Role of Exopolysaccharides and Monosaccharides in *Erwinia amylovora* Resistance to Bacteriophages

by

David R. Sjaarda

A thesis submitted to the Department of Biological Sciences

In conformity with the requirements for

the degree of Master of Science

Brock University

St. Catharines, Ontario, Canada

Abstract

Fire blight is a disease caused by the phytopathogenic bacterium *Erwinia amylovora,* an economically important pathogen in the commercial production of apples and pears. Bacteriophages have been proposed as a commercial biopesticide to relieve the pressures on apple and pear production and provide alternatives to existing biological control options. This work reports on the investigation of host resistance in the development of a phage biopesticide. Exopolysaccharide (EPS) deficient bacterial mutants were generated through recombineering to investigate the role of EPS in bacteriophage adsorption and infection. The mutants that were deficient in amylovoran production were avirulent and resistant to infection by phages of the *Podoviridae* and some of the *Siphoviridae* family. Levan deficient bacterial mutants resulted in reduced phage titers in some phages from the *Myoviridae* family. Exopolysaccharide mimetic monosaccharides were used to demonstrate that levan and amylovoran play an important role in phage attack of *E. amylovora.*

Acknowledgements

The completion of this thesis would have not been successful without the encouragement, advice and support of several people around me. My supervisors, Drs. Alan Castle and Antonet Svircev were invaluable with their continued patience and timely advice. They, with the committee members Drs. Adonis Skandalis and Andrew Reynolds, helped to shape the direction of research and the final form of this study.

The members of the Svircev and Castle labs were also instrumental in completion of this thesis. Thanks to Dwayne Roach for guidance and assistance through this study. Katherine Whybourne and Abdelbaset Yagubi helped immensely with assistance in the lab as well providing as insightful commentary and debate. Karin Schneider, Francesca Tumini and Calvin Sjaarda provided valuable technical advice.

Dr. Richard Gardiner (Biotron, University of Western Ontario) provided his time and made it possible to obtain transmission electron micrographs for this study.

Dr. David Hunter for his insight and advice on all things related to pomology and pear blossoms.

Thank you to all the AAFC staff at Vineland and the Jordan farm for making my time enjoyable. Peter Raakman, Mark Heeringa and Eric Mannapso for all their hard work moving, reconstructing, and maintaining the lab. Kathy Jensen for her tireless efforts in administration and Darlene Nesbitt and Neva Gregg encouraging discussion.

Most of all I would like to thank my wife Rachel for the never ending support and tireless dedication to all of my endeavors both academic and extra-curricular. Finally, a thank-you to my family and friends for their encouragement and support.

Table of Contents

Abstract 3
Acknowledgements 4
Table of Contents 5
List of Tables 7
List of Figures 8
Abbreviations 10
Chapter 1 Introduction 12
1.1 The “Problem” 12
1.2 Brief History of Bacteriophages 12
1.3 Properties of Bacteriophages 14
Chapter 2 Literature Review 18
2.1 Introduction 18
2.2 Historical Background 18
2.3 Etiology and Disease Symptoms 20
2.4 Pathogenesis 24
2.5 Control Measures 27
2.6 Chemical Control of Fire Blight 28
2.7 Biologics in Canada 30
2.8 Bacteriophage Life Cycle 31
2.9 Bacteriophages as Biopesticides 33
2.10 Bacteriophage-Host Resistance 36
2.11 Bacterial EPS and Bacteriophages 37
Chapter 3 Materials and Methods 40
3.1 Phage Isolates 40
3.2 Phage Growth Conditions and Media 40
3.3 Phage Titre Determination 44
3.4 Generation of Bacterial Exopolysaccharide Deficient Mutants 45
3.4.1 Amylovoran Production Measurement 48

3.5 *In planta* Bacterial Virulence Assay 49
3.5.1 Statistical Analysis 51
3.6 Bacteriophage Pre-incubation with Sugars 51
Chapter 4 Results 55
4.1 Recovery of EPS deficient mutants 55
4.1.1 *E. amylovora* EPS production 55
4.1.2 Virulence and Pathogenicity of Erwinia amylovora and the Influence of Exopolysaccarides 57
4.2 Effect of Increased EPS Production on Phage Proliferation 57
4.3 Decreased EPS production and Phage Efficiency of Plating (EOP) 63
4.4 Quantity of EPS production and Phage Population Growth 67
4.5 Effect of Supplementing Media with EPS Mimetic 70
4.6 Effect of Pretreatment with EPS Mimetic 70
Chapter 5 Discussion 76
Chapter 6 Future Considerations 90
Chapter 7 Literature Cited 92

