rinsate samples were stored at -20°C before analysis. Rinsate samples were plated on Crosse Goodman medium (CG) (Crosse and Goodman 1973) and incubated at 28°C for 48 hr at appropriate dilutions for visual CFU enumeration. The resulting colony forming units were enumerated to calculate total epiphytic bacteria, able to grow on CG, collected per treatment. *Pantoea agglomerans* and *Pseudomonas* spp. were commonly recovered from apple leaves in previous studies, therefore we chose to begin by identifying these bacteria within rinsate samples (McGhee and Sundin 2011; Yashiro and McManus 2012). A subsample of approximately 100 colonies per replicate plot (tree) were transferred to CG and Kings B media (KB) (King et al. 1954) and grown at 28°C for 24 hrs. KB plates were subjected to UV light exposure to observe fluorescent *Pseudomonas* species. Putative fluorescent *Pseudomonas* spp. colonies on KB medium and putative *Erwinia amylovora* colonies that displayed characteristic cratering morphology on CG were collected and stored for further identification. Of the remaining colonies not identified as fluorescent Pseudomonads or *Erwinia amylovora*, a subsample of 50

colonies from each replicate plot were stored for further PCR identification to estimate the percentage of *Pantoea agglomerans* colonies present in rinsate.

PCR identification of putative *Pseudomonas* spp., *Erwinia amylovora*, and *Pantoea agglomerans* colonies.

The identity of putative fluorescent *Pseudomonas* species colonies was confirmed by PCR amplification using previously described 16S rRNA *Pseudomonas* specific primers (Widmer et al. 1998). The identity of putative *Erwinia amylovora* colonies was confirmed by PCR amplification using previously described primers (McManus and Jones 1994) specific to the ubiquitous nonconjugative plasmid pEA29, found only in this bacterium (Bereswill et al. 1992; Chiou and Jones 1993; McGhee and Jones 2000). The identity of putative *Pantoea agglomerans* colonies were identified by PCR amplification with previously described primers targeting the pagR2 gene, which is an autoinducer gene specific to the *P. agglomerans* species (Braun-Kiewnick, 2012) (Table 3.1). PCR reactions were performed in 25 µl reaction volumes and consisted of 12.3 µl H_2O, 5 µl 5X Green GoTaq Flexi Buffer (Promega Corp. Madison, WI), 1 µl of forward primer, 1 µl of reverse primer, 0.5 µl 10mM dNTP mix (Promega Corp.), 2.5 µl 25mM $MgCl_2$ (Promega Corp.), 0.2 µl GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial DNA sample. PCR cycling parameters were as follows: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Annealing temperatures for the *Pseudomonas* species 16S rRNA primers, pEA29 primers, and pagR2 primers were 62°C, 54°C, and 56°C, respectively. The resulting PCR products were separated using gel electrophoresis on a 1% agarose gel in 0.5X TAE buffer (44.5 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 90 volts

for 60 minutes. To further confirm identity for each bacterial group, a subset of amplified DNA was purified using a Zymo DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) and sequenced at the Cornell Biotechnical Resource center in Ithaca, New York using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems, Waltham, MA).

Screening of bacterial epiphytes for streptomycin and kasugamycin resistance.

The colonies selected from rinsate samples for epiphyte enumeration were spot transferred to CGS (CG media amended with 100 ppm streptomycin) and CGK (CG amended with 100 ppm kasugamycin) and incubated at 28°C for 48 hrs. Colonies that grew on CGS were considered streptomycin resistant (SmR), while colonies that showed no growth were considered streptomycin sensitive (SmS). Similarly, colonies that did and did not grow on CGK were considered kasugamycin resistant and sensitive, respectively. Representative subsets of putative SmS and SmR bacterial colonies were collected and stored for further confirmation of the presence or absence of the *strA/strB* gene pair, using PCR amplification with primers developed by Russo et al. (2008) (Table 3.1). PCR amplification and gel electrophoresis followed the previously stated protocol, with exception of annealing temperatures at 56°C for *strA* primers and 53°C for *strB* primers. A representative subsample of five amplified *strA* and *strB* PCR products was purified and sequenced as previously described to confirm the identity of visualized bands.

Data analysis.

For each antibiotic resistance selection experiment, the percentage of total CFUs was determined for three major bacterial epiphyte groups: *Pseudomonas* species as a whole, *Pantoea*

agglomerans, *Erwinia amylovora,* and other miscellaneous epiphyte species. Similarly, the percentage of total antibiotic sensitive and resistant CFUs was also determined. From these numbers, the mean percentage of total CFUs was determined for each antibiotic application schedule (i.e. zero, three, five, and 10 applications). The effect of antibiotic application schedule on mean percent of total CFUs for the three major epiphyte groups was determined using Generalized Linear Mixed Models with the GLIMMIX procedure of SAS v9.4 (SAS Institute, Cary, NC), with differences in means among treatments determined using the 'lsmeans' statement of GLIMMIX at the 5% level of significance (SAS Institute, Cary, NC). The effect of antibiotic application schedules on the mean percent SmR CFUs was determined and analyzed using the same procedures.

RESULTS

Enumeration and identification of bacterial epiphytes.

Pantoea agglomerans and fluorescent *Pseudomonas* spp. (e.g. *P. putida, P. fluorescens,* and *P. syringae)* were the most frequently collected epiphytic bacteria isolated on CG in both orchards in both years of this study. *Erwinia amylovora* was rarely collected, regardless of application treatment, and was found in less than 0.1% of rinsate samples. A variety of miscellaneous epiphytic bacteria were also collected throughout this study, including several *Pantoea* spp. (e.g. *P. ananatis*), *Erwinia* spp. (e.g. *E. rhapontici*), and nonfluorescent *Pseudomonas* spp., however, no single bacterial species comprised the majority of this category. Miscellaneous epiphytic bacteria comprised no more than 20% of colonies recovered from rinsate for any of the antibiotic application schedules. Also, in terms of total epiphytic bacteria

recovered, the number of applications had no bearing on the CFU/L for each of three types of epiphytic bacteria (data not shown).

Applications of streptomycin significantly ($P < 0.0001$) altered the frequency of *Pantoea agglomerans* within the epiphytic bacterial community of the apple phyllosphere. The percentage of *P. agglomerans* bacteria isolated from streptomycin-treated trees decreased as application numbers increased from zero to 10 in both orchards in 2014 and in orchard 1 in 2015 (Figure 3.1). The percentage of *P. agglomerans* was only significantly ($P < 0.05$) lower on trees receiving 10 applications than trees receiving zero applications, with the exception of orchard 2 in 2015, where less than 20% of the total CFUs were comprised of *P. agglomerans*. Trees receiving 10 applications of streptomycin had significantly lower ($P < 0.0001$) percentages of *P. agglomerans* than trees receiving three or five applications, except for orchard 1 in 2014 where trees receiving 10 applications were statistically equivalent to those receiving five applications. For orchard 1 in 2014, *P. agglomerans* declined from 85.64 to 53.42% of the total epiphytic bacteria isolated from trees as application numbers increased from zero to 10. Orchard 2 in 2014 was similar to orchard 1, with *P. agglomerans* declining from 85.2 to 51.0% of the total isolated epiphytic bacteria as application numbers increased from zero to 10. In 2015, the trends were similar for the two orchards, although the differences in the percentage of *P. agglomerans* recovered seemed to be more strongly affected by application number. For orchard 1 in 2015, *P. agglomerans* declined from 75.2 to 17.4% of the total epiphytic bacteria isolated from trees receiving zero versus 10 applications, respectively. For orchard 2 in 2015, the percentage of *P. agglomerans* was higher after three and five applications, but was the lowest after 10 applications. In this orchard *P. agglomerans* comprised 7.4, 46.1, 34.8 and 13.5% of the total epiphytic bacteria isolated from trees with zero, three, five, and 10 applications, respectively.

The percentage of fluorescent *Pseudomonas* spp. isolated generally increased as trees received increasing numbers of streptomycin applications in both orchards in 2014 and 2015 (Figure 3.1). However, this trend of increased frequency of recovery of *Pseudomonas* spp. following increased application numbers of streptomycin was only significantly different ($P < 0.05$) between trees receiving zero and three applications, and those receiving five or 10 applications (Figure 3.1). In orchard 1 in 2014 fluorescent *Pseudomonas* spp. comprised 13.12 to 34.67% of the total epiphytic bacteria isolated from trees as application numbers increased from zero to 10. A similar trend was observed in orchard 2 in 2014 as fluorescent *Pseudomonas* spp. comprised 2.65 to 38.8% of the total epiphytic bacteria isolated from trees as applications numbers increased from zero to ten. Fluorescent *Pseudomonas* spp. comprised 9.75 to 58.25% and 11.0 to 67.5% of the total epiphytic bacteria isolated from trees receiving zero to ten applications, in orchards 1 and 2, respectively.

Applications of kasugamycin also significantly ($P < 0.05$) altered the frequency of *Pantoea agglomerans* within the epiphytic bacterial community of the apple phyllosphere (Figure 3.2). Overall, the percentage of *P. agglomerans* bacteria isolated from trees treated with kasugamycin was greater as application numbers increased from zero to 10 in orchard 1 in 2014 and both orchards in 2015. While there were significant differences between the four application regimes depending on the year and orchard, the percentage of *P. agglomerans* bacteria isolated was only consistently significantly ($P < 0.0001$) different between trees receiving 10 applications and trees receiving zero applications. In 2014 *P. agglomerans* comprised 62.1 to 100% of the total epiphytic bacteria isolated from trees as applications schedules increased from zero to 10 in orchard 1. In 2015 *P. agglomerans* comprised 48.0 to 90.8% and 37.2 to 75.0% of the total

epiphytic bacteria isolated from trees as applications numbers increased from zero to 10 in orchards 1 and 2, respectively.