List of Tables

TABLE 1. Bacteria and bacteriophages used in the study..... 42
TABLE 2. Primers used in the study 50
TABLE 3. Phages from each of *Myo-*, *Sipho-*, and *Podoviridae* enriched on *E. amylovora* wild type, Δ*rcsB* and Δ*lsc* deletion mutants of the ideal host. 68

List of Figures

FIG. 1. Schematic of protocol used to generate gene deletions by homologous recombination with an antibiotic resistance gene. 47
FIG. 2. Blossom assay protocol for the pear blossom bioassay..... 52
FIG. 3. Disease severity index for infection of pear blossoms by *Erwinia amylovora*. ... 54
FIG. 4. Amylovoran synthesis for *Erwinia amylovora* wild type and deletion mutants. . 56
FIG. 5. Disease severity caused by *Erwinia amylovora* wild type, ΔrcsB, and Δlsc deletion mutants..... 59
FIG. 6. Titres of *Myoviridae* phage ΦEa21-4, on NA and NAS media..... 60
FIG. 7. Titres of *Siphoviridae* phage ΦEa35-7, on NA and NAS media..... 61
FIG. 8. Titres of *Podoviridae* phage ΦEa1(h), on NA and NAS media 62
FIG. 9. *Myoviridae* phage ΦEa21-4 titres grown on the wild type, Δ*rcsB*, Δ*lsc* and Δ*rcsB*Δ*lsc* mutants of *E. amylovora*. 64
FIG. 10. *Siphoviridae* phage ΦEa35-7 titres grown on the wild type, Δ*rcsB*, Δ*lsc* and Δ*rcsB*Δ*lsc* mutants of *E. amylovora*. 65
FIG. 11. *Podoviridae* phage ΦEa1 (h) titres grown on the wild type, Δ*rcsB*, Δ*lsc* and Δ*rcsB*Δ*lsc* mutants of *E. amylovora*. 66
FIG. 12. Relation between amylovoran production by wild type isolates, ΔrcsB, Δ*lsc* and Δ*rcsB*Δ*lsc* mutants and phage efficiency of plating when ΦEa1(h) is grown on wild type isolates, ΔrcsB, Δ*lsc* and Δ*rcsB*Δ*lsc* mutants. 69
FIG. 13. Effect of pre-treating the phage ΦEa21-4 with fructose, arabinose or water and adding fructose to the growth medium using the wild-type and Δ*lsc* deletion mutant of *E. amylovora* Ea6-4. 71
FIG. 14. Effect of pre-treating *Myoviridae* phage ΦEa21-4 with fructose at concentrations of 0-6%; grown on *E. amylovora* Ea6-4..... 72
FIG. 15. Pre-treatment of *Podoviridae* phage ΦEa31-1 with fructose, galactose/glucuronic acid (4:1) and arabinose prior to the addition of *E. amylovora* Ea6-4..... 74

FIG. 16. Pre-treatment of *Myoviridae* phage ΦEa21-4 2% fructose at 0 to 20 minutes; grown on *E. amylovora* Ea6-4 75

Abbreviations

CFU – colony forming units

EOP – efficiency of plating

EPS – exopolysaccharide

HEP – high exopolysaccharide producing bacterial isolate

HR – hypersensitivity response

IPM – integrated pest management

LEP – low exopolysaccharide producing bacterial isolate

LPS – lipopolysaccharide

lsc – levansucrase gene

MOI – multiplicity of infection

NA – nutrient agar

NAS – nutrient agar with 2% sucrose and 1% sorbitol

NB – nutrient broth

NBSS – nutrient broth with 2% sucrose and 1% sorbitol

OD_{600} – optical density to light with a wavelength of 600 nm

PB – sodium phosphate buffer

PCR – polymerase chain reaction

PFU – plaque forming units

rcsB – regulator of capsular synthesis gene

SAR – systematic acquired resistance

T3SS – type III secretion system

Chapter 1
Introduction

1.1 The "Problem"

Plant pathogens exist in a unique, environment, causing major economic liabilities. This results in many challenges for appropriate and effective disease management. These challenges are compounded by reduced efficacy and gradual regulated disuse of conventional chemical controls; the lack of alternatives in disease resistant cultivars; and the general migration to the use of effective, yet environmentally friendly alternatives to traditional control methods (Jones et al., 2007; Svircev et al., 2011). In recent years there has been a resurgence in the development of bacteriophage based control programs in agriculture but the debate continues regarding the sustainability of phages as biological control agents (Vidaver, 1976; Stewart, 2001; Summers, 2001; Abedon, 2003; Svircev et al., 2011). Bacterial resistance to bacteriophages can be one of the most condemning criticisms of bacteriophage biopesticides. This study explores the role that the bacterial exopolysaccharide plays in phage infection and how modification of the EPS could result in resistance.

1.2 Brief History of Bacteriophages

Bacteriophages, or phages, are bacterial viruses consisting of a nucleic acid genome contained in a protein or lipoprotein capsid. Phages require the metabolism of an active, viable bacterial host, that is, they are obligate parasites (Adams, 1959; Guttman et

al., 2005). Upon successful infection the phage DNA redirects the host's metabolism to produce the phage genome and proteins. When a specific number of phage particles have assembled, the bacterium is lysed to release many new phages into the environment. This process kills the bacterial host and produces exponential phage growth of the at the cost of the bacterial host.

Control of bacterial pathogens with bacteriophages is an idea that dates back to the early 20th century. The discovery of bacteriophages is attributed to independent breakthroughs by Frederick Twort in 1915 and Felix d'Herelle in 1917 (d'Herelle, 1917; Duckworth, 1976). There was considerable debate in this regard since Twort was the first to describe the appearance of plaques on a lawn of bacteria, however d'Herelle concluded that the plaques consisted of "invisible microbes endowed with antagonistic properties against the pathogen" (d'Herelle, 1917; Duckworth, 1976; Summers, 1999; Summers, 2001). D'Herelle called the "invisible microbes" bacteriophage from the Greek word *phagein* which means "eater" and invented the idea of using phage to kill the dysentery bacillus plaguing the French army during the First World War (d'Herelle, 1917; Duckworth, 1976). In essence this was the first example of biological control of a human pathogen by phages and one of the pioneering experiments into phage therapeutics.

D'Herelle's extensive work with phages in France and India received much attention and interest in the potential of the "d'Herelle phenomenon" (Davison, 1922; Summers, 1999). However, the interest and excitement in this new treatment of bacterial diseases quickly waned for two main reasons: unsuccessful phage treatments and the advent of antibiotics in the late 1920s and their subsequent development into the 1940s (Flemming, 1929; Summers, 2001; Clark and March, 2006). Phage therapies, due to the

lack of understanding of the mechanism and host range of the specific phage, met with disappointing results and combined with the sale of unproven remedies in the United States tarnished the reputation of phage therapy (Summers, 2001). When antibiotics became more available and cost effective to produce in the late 1930s, phage therapy was forgotten in all but a few areas of the world (Clark and March, 2006). In the West the use of phages were directed to more fundamental research. Phages, due to their small genomes and ease of manipulation, became the basis of molecular research to facilitate the early studies of genomics and proteomics. Phage therapy research slowly progressed in countries in Eastern Europe after World War II at the Eliava Institute that d'Herelle had founded and in a few other studies sponsored by the World Health Organization (Monsur et al., 1970; Summers, 1999). However, these studies were often inconclusive do to the lack of proper controls, shoddy experimental design or ineffective patient monitoring (Luria and Delbrück, 1943; Summers, 2001).

1.3 Properties of Bacteriophages

There are several properties that make phage valuable as a biological control agent. Phages can be bactericidal, can increase in number over the course of the treatment, and are often highly specific as to not affect the microbial flora of the treatment organism or environment. Phages are often as effective against antibiotic resistant bacterial as antibiotic sensitive strains, are often ubiquitous in a given environment, easy to isolate, seem to have the ability to disturb biofilms, and often have low inherent toxicities (Summers, 1999; Summers, 2001; Nagy et al., 2012).