In contrast with *P. agglomerans* in these orchards and their response to streptomycin applications presented above, the percentage of fluorescent *Pseudomonas* spp. isolated from trees treated with kasugamycin decreased with increasing application numbers in orchard 1 in both years (Figure 3.2). In 2014, fluorescent *Pseudomonas* spp. fell from 24.7% of the total epiphytic bacteria isolated from trees receiving zero applications to undetectable levels after five or 10 applications (Figure 3.2). In 2015, fluorescent *Pseudomonas* spp. similarly fell from 17.8 to 1.0% of the total epiphytic bacteria isolated from trees receiving zero versus ten applications, respectively (Figure 3.2). This pattern of decreasing recovery of *Pseudomonas* spp. following increasing numbers of applications was not observed following the application schedule in orchard 2 in 2015, where there were no significant differences among treatments.

Across both years and orchards, there were no trends observed between application schedule and the percentage of miscellaneous epiphytic bacteria, regardless of the antibiotic used, as many of the miscellaneous epiphytes were only recovered in single year or experiment. Even when placed together in the 'miscellaneous' category, there were still no significant trends between application schedule and epiphyte recovery for either antibiotic or orchard (Figure 3.1, Figure 3.2).

Streptomycin and kasugamycin resistance screening of bacterial epiphytes.

No kasugamycin resistant bacteria were recovered in any orchard in any year of this study, as all isolated epiphytic bacteria failed to grow on CG medium amended with 100 ppm kasugamycin. Streptomycin resistant (SmR) epiphytic bacteria were present in high frequencies

in both orchards and years prior to the application of antibiotics, and continued to increase in frequency as streptomycin applications continued until they comprised all to nearly all of the population (Table 3.2). In contrast, the percentage of SmR epiphytic bacteria decreased as trees received increasingly higher numbers of kasugamycin applications. For example, before applications began, SmR bacteria comprised 85% of the total epiphytic bacteria isolated from trees in orchard 1 in 2014 and orchard 2 in 2015, but these frequencies fell to 20 and 17%, respectively, after 10 kasugamycin applications. This trend was similar, but less pronounced in orchard 1 in 2015, with SmR epiphytic bacteria comprising 96% of the total epiphytic bacteria isolated from trees before spraying and 72% after 10 kasugamycin applications. Across all orchards, trees with 10 applications had significantly lower percentages SmR epiphytic bacteria than trees with zero applications (P values < 0.0001) (Table 3.2). While there were significant differences between the four application schedules depending on the year and orchard (data not shown), the percentage of SmR epiphytic bacteria isolated was only consistently significantly (P < 0.0001) lower for trees receiving 10 applications compared to trees receiving zero applications.

DISCUSSION

The frequent recovery of *Pantoea agglomerans* and fluorescent *Pseudomonas* spp. from all orchards was not surprising, as these bacteria are commonly recovered from apple blossoms and leaves in similar studies of apple phyllosphere bacteria communities (McGhee and Sundin 2011; Yashiro and McManus 2012). Despite the fact that there was active blossom and shoot blight in the orchard (data not shown), it was somewhat surprising to have recovered such a low abundance of *Erwinia amylovora* (presence in < 0.1% of rinsate samples). However, the relative

rarity of *Erwinia amylovora* may be not considered an anomaly given that *E. amylovora* has previously been described as an unsuccessful epiphyte (Bonn 1981; Manceau et al. 1990; Ockey and Thomson 2006). Because no trends were observed regarding the 'miscellaneous' bacteria category, effects of antibiotics on these bacteria were considered minor within orchards used in this study.

The alteration in the epiphytic bacteria community structure, which occurred in response to increasing numbers of streptomycin and kasugamycin applications, was not anticipated. We hypothesized that antibiotic applications would affect the proportion of resistant bacterial epiphytes alone, but the changes in relative abundance of the different species was surprising. The decrease in the relative abundance of *P. agglomerans,* regardless of streptomycin resistance, after increasing exposure to the antibiotic has not been reported. Interestingly, *P. agglomerans*, syn. *Erwinia herbicola*, has been found to be closely related to and often associated with *Erwinia amylovora* in several studies (Kwon et al. 1997; Riggle and Klos 1972). Although *P. agglomerans* is well known for its competitive inhibition of *E. amylovora* growth (Vanneste et al. 1992), the decrease in the relative abundance of *P. agglomerans* after five or more streptomycin applications did not lead to an increased recovery of epiphytic *E. amylovora*. Nevertheless, it is unknown whether the decrease in the overall *P. agglomerans* population would affect the establishment of *E. amylovora* populations in the following growing season.

Conversely, increasing numbers of kasugamycin applications resulted in an increase in the relative abundance of *P. agglomerans*. Although the total CFU/L of epiphytic bacteria declined with increasing applications of kasugamycin (data not shown), an increased frequency of *P. agglomerans* in the phyllosphere in response to kasugamycin applications may be disconcerting. *P. agglomerans* is theorized to be the source from which *E. amylovora* acquired

the streptomycin resistance genes *strA* and *strB* (Chiou and Jones 1993, McGhee and Sundin 2011). If *P. agglomerans* is the predominant species in the apple phyllosphere following kasugamycin application, these bacteria could serve as concentrated source of horizontally transferred streptomycin resistance genes, or even kasugamycin resistance genes, should they develop in the future. However, it is important to note that although *P. agglomerans* is the prominent epiphyte, the population was reduced by nearly 1000-fold after ten applications of kasugamycin (data not shown).

Changes in the relative abundance of fluorescent *Pseudomonas* spp. after kasugamycin applications were also evident. In some cases, fluorescent *Pseudomonas* spp. were no longer detectable after multiple applications within a single season. This led us to infer that perhaps differential sensitivity to kasugamycin exists among bacterial species within the epiphytic community. Preliminary results have shown that *Pseudomonas syringae* pv. *papulans* isolates were not able to grow on CG with kasugamycin concentrations exceeding 50 ppm, while *P. agglomerans* isolates were able to grow on 100 ppm (Tancos and Cox, unpublished). Kasugamycin is not currently registered for management of tree fruit diseases caused by *Pseudomonas* spp., such as blister spot (causal agent *Pseudomonas syringae* pv. *papulans*), but kasugamycin products could prove useful for this purpose and have provided effective disease control in trials on bacterial speck of tomato (*Pseudomonas syringae* pv. *tomato*), bacterial leaf spot of parsley (*Pseudomonas syringae* pv. *coriandricola*), and bacterial blight of lilac (*Pseudomonas syringae* pv. *syringae*) (Vallad et al. 2014, Miller et al. 2013, Pscheidt and Bassinette 2011). Certainly, future field efficacy trials on blister spot of apple would be necessary to validate such speculation.

Regardless of the species, nearly all of the epiphytic bacteria recovered before antibiotic treatments were deemed streptomycin resistant, even though streptomycin had been used only on a limited basis in these orchards over the past 10 years. Streptomycin sensitivity was considered rare across all treatments with less than 5-20% of epiphytic bacteria recovered being streptomycin sensitive. In both orchards receiving streptomycin applications over both years, streptomycin resistant epiphyte recovery was significantly lower ($P < 0.05$) for the trees with no applications than trees that received three, five, or 10 applications (Table 3.2). Although we believe that applications of kasugamycin should not select for streptomycin resistant phenotypes, increasing numbers of kasugamycin applications nevertheless resulted in a lower percentage of streptomycin resistant bacterial epiphytes recovered. This may imply that there is a fitness cost associated with streptomycin resistance, however this observation could simply be an artifact resulting from the decrease in total epiphytic bacteria following schedules of increased applications of kasugamycin. Further studies are necessary to elucidate the effects of kasugamycin on the stability of streptomycin resistance in epiphytic bacteria.

While the recovery of streptomycin resistant bacteria was commonplace, no kasugamycin-resistant bacterial colonies were recovered from any orchard, even following 10 applications of the antibiotic. Schedules with excessive applications (five and 10 applications) of kasugamycin did not influence the selection or recovery of kasugamycin resistant epiphytes. This observation leads to the supposition that kasugamycin resistant bacteria are not currently present in the phyllosphere in these orchards. Nevertheless, additional sampling from additional sources, such as orchard soil, would be necessary to eliminate the possibility of kasugamycin resistant bacteria in these orchards. Kasugamycin resistant bacteria have been previously isolated in Michigan orchards from soil samples (McGhee and Sundin 2011), however it is not known

whether kasugamycin resistant bacteria in orchard soils or others niches would have an effect on bacterial epiphytes in the phyllosphere.

The observed changes in community structure observed following antibiotic application schedules were generally consistent in both orchards in 2014 and 2015, regardless of whether applications were initiated at 80% bloom or during shoot elongation. This suggests that the observed changes in the community structure were not simply due to seasonal host development or environmental factors such as temperature or rainfall. The only exception to this would be for percentage of fluorescent *Pseudomonas* spp. following increased numbers of kasugamycin applications in orchard 2 in 2015. Since this schedule for kasugamycin application (during shoot elongation) was not done in 2014, we cannot rule out the potential influence of seasonal factors on the relative abundance of fluorescent *Pseudomonas* spp. for the 2014 season, or in general.

In summary, our results demonstrated that even if active fire blight is established in plantings, *E. amylovora* may not be highly abundant in the epiphytic community of the apple phyllosphere. Also, even 10 applications of streptomycin after bloom did not result in recovery of streptomycin resistant *E. amylovora*. However, other common bacterial epiphytes, such as *Pseudomonas* spp., which reside on apple foliage and harbor streptomycin resistance genes, became more abundant in trees after receiving increasingly higher numbers of streptomycin applications. Such observations support the recommendation that streptomycin use after bloom should be minimal and reserved for trauma events, even on nursery and non-bearing trees. Lastly, applications of kasugamycin are highly effective at reducing overall epiphytic bacterial populations, especially in regards to *Pseudomonas* spp., and may even be effective at reducing the overall abundance of bacterial epiphytes with streptomycin resistance in the phyllosphere.