Once a lytic phage successfully penetrates the cell wall of a susceptible bacterium, the bacterium is unable to regain viability. This is due to the degradation of the bacterial genome and the hijacking of the cellular processes for the replication of the bacteriophage (Guttman et al., 2005). In contrast some antibiotics, such as tetracycline, are merely bacteriostatic and may lead to development of resistance (Summers, 2001; Loc-Carrillo and Abedon, 2011). Phages have highly specific modes of action that are unrelated to the action of antibiotics, making the phage equally effective against both antibiotic resistant and susceptible bacteria (Chanishvili et al., 2001; Capparelli et al., 2010; Gupta and Prasad, 2011). These highly specific modes of action make bacteriophage specific to one or a few closely related bacterial species. Therefore, treatment with bacteriophage will not significantly alter the levels of healthy environmental flora, as does the application of broad spectrum antibiotics (Van Den Bogaard and Stobberingh, 2000; Loc-Carrillo and Abedon, 2011). Loss of the competitive and inhibitory effects of beneficial flora could lead to the subsequent superinfection by resistant pathogenic bacteria in the biotic void (Carlton, 1999; Summers, 2001). Bacteriophages are abundant in the environment, are easily isolated, purified, and cultured using well established protocols (Adams, 1959; Guttman et al., 2005). Phages are self-replicating and self limiting; with a single application, a population of phage will exponentially increase as long as there is a susceptible, actively growing host in that environment and be quickly inactivated in the absence of a host (Inchley, 1969; Geier et al., 1973; Davies and Breslin, 2003; Boerlin, 2010). Finally, the intimate association of phage with host cells enables phage to penetrate into areas that other chemicals may not be able to and the phage is able to populate specifically where

the host is located (Loc-Carrillo and Abedon, 2011). As the understanding of the phage host interactions increases the more feasible using phage therapy becomes.

Researchers have identified potential problems with phage therapy and treatment of phytopathogens (Okabe and Goto, 1963; Vidaver, 1976; Jones et al., 2007). More specifically, phage therapy presents the potential to increase the incidence of phage resistant bacterial hosts due to biological pressure and phages changing from lytic to temperate and transfer of virulence genes from pathogenic to non-pathogenic hosts (Okabe and Goto, 1963; Vidaver, 1976; Marks and Sharp, 2000; Jones et al., 2007). Phages, through the process of transduction, could introduce pathogenicity factors, antibiotic resistance genes, or other genes of interest from one host to another. There is evidence that phage treatment efficacy could be reduced by abortive infection which would prevent replication of phages in susceptible hosts (Duckworth, 1987). Additionally, using phage biologicals in variable environmental conditions (i.e. the orchard phyllosphere or rhizosphere) introduces an increased level of complexity that could lead to variable efficacy with phage biologicals (Vidaver, 1976; Gill and Abedon, 2003).

This study aims to investigate host resistance in the development of a phage biopesticide through study of *E. amylovora* cell components bacteriophages may utilize to gain access to the bacterium using EPS deficient bacteria and EPS mimetics. The hypothesis, given the significant differences in host infection efficiencies, is that *E. amylovora* bacteriophages use the EPS produced by the bacterium as receptors. Further, without the EPS the infectivity of some phages may be reduced; addition of EPS mimetics may decrease the phage infection efficiency.

Appreciating the criticisms of bacteriophage biocontrol due to resistance etc., understanding the mechanisms of action of the phage and using obligately lytic phage in treatment are keys to a successful therapy. Effective application techniques are required to protect the phage from environmental stressors and control the pathogen at the appropriate time in the disease cycle. Mixtures of carefully chosen, phenotypically and/or genotypically different phages are also recommended to prevent host resistance due to variation and lysogeny that are often observed in many phage-based biological control applications.

Chapter 2
Literature Review

2.1 Introduction

Erwinia amylovora (Burrill) Winslow et al. is the causal agent of fire blight, a serious bacterial disease of rosaceous plants. The Gram negative, motile, rod-shaped bacterium of the *Enterobacteriaceae* family leads to high commercial losses in the production of economically important fruit crops belonging to the *Malus* (apple) and *Pyrus* (pear) *spp.* (Arthur, 1885; European and Mediterranean Plant Protection Organization, 1983; Holt et al., 1994; Momol and Aldwinckle, 2000; Aksyuk and Rossmann, 2011). The pathogen was first discovered in 1880 by Thomas J. Burrill at the University of Illinois, and named *Micrococcus amylovorus*. Five years later Koch's postulates were completed and the causal agent of fire blight was recognized as the first bacterium proven to cause disease in plants (Burrill, 1883; Arthur, 1885).

2.2 Historical Background

E. amylovora is thought to be native to North America with first reports of disease symptoms dating back to 1780 in New York State (Denning, 1794). The pathogen spread to many parts of the United States and Canada by the end of the nineteenth century (Denning, 1794; van der Zwet and Keil, 1979; Bonn and van der Zwet, 2000). Fire blight originated in wild plants such as hawthorn, wild ash, and crab apple; spreading to domesticated forms of apple and pear when orchards were planted by 16th century

pioneers (van der Zwet and Keil, 1979; Thomson, 2000). In the 20th century, fire blight was confirmed in New Zealand (1919), Mexico (1943), United Kingdom (1958) and Egypt (1964). The disease spread from these continental foci (especially Egypt and England) to most of Europe and some of Africa and the Middle East (van der Zwet and Keil, 1979; Johnson and Stockwell, 1998; Bonn and van der Zwet, 2000; Kazempour et al., 2006; Lagonenko et al., 2008; Végh et al., 2012). This transcontinental spread is mainly attributed to human activity and the transfer of infected plants or plant tissue from one area to another (Crosse et al., 1958; van der Zwet and Keil, 1979; European and Mediterranean Plant Protection Organization, 1983). The initial spread of *E. amylovora* to England most likely resulted from infected fruit, budwood or nursery stock being brought to London (Crosse et al., 1958). Meijneke (1974) hypothesized that the bacteria spread from England to Europe via migrating birds (van der Zwet and Keil, 1979; Bonn and van der Zwet, 2000). In Ontario, fire blight was first observed in Niagara in 1840 and has spread to most of the province, with the most severe infection south of a line between Sarnia and Oakville (OMAFRA, 2011).

The greatest concern of fire blight is the economic impact caused by the loss of commercial fruit, especially apples and pears. In Canada, apples and pears are mainly grown in Ontario (6232 ha. and 407 ha. resp.) and British Columbia (3355 ha. and 216 ha. resp.) with smaller acreages in Quebec and Nova Scotia (Statistics Canada, 2010). In Canada approximately 346,677 metric tonnes of apples and 7833 metric tonnes of pears are produced for a total market value of $142,333,000 of apples and $6,892,000 of pears (Statistics Canada, 2010). In one growing season it is possible to lose entire orchards from the effects of fire blight (European and Mediterranean Plant Protection

Organization, 1983; Johnson and Stockwell, 1998; Bonn and van der Zwet, 2000). In fact, some regions of California have been forced to remove all apple and pear orchards due to the impact of fire blight (Holtz, et al., 2008). In 1998, the economic impact of fire blight in Washington and Oregon alone was estimated at $68 million (Johnson and Stockwell, 1998; Bonn and van der Zwet, 2000). A fire blight epidemic swept through southwest Michigan in 2000 causing a reported $42 million in total losses over five years (Longstroth, 2001). However, it is very difficult to accurately determine the cost of an outbreak on a certain country or region due to a number of factors. Fire blight is an epiphytic disease and occurs very sporadically, its effects vary from year to year depending on the type of season, and the effects are not limited to one year (van der Zwet and Keil, 1979; Johnson and Stockwell, 1998; Bonn and van der Zwet, 2000).

2.3 Etiology and Disease Symptoms

Plant infection with *E. amylovora* has five generally accepted stages: blossom, shoot, rootstock, canker, and trauma blight (van der Zwet and Beer, 1995). The type of blight depends on the distinct symptoms and pathogen location (van der Zwet and Keil, 1979). *E. amylovora* is a necrotic wilt pathogen that begins epiphytically on the plant epithelium. Once it infects the plant it moves quickly through the vascular system causing lesions in. This results in the characteristic symptoms of fire blight, scorched appearance of leaves on affected branches, and a typical "shepherd's crook" appearance of succulent shoots (European and Mediterranean Plant Protection Organization, 1983; van der Zwet and Beer, 1995). The inherent nature of a disease cycle is that it has no definable beginning or end. Primary infections occur in early spring in the form of blossom blight

due to the susceptibility of the blossom to pathogen colonization. The pathogen is spread by insect vectors from the bacterial "ooze" produced by the cankers that formed during the previous year's infection of woody tissues to susceptible blossoms (Schroth et al., 1974; van der Zwet and Keil, 1979). The blossoms first appear water soaked, secreting ooze; then wilted and shriveled; and finally turning blackish brown, clinging to the branch for long periods of time (van der Zwet and Keil, 1979; van der Zwet and Beer, 1995).