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TABLES

Table 3.1: Primers used to confirm epiphyte identity and presence of streptomycin resistance genes

Target[a]	Primer[b]	Sequence (5'- 3')	Source
Pseudomonas spp. 16S rRNA	Ps-F	GGTCTGAGAGGATGATCAGT	Widmer et al. 1998
	Ps-R	TTACCTCCACCTCGCGGG	
P. agglomerans pagR2	PagRrt2-F	ACGGTGCGTTCCGCAATA	Braun-Kiewnick et al. 2012
	PagRrt2-R	GGCGCCGGGAAAACATAC	
E. amylovora pEA29	AJ75	CGCA TTCACGGCTTCGCAGAT	McManus and Jones. 1994
	AJ76	AACCGCCAGGATAGTCGCATA	
strA	strA406-F	TGACTGGTTGCCTGTCAGAG	Russo et al. 2008
	strA406-R	CGGTAAGAAGTCGGGATTGA	
strB	strB403-F	ATCGCTTTGCAGCTTTGTTT	Russo et al. 2008
	strB403-R	CGTTGCTCCTCTTCTCCATC	

a. Gene or sequence target of the primer set

b. Name of the primer used as given in the original source literature

Table 3.2: Percentage of epiphytes resistant to streptomycin after antibiotic applications

			Application schedule and percentage of total SmR epiphytes			
Antibiotic[a]	**Year**	**Orchard**	**Zero[b]**	**Three**	**Five**	**Ten**
streptomycin	2014	1	98.84 ± 0.86[c]	98.08 ± 1.08	100 ± 0	100 ± 0
" "	2014	2	79.5 ± 6.46	95.5 ± 0.64	93.25 ± 3.47	97.0 ± 1.22
" "	2015	1	83.25 ± 6.66	88.25 ± 2.95	93.5 ± 2.5	97.5 ± 1.44
" "	2015	2	43.25 ± 22.65	96.25 ± 0.65	95.25 ± 1.44	99.25 ± 0.48
kasugamycin	2014	1	85.25 ± 1.31	79.5 ± 5.44	76.0 ± 3.24	20.0 ± 12.24
" "	2015	1	96.25 ± 1.05	90.75 ± 1.49	76.75 ± 2.41	71.5 ± 2.98
" "	2015	2	85.75 ± 6.60	76.0 ± 10.21	73.75 ± 8.46	17.0 ± 1.47

a. Antibiotic applied with streptomycin applications made using Agri-Mycin 17 and with kasugamycin applications made using Kasumin 2L

b. Number of antibiotic applications in the treatment schedule applied to apple trees

c. Mean percentage of total sampled bacterial epiphytes able to grow on CG amended with 100 ppm streptomycin. Values are the mean and standard errors of 10 batches of five leaves with four replicates.

FIGURES

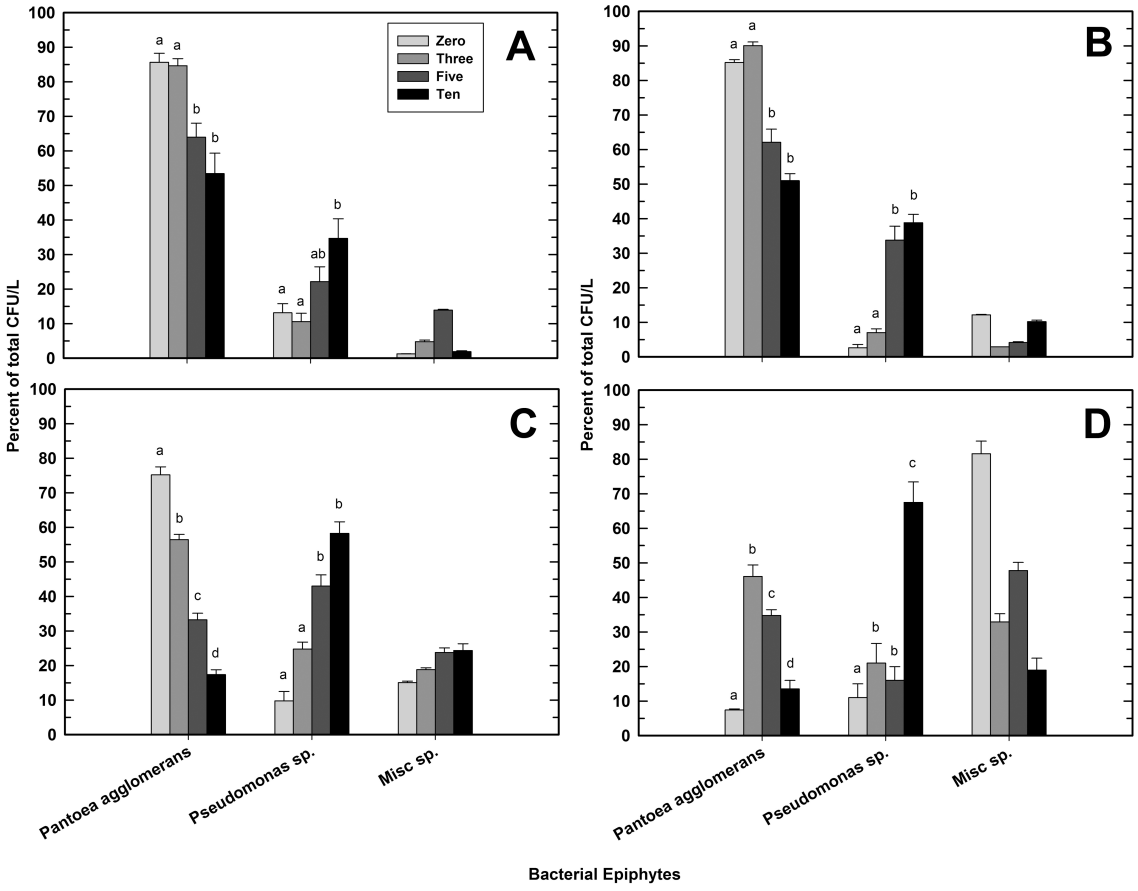

Figure 3.1: Percent of total CFU/L for *Pantoea agglomerans*, *Pseudomonas* spp., and 'miscellaneous' epiphytes for trees receiving zero, three, five, and ten applications of streptomycin (Agri-Mycin 17). Values are the mean and standard errors of 10 batches of five leaves with four replicates for: orchard 1 in 2014 (A.), orchard 2 in 2014 (B.), orchard 1 in 2015 (C.), and orchard 2 in 2015 (D.). Bars denoted by the same letter are not significantly different as denoted by the 'lsmeans' statement of the GLIMMIX procedure in SAS v9.4 at the 5% level of significance.

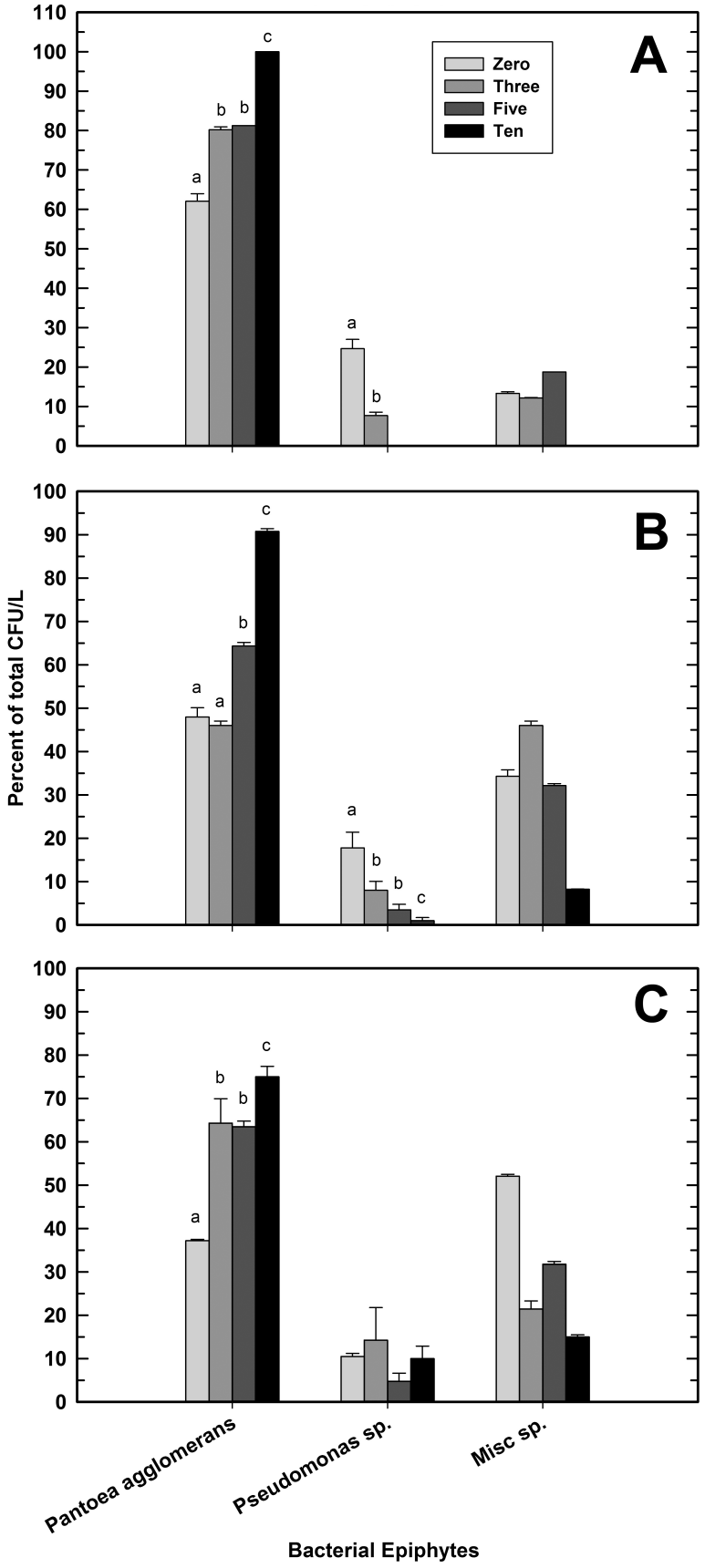
A
B
C
Zero
Three
Five
Ten
Percent of total CFU/L
Pantoea agglomerans
Pseudomonas sp.
Misc sp.
Bacterial Epiphytes

Figure 3.2: Percent of total CFU/L for *Pantoea agglomerans*, *Pseudomonas* spp., and miscellaneous epiphytes for trees receiving zero, three, five, and ten applications of kasugamycin (Kasumin 2L). Values are the mean and standard errors of 10 batches of five leaves with four replicates for: orchard 1 in 2014 (A.), orchard 1 in 2015 (B.), and orchard 2 in 2015 (C.). Bars denoted by the same letter are not significantly different as denoted by the 'lsmeans' statement of the GLIMMIX procedure in SAS v9.4 at the 5% level of significance.

CHAPTER 4

FIRE BLIGHT SYMPTOMATIC SHOOTS AND THE PRESENCE OF *ERWINIA AMYLOVORA* IN ASYMPTOMATIC APPLE BUDWOOD[4]

ABSTRACT

Erwinia amylovora, the causal agent of fire blight, causes considerable economic losses in young apple plantings in NY on a yearly basis. There are concerns that the bacterial pathogen is being spread through the use of infected budwood during clonal propagation of trees. The use of clean budwood for propagation is essential, but may be confounded by the presence of *E. amylovora* in nursery source trees that appear to have no fire blight symptoms at the time of collection. The use of infected budwood, especially by commercial nursery operations, could in part be the cause of fire blight outbreaks that often occur in young apple plantings in NY. Our goal was to investigate the presence of *E. amylovora* in asymptomatic budwood from nursery source plantings as it relates to the presence of fire blight. From 2012 to 2015, apple budwood was collected from two commercial budwood source plantings of 'Gala' and 'Topaz' at increasing distances from visually symptomatic trees. From these collections, internal contents of apple buds were analyzed for the presence of *E. amylovora*. *E. amylovora* was detected in asymptomatic budwood in trees more than 20 m from a center point tree with fire blight

[4] Tancos, K. A., Borejsza-Wysocka, E., Breth, D., and Cox, K. D. Fire blight symptomatic shoots and the presence of *Erwinia amylovora* in asymptomatic apple budwood. Plant Disease. *Submitted.*

symptoms. In some seasons, there were significant ($P < 0.05$) differences in the incidence of *E. amylovora* in asymptomatic budwood collected from the center point tree and those at greater distances. In 2014 and 2015, the mean *E. amylovora* CFUs/L recovered from budwood in both the 'Gala' and 'Topaz' plantings were significantly lower in budwood collected 20 m from the center point tree. Further investigation of individual bud dissections revealed that *E. amylovora* was present even in the meristem tissue of buds. Results from the study highlight the short comings of current budwood collection practices and the need to better understand the presence of *E. amylovora* in bud tissues to ensure the production of pathogen free apple trees.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora,* is responsible for devastating losses in plantings of apple (*Malus* × *domestica* Borkh.) worldwide (Vanneste 2000). In recent years, New York apple growers have experienced by up to 50% tree losses in newly planted orchards to the sudden and unexpected emergence of fire blight following bloom (Breth 2008). Such losses are expected to increase as older orchards are converted to high-density plantings consisting of highly-susceptible apple varieties, which are planted to meet consumer demand (Breth 2008). Fire blight production losses occur in the form of direct reductions in harvestable fruit from blossom blight and fruit bearing wood from shoot blight. Moreover, *E. amylovora* may spread systemically from blighted blossoms and blighted shoots to the central leader and rootstock of the tree, causing rootstock blight and eventually tree death (van der Zwet et al. 2012). Hence, management of blossom blight is essential for fire blight management because the potential for spread of infection to additional tissues may lead to complete trees loss. Presently,

the aminoglycoside antibiotics streptomycin and kasugamycin are the most effective and widely used means protecting apple blossoms from infection *E. amylovora* (McManus et al. 2002; McGhee and Sundin 2011; Sundin 2014).

As infections progress into shoot tissues, it becomes difficult and labor intensive to control the spread of *E. amylovora* within the tree. Antibiotics, such as streptomycin, have very limited systemic activity making them ineffective when applied for shoot and rootstock blight. However, streptomycin is effective at reducing shoot blight when applied after trauma events that create bacterial entry points in green tissues (van der Zwet et al. 2012). The use of the growth regulator prohexadione-calcium has shown to be fairly effective in management of shoot blight by thickening the cell walls of cortical parenchyma to the point at which it may impede the movement of the pathogen (McGrath et al. 2009). The thickening of cell walls further slows young vigorous shoot growth and therefore reduces the amount of susceptible tissue available for infection (McGrath et al. 2009; Yoder et al. 1999). Once infection has become established, the only remaining control method is to prune out blighted shoot tissues. Removal of blighted shoots by cutting back into asymptomatic tissues of the previous year's shoot may help reduce disease and prevent spread to other trees (Agnello et al. 2016).

Removal of blighted shoots, also referred to as strikes, typically occurs during dormant pruning, or throughout the growing season as time permits. Pruning fire blight throughout the growing season is a common practice employed in nursery operations to ensure the production of disease free planting material. It is recommended that fire blight strikes on nursery trees be removed as they appear throughout the period of terminal growth to minimize the spread of infection (Agnello et al. 2016). Although the removal of symptomatic tissues may reduce fire blight inoculum, it does not ensure the complete eradication of the pathogen from the tree. *E.*

amylovora may persist as an endophyte (Crepel et al. 1996; Crepel and Maes, 2000; Ge and van der Zwet, 1995, 1996; Keil and van der Zwet, 1972a; Momol et al. 1994, 1998; van der Zwet, 1996) and not produce characteristic fire blight symptoms. The occurrence of asymptomatic fire blight infection has been documented in several studies (Keil and van der Zwet 1972; McManus and Jones 1994; Smith 2002; van der Zwet 1983, 1996). Most recently in Washington State, Smith (2002) observed that nursery trees propagated with asymptomatic budwood led to the loss of nearly 50,000 trees over three years in an individual nursery. Such losses associated with asymptomatic budwood are especially disconcerting because new trees could remain symptomless for several years and be shipped to commercial orchards without knowledge of latent infections. Once planted in a commercial orchard, trees with such latent infections could develop symptoms during favorable weather and vigorous growth, and may lead to infection of nearby established trees and continued losses (Smith 2002; van der Zwet 1983, 1996).

Selection of pathogen free budwood is essential to prevent the infection of newly propagated trees and the subsequent spread of *Erwinia amylovora* to commercial orchards. The practice of selecting budwood varies across nursery operations, but typically involves the collection bud sticks from current season's terminal growth in early fall. Nursery source collection ideally takes place in plantings that appear healthy and have no active fire blight symptoms (Agnello et al. 2016; Breth 2008). However, commercial demand for buds from certain cultivars with low availability may lead to relaxed selection practices. Additionally, the removal of shoot strikes throughout the season, a common management practice for fire blight, complicates collection of pathogen-free budwood due to the absence of characteristic fire blight symptoms, which may otherwise have been easily avoided. Hence, it is essential to keep a detailed record of fire blight occurrence in orchards used for nursery source materials.

The relationship of fire blight strikes and collection of infected budwood from the same tree has been studied (Smith 2002; van der zwet 1996), however, the presence and quantity of *E. amylovora* in the buds of asymptomatic trees is still largely unknown. It is unknown whether surrounding symptomless trees are truly a reliable source of *Erwinia amylovora* free budwood. In order to better understand sudden and unexpected outbreaks of fire blight in young plantings in NY, we endeavored to investigate the relationship between the proximity of symptomatic and asymptomatic trees and the recovery of *E. amylovora* from budwood. The effect of apple cultivar on the presence of *E. amylovora* in budwood is also unknown and in this regard, we examined asymptomatic buds of 'Topaz' and 'Gala' apples, both of which are susceptible to fire blight (Korba et al. 2008; Rosenberger 2003).

We hypothesize that asymptomatic trees located closer to symptomatic trees will have a greater incidence of *E. amylovora* in buds than those farther away them. Furthermore, we hypothesize that different cultivars will differ in the incidence of *E. amylovora* in buds as it relates to proximity of fire blight symptoms. Regardless, of whether these hypotheses are refuted or supported, the outcomes of this study should better help to explain the development of fire blight in young apple plantings in NY and further highlight the deficiencies in budwood collection practices that should be improved to ensure the propagation of pathogen-free trees.

METHODS AND MATERIALS

Collections of asymptomatic budwood.