The primary inoculum source is a reservoir of bacteria in a canker surviving from the previous growing season. Brown cankers develop on infected trunks due to the pathogen induced collapse of the cortex parenchyma (van der Zwet and Keil, 1979). When the growing season is coming to an end, the multiplication of the bacteria slows and the cankers begin to form, harbouring populations of *E. amylovora* as the major source of inoculum for the following spring (Beer and Norelli, 1977; Biggs, 1994; van der Zwet and Beer, 1995). Cankers begin to exude characteristic bacterial laden droplets known as ooze (Schroth et al., 1974; van der Zwet and Beer, 1995). Bacteria can remain viable in the ooze for more than a year provided the relative humidity is below 45% (Hildebrand, 1939; van der Zwet and Reiber, 1994).

The bacterial inoculants from the ooze may be disseminated to other plants or parts of the same plant through the actions of wind, rain, insects, and birds. Opening of blossoms and new succulent shoot growth occurs with the warm weather and increased precipitation that characterizes spring. The onset of spring attracts insects to the new growth on the plants. Pollinating insects (i.e. bees) have long been proven to be carriers of *E. amylovora* from infected blossom to infected blossom (Emmett, 1971; Emmett and

Baker, 1971; Schroth et al., 1974; Alexandrova et al., 2002). There only needs to be a small number of initially infected blossoms to create epidemic blossom blight (Billing, 2008; Billing, 2011). Pollinating insects have been reported to have up to 10^5 bacterial cells each and can persist on these insects for up to six days (Ark and Thomas, 1936; Schroth et al., 1974). This is important because it has been shown that there needs to be a minimum population of 10^4 colony forming units per millilitre (CFU/ml) on the blossoms for the bacterium to take hold and a few bacterial cells on the plant is not enough to cause disease (Schroth et al., 1974). There are also instances where birds have spread fire blight. Migratory birds (i.e. starlings) are thought to have brought the pathogen from the coast of England to the other parts of Europe, especially Poland and the Netherlands (Meijneke, 1974; Schroth et al., 1974). Avian vectors could account for some long distance spread from orchard to orchard.

The flower stigma provides a suitable surface and environment for the growth of *E. amylovora* (van der Zwet and Keil, 1979). The blossoms appear to be the most susceptible on the first day after opening, and become increasingly resistant for five days (Thomson and Gouk, 2003; Pusey and Curry, 2004; Pusey et al., 2009). Work by Azegami et al. (2008) hypothesizes that the anther of the developing pear blossom could be the "tinder" that feeds the early blossom blight. From the anther, the bacterium could move the stigma, which provides a moist, sugar rich environment that suits the needs of the bacterium in a comparatively arid orchard canopy (Stockwell et al., 1999; Billing, 2007; Azegami et al., 2008). Most researchers agree however, that that the bacteria are directly deposited on the stigma not on the anther (Johnson and Stockwell, 1998; Stockwell et al., 1999; Pusey and Curry, 2004). On the stigma the bacteria may grow

epiphytically, reaching concentrations of 10^7 CFU. Facilitated by rain or dew the pathogen moves down the style into the flower cup or hypanthium (Spinelli et al., 2005; Johnson et al., 2008; Farkas et al., 2012). The actual infection of the blossom occurs when the bacteria enter the nectary stomata (nectarthodes) located on the surface of the hypanthium (Schroth et al., 1974; Spinelli et al., 2005; Pusey et al., 2009). Following invasion of the nectarthode, *E. amylovora* continues to invade and multiply in the intercellular spaces causing the blossoms to take on a water-soaked, necrotic appearance.

The infection of succulent shoot or twig tissue is the result of either secondary infection from blossoms and/or the populations of *E. amylovora* spreading to the vegetative tissue due to biting and piercing insects (eg. aphids). Blighted twigs often form a characteristic "shepherd's crook" at their tips (van der Zwet and Beer, 1995; Steiner, 2000).