Over the course of four seasons from 2012 to 2015 budwood sticks were collected from two commercial apple orchards commonly used for nursery source material in Ontario and

Wayne counties in western New York. The orchards were both planted to mature trees (> 10 years). The orchard in Ontario County was planted to Topaz apples on B.9 rootstocks, while the orchard in Wayne County was planted to 'Gala' apples on M.9 rootstocks. Budwood collections took place following terminal bud set in late August to early September each year with specific timing depending on the seasonal and local development of terminal buds. Prior to collection, orchards were observed for fire blight symptoms to determine spatial distribution of fire blight for the purpose of establishing sampling blocks with individual symptomatic trees. In each planting for each season, an individual tree with fire blight symptoms was designated as a center point for budwood stick collection. Trees in a radius of 50 m from the center point tree were intensely scouted for fire blight symptoms to ensure the center point tree was the only visible fire blight source within the sampling block. Ten budwood sticks from the current season's growth, containing a minimum of 5 buds each, were randomly collected from individual trees in three replicate sampling blocks under three fire blight proximity treatments: 1) asymptomatic tissue on the center point tree, less than 1 meter away from fire blight symptoms (referred to as < 1 m), 2) branches on an adjacent asymptomatic tree that was greater than 1 meter from the center point tree (referred to as > 1 m), and 3) an asymptomatic tree 20 meters from the symptomatic center point tree (referred to as > 20 m) (Figure 4.1). Following collection, samples were packaged individually in plastic bags, stored on ice, and immediately transported to the New York State Agricultural Experiment Station for processing.

Isolation and identification of *E. amylovora* from budwood.

Budwood sticks were stored at 4°C in individual plastic bags for up to 48 hr prior to isolation of *E. amylovora.* Five buds per budwood stick sample were removed and surface

sterilized in a 15% Clorox solution for 15 minutes. Buds were rinsed three times with sterile water and transferred into a single 2.0 ml microcentrifuge tube. A sterile steel bead and 1 ml of 1X PBS buffer were added to the tube and the tube was placed in a Tissuelyser (Qiagen Inc., Valencia, CA) for 2 minutes to grind samples. The macerated bud solution was then plated on Crosse Goodman media (CG) (Crosse and Goodman 1973) at appropriate dilutions for visual differentiation of single colonies and incubated at 28°C for 48 hr. The presence of *E. amylovora* colonies that displayed characteristic cratering morphology on CG was noted for each five-bud composite samples and used to calculate the incidence of infected budwood sticks for each replicate tree in the three proximity treatments. In 2014 and 2015, *E. amylovora* colony forming units (CFUs) were enumerated for collections made from both the 'Topaz' and 'Gala' orchards. Following morphological characterization, a subset of colonies with representation across all treatments, cultivars, and seasons was set aside and stored at -80°C for subsequent molecular identification.

Localization of *E. amylovora* within individual buds.

To determine whether *E. amylovora* was present in actual meristematic tissues or just in deep crevasses of bud scales, dissections were made from a subset of representative buds from all proximity treatments and both cultivars in 2012. A total of 18 intact buds were removed from budwood sticks and surface sterilized in a 15% Clorox solution for 15 minutes, and subsequently rinsed 3 times with sterile water. Using sets of individual sterile dissection needles, individual bud scales were carefully removed with one needle per bud scale to separate the meristem tissue from the other tissues of the buds (Figure 4.2). The meristem was then surface sterilized using the same aforementioned procedure. As described in the preceding section, the meristem tissue

was then subjected to grinding, and the macerated meristem solution was subsequently plated on CG medium at appropriate dilutions for visual differentiation of single colonies. After 48 h of incubation at 28°C, colonies were observed for *E. amylovora* characteristic cratering morphology. The colonies where then stored at -80°C for subsequent molecular identification.

PCR and sequencing confirmation of *Erwinia amylovora.*

The identity of putative *E. amylovora* colonies with cratering morphology on CG was confirmed by PCR and sequencing of a portion of the ubiquitous nonconjugative plasmid pEA29, which has been shown to be reliable marker to confirm the identity of *E. amylovora* (Bereswill et al. 1992; Chiou and Jones 1993; McGhee and Jones 2000). Specifically, individual colonies were placed in 500 µl of H_2O and PCR amplified with previously described primers AJ75 (5'-CGTATTCACGGCTTCGCAGAT-3') and AJ76 (5'-ACCCGCCAGGATAGTCGCATA-3') (McManus and Jones 1995). PCR reactions, 25 µl in volume, consisted of 12.3 µl H_20, 5 µl 5X Green GoTaq Flexi Buffer (Promega Corp., Madison, WI), 1 µl each of forward and reverse primer, 0.5 µl 10 mM dNTP mix (Promega Corp.), 2.5 µl 25mM MgCl2 (Promega Corp.), 0.2 µl GoTaq G2 Flexi DNA Polymerase (Promega Corp.), and 2.5 µl of bacterial suspension sample. Cycling parameters consisted of 5 minutes at 94°C followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. PCR products were separated using gel electrophoresis with 1% agarose gels in 1x TBE buffer (44.5 mM Tris-borate, 1 mM EDTA, pH 8.0) at 90 volts for 60 minutes. PCR products were purified for subsequent sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research, Irvine, CA). Purified products were sequenced at the Cornell Biotechnical Resource center in

Ithaca, New York using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems, Waltham, MA).

Analysis of budwood isolation data.

For each of the four seasons (2012 to 2015) and both 'Gala' and 'Topaz' orchards, the incidence of budwood sticks with buds infected by *E. amylovora* was determined for each of the three fire blight proximity treatments (i.e. <1 m, >1 m, and >20 m) in each of the replicate sampling blocks. Incidence was scored using a binary scale with "1" denoting internal presence and "0" indicating the absence of *E. amylovora*. The effect of proximity of fire blight symptoms on the frequency of buds with *E. amylovora* was determined using the FREQ procedure of SAS v9.4 (SAS Institute, Cary, NC) with Pearson's chi-square statistics. Individual planned comparisons of infection frequencies between fire blight proximity treatments were conducted by Wilcoxon Two-Sample tests using the NPAR1WAY procedure of SAS v9.4. In 2014 and 2015, total *E. amylovora* CFUs/L were enumerated from bud isolations and the mean CFUs/L were found across replicate sampling blocks for each the three treatment conditions. The effect of proximity of fire blight symptoms on mean *E. amylovora* CFUs/L obtained from buds was determined using Generalized Linear Mixed Models with the GLIMMIX procedure of SAS v9.4 (SAS Institute, Cary, NC). Differences in the mean percent of total CFUs for each epiphyte group between antibiotic application schedules were determined using the 'lsmeans' statement of GLIMMIX at the 5% level of significance (SAS Institute, Cary, NC).

RESULTS

Incidence of *Erwinia amylovora* in asymptomatic budwood.

Erwinia amylovora was isolated from buds on budwood sticks collected from commercial orchards in all four years from 2012 to 2015. Putative *E. amylovora* colonies all displayed the characteristic cratered appearance on CG, and representative colonies selected for PCR using primers AJ75 and AJ76 all produced the expected 840-bp band indicative of pEA29. Sequencing of the 840-bp PCR products and subsequent BLAST searches using the Genbank NCBI database (http://www.ncbi.nlm.nih.gov) revealed that these products were over 99% homologous to known sequences for *Erwinia amylovora* pEA29 (e.g. accession no. FN434114).

In the 'Topaz' orchard, the mean incidence of *E. amylovora* in buds was often lower on trees that were further from the center point trees in all years of this study (Figure 3). The clear exception to this trend was the 2013 collection where the incidence of bud infection on center point tree and trees > 20 m away were nearly identical. In 2012 the mean incidence of *E. amylovora* in buds on the center point trees (< 1 m) was 40% compared to 13% for trees more than 20 m from the center point trees. However, those differences were not statistically significant due to a high level of variability among replicates. In 2014 and 2015, there were significant differences in the incidence of bud infection among all three fire blight proximity treatments.

In the 'Gala' orchard, the trend of a lower mean incidence of *E. amylovora* in buds on trees further from the symptomatic center point tree was less consistent over the four years of the study. In 2012 and 2015, there were significant ($P < 0.05$) differences between the incidence of *E. amylovora* in buds on the center point trees (2012, 80%; 2015, 77%) compared with trees

more than 20 m away (2012, 33%; 2015, 0%) (Figure 4.3). In 2013 and 2014, a lower mean incidence was observed between the center point tree (2013, 93%; 2014, 100%) and trees more than 20 m away (2013, 60%; 2014, 97%), but these differences were not significant.

Quantification of *Erwinia amylovora* colony forming units in buds.

After observing differences in the incidence of *E. amylovora* in buds among the three fire blight proximity treatments in 2012 and 2013, *E. amylovora* colony forming units (CFUs) were enumerated for collections made from both the 'Topaz' and 'Gala' orchards in 2014 and 2015. Similar to the trend with the incidence of bud infection, buds on the center point trees had higher numbers of *E. amylovora* CFUs than those from trees further away. In the Topaz orchard, *E. amylovora* CFUs in buds ranged from 3.67e+3 to 1.42e+5 CFUs/L across all proximity treatments over both years. In 2014, there were significant differences ($P < 0.05$) in *E. amylovora* CFUs in buds between the center point trees (1.42e+5 CFUs/L, < 1 m) and trees more than 20 m away (3.53e+4 CFUs/L, > 20 m) (Figure 4.4). In 2015, there were significant differences ($P < 0.05$) in *E. amylovora* CFUs/L between all fire blight proximity treatments (Figure 4.4). In the 'Gala' orchard, *E. amylovora* CFUs in buds in 2014 ranged from 4.10e+5 to 9.73e+5 CFUs/L across all proximity treatments. In 2015, *E. amylovora* CFUs recovered from buds were lower than those recovered in 2015, which ranged from 0.0 to 1.21e+5 CFUs/L. In 2014, there were significant differences ($P < 0.05$) in *E. amylovora* CFUs between all fire blight proximity treatments, but in 2015, there were only significant differences ($P = 0.00$) in *E. amylovora* CFUs in buds between center point or adjacent trees (1.42e+5, < 1 m) and trees more than 20m away (3.53e+4, > 20 m) (Figure 4.4).

Localization of *E. amylovora* within individual buds.

Following dissection, removal of the meristematic tissues, and surface sterilization, putative colonies of *E. amylovora* with a cratered appearance on CG were obtained from all 18 bud meristems. PCR of the colonies using primers AJ75 and AJ76 resulted in the expected 840-bp band indicative of pEA29. Sequencing of the 840-bp PCR products and subsequent BLAST searches using the Genbank NCBI database (http://www.ncbi.nlm.nih.gov) revealed that these products were over 99% homologous to known sequences for *Erwinia amylovora* pEA29 (e.g. accession no. FN434114).

DISCUSSION

Buds from asymptomatic shoots on 'Gala' and 'Topaz' apples trees from two commercial nursery source plantings were found to harbor the fire blight pathogen *E. amylovora*. Over the four growing seasons, the incidence of *E. amylovora* in buds on asymptomatic shoots was typically highest from trees with symptomatic tissues and lower on trees further away. At the same time, there was a considerable variability in the incidence of *E. amylovora* for individual trees in all proximity treatments across all years for both the 'Gala' and the 'Topaz' orchards. Such variability may have overshadowed statistically significant differences that would have been more apparent with a greater number of sampling plots. However, the conditions of the proximity treatments and the size of orchard blocks limited the number of possible plots to three. One might be tempted to suggest that differences in seasonal weather could have been factor in variability or, in some instances, uniformly high levels of incidence across all proximity treatments. However, instances of excessive variability or uniformly highly levels of bud

infection occurred in different years for the two cultivars (Figure 4.3). For example, there was > 95% incidence of bud infection for all proximity treatments for the 'Topaz' orchard in 2014, but not the 'Gala' orchard. Variability or uniformly high incidences in bud infection among treatments could be due to the fact that the symptomatic center point tree, used as a reference for bud stick collection, may not have been the only source of *E. amylovora* present in the orchard. Although no additional fire blight symptoms were found during scouting efforts, it is possible that small undetectable cankers left over from previous seasons infections served as an inoculum source on seemingly healthy trees. While we did have assurance from the grower that no strikes were removed and no summer pruning was conducted during the seasons study, small cankers (< 3-5 cm) could have been missed. It could simply be the case that apple trees within these orchards contained endophytic *E. amylovora* populations that served as additional cryptic sources inoculum. Indeed, it is well documented that *E. amylovora* may live endophytically without producing blight symptoms for several months at a time, and potentially longer depending on the cultivar and age of the tree (Crepel et al. 1996; Crepel and Maes, 2000; Rosen 1929, 1933, 1936, Ge and van der Zwet, 1996, Keil and van der Zwet, 1972, Lelliot 1973, van der Zwet, 1996). Overall, the data from the current study suggest that there may a trend between the incidence of bud infection and proximity to fire blight symptoms. At the same time, there appears to be variability among trees in the level of endophytic *E. amylovora* in buds. In the end, collecting budwood from trees with close proximity to fire blight symptoms may increase the risking of collecting asymptomatic buds.

Overall, little is known about the presence of *E. amylovora* in apple buds. The phenomena of endophytic *E. amylovora* in apple buds was first documented by Baldwin and Goodman (1963) who found that *E. amylovora* in dormant buds of 'Jonathan' apples in Missouri.

Durek and Morand (1975), and Bonn (1979) made similar isolations from healthy apple and pear buds, and Calzolari et al. (1982) isolated *E. amylovora* from imported budwood 10 months prior to establishment of the disease in Italy. In the current study, we found asymptomatic infection of buds to be quite prevalent in two orchards in western NY where fire blight outbreaks are commonly observed in each year. It's not known whether such levels of bud infection occur in apple production regions where fire blight rarely occurs, but such information could be ascertained in a follow-up study.

In contrast to the early studies simply documenting the recovery of *E. amylovora* from buds (Baldwin and Goodman, 1963; Durek and Morand, 1975), we found that *E. amylovora* was present beneath the bud scales within the meristematic tissues. Because *E. amylovora* was isolated from the inner meristematic tissues of the bud following rigorous surface sterilization, it may be that the bacterium simply washed into the bud from a source of active infection within the orchard block or it could be that there are endophytic populations of *E. amylovora* presiding within trees asymptomatically, and these bacteria may move systemically to into the bud tissues. Indeed, such an occurrence could explain a lack of a trend between proximity to shoot blight and the incidence of bud infection. While the endophytic growth and systemic movement of *E. amylovora* within a tree has been recognized (Crepel et al. 1996; Crepel and Maes, 2000; Ge and van der Zwet 1995, 1996; Keil and van der Zwet 1972; Lelliot 1973; Momol et al. 1994, 1998; Rosen 1929, 1933, 1936; van der Zwet 1996), the movement of these bacteria from shoot tissue into the meristem of buds has yet to be established. To definitively demonstrate the movement of *E. amylovora* from shoots into bud tissues, follow-up studies using microscopy techniques with green fluorescent protein or similarly marked *E. amylovora* strains would be necessary.

While our bud infection, and potentially meristem infection, results may be explained by

the systemic internal movement of *E. amylovora*, our data on the abundance (CFUs/L) of *E. amylovora* found in buds suggest that visible shoot blight may be a source of external inoculum for bud infection. In 2014 and 2015 there was a clear trend of higher CFUs/L of *E. amylovora* in buds with increasing proximity to trees with fire blight symptoms. It may be that the increased presence of *E. amylovora* in these buds is a direct result of the active inoculum from nearby shoot blight. Alternatively, it may be that active shoot blight is simply an indicator of high levels of endophytic *E. amylovora*, since some form of weather related trauma (e.g. thunderstorm) would be needed in order for such external shoot blight inoculum to contribute to infection. While this trend of higher CFUs of *E. amylovora* in buds closer to fire blight symptoms was observed in both cultivars, there were differences between the two cultivars. In both years, the CFUs/L recovered from 'Gala' buds were much higher than those for 'Topaz' buds. These results suggest that cultivars may differ in their ability to support endophytic populations of *E. amylovora* in their bud tissues. However, additional controlled studies would be required to elucidate the contributions of internal and external infection of buds and the relative potential for different cultivars to support endophytic populations of *E. amylovora* in their buds.

In summary, our studies highlight the disparities in apple budwood selection protocols for the clonal propagation of trees. Clearly, the avoidance of symptomatic trees is of the upmost importance for selecting budwood, however, there is a risk of endophytic *E. amylovora* in buds regardless of distance from these trees. While trends suggest that there are lower incidences and populations of *E. amylovora* in trees > 20 m from a symptomatic tree, this does not guarantee the propagation of pathogen free trees from these materials. Future studies are needed to determine the mechanism of bud infection and determine the outcomes using infected budwood in the propagation of apple trees. Given the near endemic presence of *E. amylovora* in NY apple

orchards, it will be specifically imperative to investigate the relationship between endophytic population size and subsequent infection of newly propagated trees.

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FIGURES

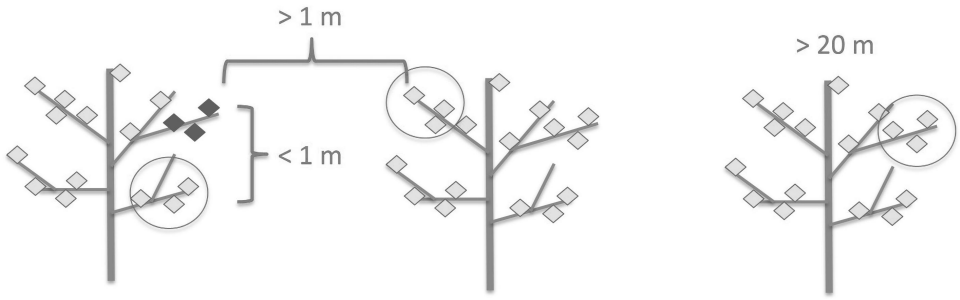

Figure 4.1: Representation of the budwood stick collection practices for three fire blight proximity treatments where budwood sticks where collected either: on the center point tree less than 1 meter away from fire blight symptoms (<1 m); from branches on an adjacent tree that was greater than 1 meter from the center point tree (> 1 m); and from a tree 20 meters from the symptomatic center point tree (> 20 m). Diamonds represent leaves with pale grey diamonds indicating healthy leaves and dark grey diamonds indicating fire blight infection.

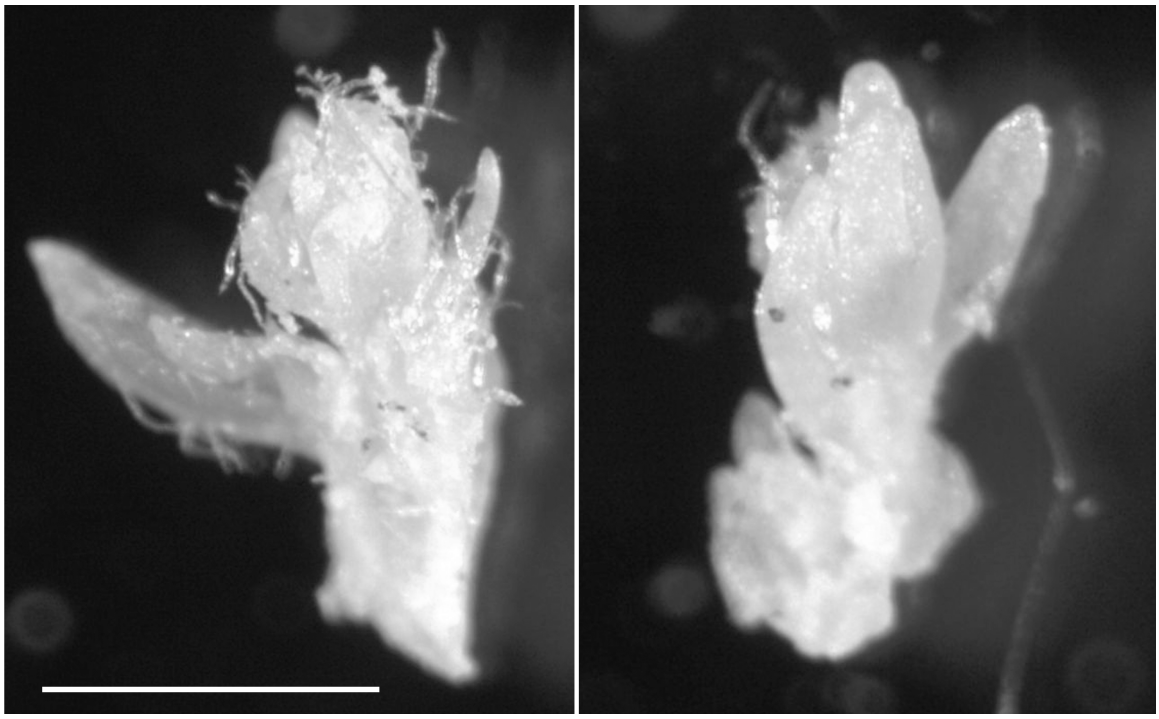

Figure 4.2: Example of a dissected apple bud after removal of outer bud scales by individual dissection needles prior to surface sterilization. Bar is the left panel is 3 mm.