Rootstock blight occurs when the pathogen travels through the woody tissue to the rootstock and/or the infection via suckers. Suckers, like tender shoots, are often more susceptible to damage by weather and subsequent infection by *E. amylovora* (Vanneste and Eden-Green, 2000). Rootstock blight is the most destructive type of blight, is untreatable and often causes the immediate death of the tree (van der Zwet and Keil, 1979; Russo et al., 2008). Trauma blight results from the infection wounds caused by forces that damage the protective layer of the branches, twigs or young growing shoots. Trauma blight most commonly results after hail or wind damage (Schroth et al., 1974; Johnson and Stockwell, 1998; OMAFRA, 2011).

2.4 Pathogenesis

Control of the fire blight pathogen by biologicals can occur only during bloom on the surface of the stigma and hypanthium. Reducing the populations of bacteria on the open blossom is paramount to controlling the spread and preventing the establishment of the pathogen in the plant tissue.

Once inside the plant tissue, in the apoplast of the xylem, the pathogen uses two main pathogenicity processes. The first is the production of the hypersensitivity response proteins (*hrp*) in conjunction with a type III secretion (T3SS) pathogenicity island (PAI1) and second is the presence of an exopolysaccharide capsule (Oh and Beer, 2005; Lalli et al., 2008; McNally et al., 2012). The hypersensitivity response proteins are coded on the *hrp* gene cluster and function through the T3SS to deliver proteins from the bacterium to the apoplast or cytoplasm of the cells of the host plant (Oh and Beer, 2005). Once in the host cell, effector proteins function to suppress host defenses and alter host metabolism (Lalli et al., 2008). The *hrp* gene cluster contains two main regulatory factors called HrpS and HrpX/HrpY which control Hrp L, in turn controlling expression of all known *hrp* genes. These factors are expressed *in planta* only under conditions of low nutrients, low pH, and specific water potential and temperature (Oh and Beer, 2005). It is estimated that the plant pathogen secretes at least 12 and upwards of 30 proteins into the plant host cell or apoplast through the T3SS including the essential virulence pathogens HrpN and disease specific protein A and E (DspA/E) (Barny et al., 1990; Oh and Beer, 2005; Lalli et al., 2008; Khan et al., 2012). HrpN is very abundantly secreted during the infection process and appears to interfere with ion channels in the plasma membrane of the host cells (Wei et al., 1992). Additionally, HrpN protein is able to interfere with

mitochondrial function by disrupting ATP synthase, a channel protein in the mitochondria coupled to the synthesis of ATP (Wei et al., 1992; Oh and Beer, 2005; Kong and Li, 2011). The *dsp* mutants are avirulent, and it is understood that the transcription of those genes is associated with electron transport in Photosystem I, but the exact mechanism of action is still unclear (Oh and Beer, 2005; Sinn et al., 2008; Kong and Li, 2011).

In addition to these pathologies, *E. amylovora* also produces an exopolysaccharide capsule to enhance virulence and pathogenicity. *E. amylovora* exopolysaccharides, amylovoran and levan, form a capsule around the cell (Wilson et al., 1970; Geider et al., 1993; Geider, 2000). In the late 1930s experiments found that "ooze" produced by the bacterial cells caused characteristic wilt symptoms in pear shoots (Hildebrand and Heinicke, 1937; Hildebrand, 1939) These EPS form a major component of ooze in diseased tissue and is essential to the pathogenicity of *E. amylovora* as EPS deficient mutants are avirulent (Bennett and Billing, 1978; Bennett, 1980; Geider et al., 1993; Oh and Beer, 2005).

Amylovoran is an heterogeneous, acidic polysaccharide made up of repeating subunits of four galactose molecules linked to a glucuronic acid residue (Bennett and Billing, 1978; Geier and Geider, 1993; Nimtz et al., 1996). The *ams* region of the genome controls the production of this EPS with *rcsA* and *rcsB* genes being required for synthesis (Bugert and Geider, 1995; Bugert et al., 1996; Langlotz et al., 2011; Wang et al., 2012). Mutation in either of the two genes results in a reduction or elimination of amylovoran production and virulence (Bugert et al., 1996; Bereswill and Geider, 1997).