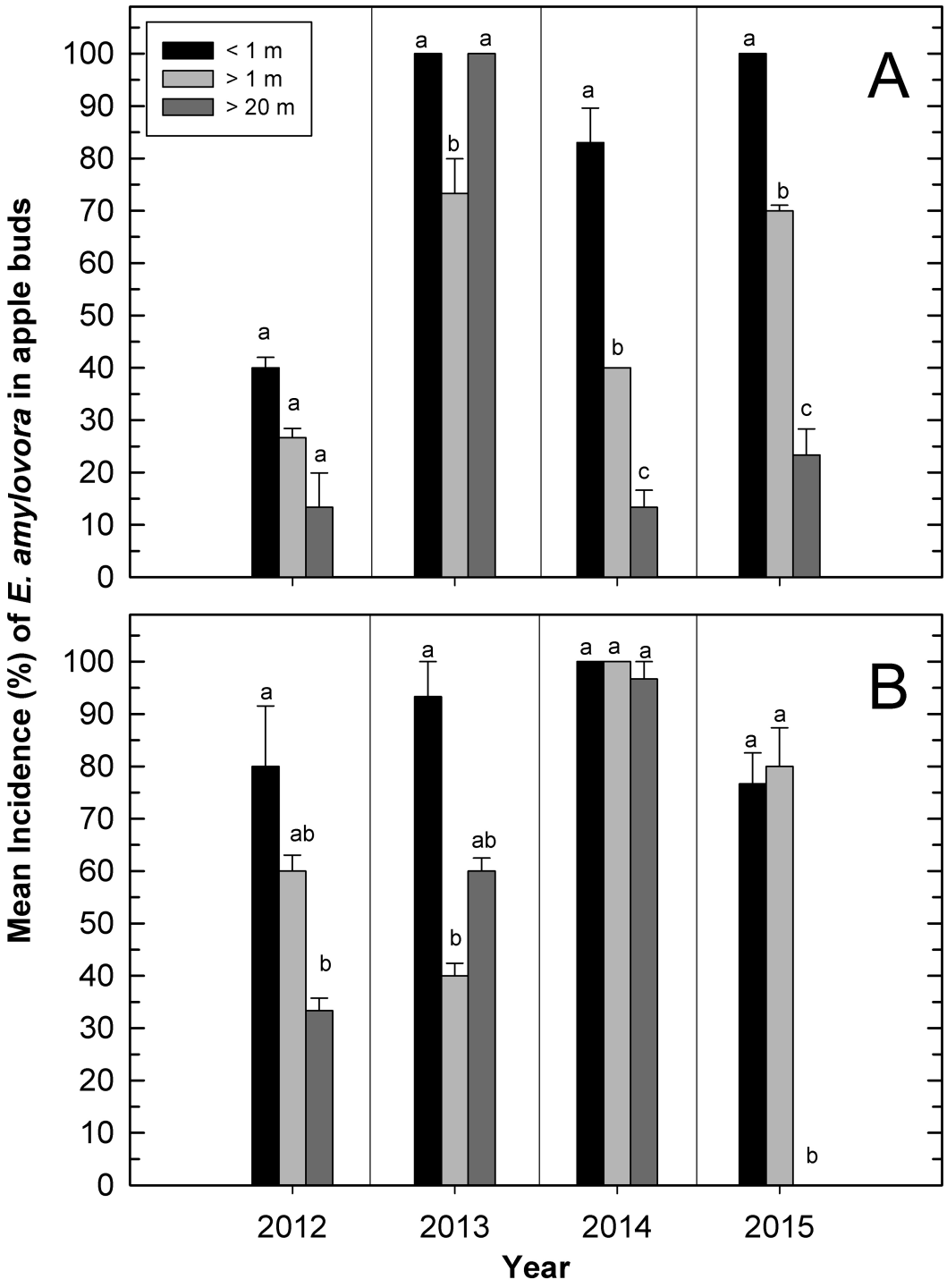
< 1 m
> 1 m
> 20 m
A
B
Mean Incidence (%) of E. amylovora in apple buds
Year
2012
2013
2014
2015
0
10
20
30
40
50
60
70
80
90
100

Figure 4.3: The incidence of *Erwinia amylovora* in asymptomatic budwood collected from 2012 to 2015 from trees at different proximities to a center point tree with fire blight where: < 1 m represents budwood sticks on the center point tree less than 1 meter away from fire blight symptoms; > 1 m represents budwood sticks from branches on an adjacent tree that was greater than 1 meter from the center point tree; and > 20 m represents budwood sticks from a tree 20 meters from the symptomatic center point tree. Values are the mean and standard error of ten budwood sticks across three replicate sampling plots for *Malus* × *domestica* cvs. 'Topaz' (A) and 'Gala' (B). Bars denoted by the same letter are not significantly different at the 5% level of significance.

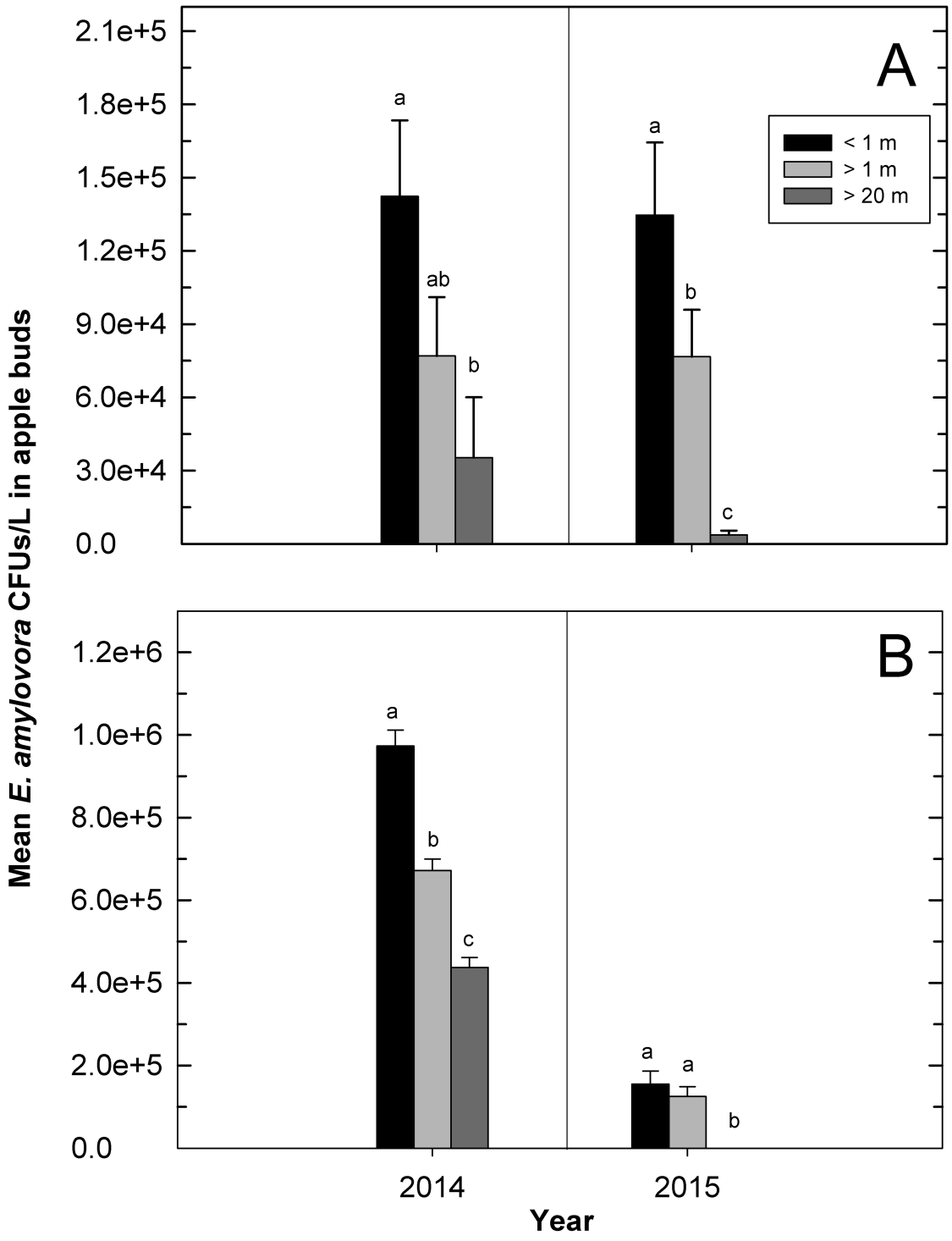
A
B
< 1 m
> 1 m
> 20 m
Mean E. amylovora CFUs/L in apple buds
2.1e+5
1.8e+5
1.5e+5
1.2e+5
9.0e+4
6.0e+4
3.0e+4
0.0
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
a
ab
b
a
b
c
a
b
c
a
a
b
2014
2015
Year

Figure 4.4: *E. amylovora* CFUs/L recovered from asymptomatic budwood collected from 2012 to 2015 from trees at different proximities to a center point tree with fire blight symptoms where: < 1 m represents budwood sticks on the center point tree less than 1 meter away from fire blight symptoms; > 1 m represents budwood sticks from branches on an adjacent tree that was greater than 1 meter from the center point tree; and > 20 m represents budwood sticks from a tree 20 meters from the symptomatic center point tree. Values are the mean and standard error of ten budwood sticks across three replicate sampling plots for *Malus* × *domestica* cvs. 'Topaz' (A) and 'Gala' (B). Bars denoted by the same letter are not significantly different at the 5% level of significance.

CONCLUSIONS

Fire blight, caused by *Erwinia amylovora,* continues to be a significant threat to the apple industry in the northeastern United States, as well as apple growing regions world-wide (Vanneste 2000; van der Zwet 2012). Although streptomycin has provided growers in the northeast with the most effective management of this disease for several decades (McManus et al. 2002), the development of antibiotic resistance in western New York orchards has created new concerns about the longevity of this antibiotic for fire blight management. The primary objectives of my dissertation were to provide insight into the current status of streptomycin resistant (SmR) *Erwinia amylovora* in New York, in regards to location, diversity, and possible origins of SmR isolates, and also to investigate how common practices may contribute to the development and spread of SmR *Erwinia amylovora* throughout the state.

My research began with a survey of *E. amylovora* from New York orchards where outbreaks had been reported in 2011 to 2014. The isolation, identification, and streptomycin resistance characterization of these isolates provided critical information on the prevalence of SmR *E. amylovora* in NY. The results of this study (Chapter 1) revealed that SmR isolates are found in apple growing regions in central and western NY, but are absent from the apple growing regions of eastern NY (Lake Champlain, the Hudson Valley and Long Island). Furthermore, the vast majority of SmR *E. amylovora* isolates contained the streptomycin resistance genes *strA* and *strB*. These genes are most commonly found in SmR isolates from the midwestern and eastern United States (McManus and Jones 1994; McGhee et al. 2011), and are common in apple phyllosphere dwelling epiphytes, such as *Pantoea agglomerans* and various *Pseudomonas* species (Burr 1988, 1993; Chiou and Jones 1991). This result led us to infer that

perhaps streptomycin resistance was acquired within local orchards or that SmR isolates could have been introduced to NY from other locations in the eastern US. Although SmR *E. amylovora* isolates with a point mutation at codon 43 of the *rpsL* gene, another mechanism of streptomycin resistance found in *E. amylovora* (Chiou and Jones 1995), are rarely found outside of the western United States (McGhee and Sundin 2011), two such isolates were recovered from NY orchards. Based on this new information, it was hypothesized that streptomycin resistance in local populations could partially be due to the movement of contaminated plant material from the western US. Consequently, the next objective was to investigate the diversity of the isolates (from research presented in Chapter 1) to elucidate possible origins of SmR *E. amylovora* isolates in NY.

Investigating diversity within *E. amylovora* has been challenging due to the high homology (~99.8%) among isolates worldwide (Sebaihia et al. 2010; Smits et al. 2010) resulting from the relatively recent distribution of this pathogen from its center of origin in the eastern United States (van der Zwet 2012). Recent studies have utilized CRISPR spacer arrays to explore diversity within a bacterial species where previous techniques have proven insufficient due to high homology among isolates (Almendros et al. 2014; Barros et al. 2014; Shariat et al. 2013; McGhee and Sundin 2012; Rezzonico et al. 2011). CRISPR spacer arrays are comprised of short DNA segments, which are acquired in a polar manner by a bacterium over time from their surrounding environment (Horvath et al. 2008). Because of their polar (or cumulative) arrangement, CRISPR spacer arrays can be used to investigate diversity within a bacterial species based upon an inferred geographical record (Horvath et al. 2008). In Chapter 2, CRISPR spacer arrays (CR1, CR2, and CR3) of SmR and streptomycin sensitive (SmS) *E. amylovora* isolates, from our previous NY survey collections, were sequenced and concatenated to created

unique CRISPR profiles for isolates. The CRISPR profiles of NY SmR isolates were compared to NY SmS isolates, as well as to previously determined CRISPR profiles of isolates collected world-wide (McGhee and Sundin 2012). Using these comparisons, evidence was found that supports both the introduction of SmR isolates from other regions of the US, such as the western US and Michigan, and also the development of SmR isolates in local NY orchards, which may be due to the overuse of streptomycin for fire blight management. It was hypothesized that the long history of streptomycin use in NY, coupled with the common misuse of this antibiotic for shoot blight control, could have led to the development of SmR *E. amylovora* in local orchards. These discoveries inspired the next objective (Chapter 3), which was to further explore the effects of streptomycin and kasugamycin, a newly approved antibiotic for fire blight management, on bacterial epiphytes in the apple phyllosphere.

Current management recommendations for streptomycin use focus on the control of blossom blight, where the antibiotic is applied to open blossoms, and furthermore, stress the importance of avoiding applications for shoot blight control (Agnello et al. 2016). Streptomycin is a preventative bactericide that displays little to no systemic activity, and therefore cannot offer effective shoot blight management because the bacteria has already become established within the tree (Agnello et al. 2016; van der Zwet 2012). However, nursery operations and commercial growers may be tempted to continue streptomycin use throughout the season in an effort to prevent *E. amylovora* from becoming established in valuable young plantings (Breth 2008). Studies have linked historical streptomycin application records in individual orchards to the later recovery of SmR *E. amylovora* (Loper et al. 1991; Yashiro and McManus 2012). It has also been established that common apple epiphytes, such as *Pantoea agglomerans* and a variety of

Pseudomonas spp., may harbor streptomycin resistance genes *strA* and *strB*, which are found commonly in SmR isolates in the eastern US (Burr 1988, 1993; Chiou and Jones 1991).

The goal of Chapter 3 was to explore the direct effects of antibiotic applications on the epiphytic bacterial community in the apple phyllosphere within a growing season. In a study repeated in two seasons, streptomycin and kasugamycin were applied in increasing application numbers starting at both bloom and during the shoot elongation phase. The epiphytic bacterial community was sampled at the completion of applications each season in order to assess the community structure in regards to prominent bacterial species and streptomycin resistance status. Excessive applications of streptomycin led to an overall increase of the portion of SmR epiphytes in the phyllosphere, as was expected, and an unexpected increase in the proportion *Pseudomonas* spp. recovered. Interestingly, excessive kasugamycin applications resulted in a decrease in the proportion of SmR bacteria and an overall increase in the proportion of *P. agglomerans*. Although further studies may be necessary to better understand the impact of these findings, results imply that: 1) these antibiotics may have differential effects on certain bacteria, 2) the overuse of streptomycin may increase overall SmR bacteria within a single season, and 3) applications of kasugamycin may aid the reduction of overall SmR bacteria within orchards. Furthermore, the sustainable use of these antibiotics might be achieved through rotation and minimal use, and the strict avoidance of use after bloom.

While the overuse of streptomycin for fire blight control may have contributed to the development of SmR *E. amylovora* isolates in NY, the introduction of SmR isolates from other regions of the country is disconcerting. The movement of infected nursery trees from regions were SmR *E. amylovora* outbreaks have occurred may be partly responsible for the establishment of SmR isolates in NY. NY growers commonly source trees from nurseries in the

western and midwestern US (Washington, California, and Michigan) (Miller and Schroth 1972; Coyier and Covey 1975; McManus and Jones 1994). Nurseries clonally propagate trees using budwood from apparently healthy trees. Although nurseries, especially budwood source blocks, are scouted intensely for fire blight symptoms, pathogen free tree propagation can be complicated by the presence of asymptomatic *E. amylovora* (Keil and van der Zwet 1972; McManus and Jones 1994; Smith 2002; van der Zwet 1983, 1996). Although budwood selection practices may vary depending on the individual nursery operations, trees with fire blight symptoms and the adjacent trees are avoided when selecting budwood. Given that these collection guidelines are not enforced by any agency and there are no fire blight certification programs, it is unknown if any nursey source trees are truly fire blight free. Consequently, a final study (Chapter 4) was conducted to discern if a relationship exists between fire blight symptoms and the recovery of *E. amylovora* from asymptomatic budwood.

In Chapter 4, the buds of apple trees, collected at defined distances from a center point tree with fire blight, were tested for the presence of *E. amylovora*. Results demonstrated that trees more than 20 m from a symptomatic tree may have lower incidences and lower populations of *E. amylovora*. However, *E. amylovora* was still recovered from several of these distant asymptomatic trees. Avoiding symptomatic trees is critical, yet the risk of endophytic *E. amylovora* in buds is still present, regardless of the distance from symptomatic trees. Importantly, there is no guarantee that pathogen free trees can be propagated from these materials. Research to better understand the relationship between *E. amylovora* population size and subsequent infection of newly propagated trees is needed, as is developing advanced pathogen screening practices for propagation materials.

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FINAL THOUGHTS

Fire blight will continue to be a significant threat to New York apple growers, especially if SmR *E. amylovora* becomes more prominent. Growers must become actively involved in the prevention and management of this disease on a state and regional level to cooperatively mitigate the spread of streptomycin resistance. This can be enhanced by the responsible and informed use of streptomycin and new antibiotic products, such as kasugamycin. The scientific community must educate growers on proper use of chemical controls and the severe consequences of product misuse. Although many growers rely extensively upon the use of streptomycin, alternative control strategies must be explored and adopted to promote the sustainable management of fire blight and secure the future of the NY apple industry.