In the presence of sucrose, *E. amylovora* also produces a fructose homopolymer called levan, an important virulence factor (Gross et al., 1992; Belleman and Geider, 1992; Bellemann et al., 1994). This production takes place extracellularly through the action of the enzyme levansucrase, encoded by the *lsc* gene (Gross et al., 1992; Geier and Geider, 1993; Geider et al., 1993). Additionally, levansucrase reduces the high osmotic pressure in nectar by converting sucrose into levan which has a low osmotic pressure (Gross et al., 1992).

EPS play a complex and multifaceted role in the interaction between the pathogen and its environment (Koczan et al., 2009; 2011). In addition to being important to bacterial virulence and pathogenicity (Ayers et al., 1979; Steinberger and Beer, 1988; Leigh and Coplin, 1992; Bogs et al., 1998; Lee et al., 2010; Schollmeyer et al., 2012) the resultant capsular layer may help shield the bacterium from host defenses; (Gross et al., 1992; Geider et al., 1993) may be a vital component of biofilm production; and binds water (Koczan et al., 2011), nutrients and ions to keep them in close contact with the cell (Sutherland, 1972; Goodman et al., 1974; Sutherland, 1988; Bellemann et al., 1994; Hughes et al., 1998b; Schollmeyer et al., 2012). Koczan et al. (2011) demonstrated that in *E. amylovora*, both levan and amylovoran are essential for the formation of the biofilm. Amylovoran deficient cells of *E. amylovora*, did not attach to artificial growth substrate to initiate biofilm production and cells deficient in levan has reduced biofilm production. Additionally, as in most biofilms, the EPS protects the cell from desiccation by keeping the nutrients contained in the highly hydrated EPS close to the cell (Sutherland, 1972; 1988; Whitfield, 1988; Leigh and Coplin, 1992) The EPS may provide protection for

bacterium from plant host defenses by masking cell receptors that generate an immune response (Whitfield, 1988). In *Pseudomonas solanocearum*, the non-virulent bacteria appear to be immobilized by the host cells recognizing components of the bacterial cell surface. The host lectin-lipopolysaccharide (LPS) interaction seems to be inhibited by the production of EPS. These mechanisms of pathogenesis make *E. amylovora* very harmful and therefore it is essential that effective control strategies are developed to combat the spread of fire blight.

2.5 Control Measures

There are many factors that affect the prevalence of disease in the orchard. The spread of the disease is affected by climate, geography, and weather patterns. Additionally, the overall health of an orchard and the host susceptibility also affects the spread of *E. amylovora.* Fire blight is a good example of a disease that should be controlled by an integrated pest management (IPM) system. Once the pathogen becomes established in an orchard it is very difficult to eradicate without destroying the entire orchard. In the IPM system, growers use a combination of disease forecasting models, good orchard management, chemical control, and biological control agents.

The optimum growing temperature for *E. amylovora* is 21 - 28° C (Schroth et al., 1974; van der Zwet and Keil, 1979). Two computer programs, *MARYBLYT* and *Cougarblight*, use environmental parameters such as temperature, moisture, and blossom age to predict when optimum conditions are occurring for the spread of disease and there is a high risk of *E. amylovora* infection (Johnson et al., 2004).

Sound orchard management can be achieved by good orchard site and variety selection, managing tree vigor, and removal of diseased tissue. Prevention of fire blight should be considered even before an orchard is planted. The planned orchard should be on a site that has good drainage and light, balanced soil. Poor drainage and soil quality make the trees much more susceptible to fire blight (van der Zwet and Keil, 1979; van der Zwet and Beer, 1995; Toselli et al., 2002; OMAFRA, 2011). Additionally, new orchards should be planted with varieties that have resistant rootstocks and scions, if available, and the grower should expect to prioritize the surveillance and control of fire blight (Quamme et al., 1990; van der Zwet and Beer, 1995; Calenge et al., 2005; Russo et al., 2007; 2008).

Dormant pruning can be very a effective means of controlling infection and maximizing fruiting capacity (van der Zwet and Keil, 1979; OMAFRA, 2011). There is also a direct correlation between tree vigour and pathogen susceptibility (van der Zwet and Beer, 1995; Blachinsky et al., 2006). Tree vigour should be managed through timely pruning, minimal nitrogen application, and application of growth regulators to minimize the physiological changes that would make the trees more susceptible to infection (Costa et al., 2001; Spinelli et al., 2007; Spinelli et al., 2007). The other key to disease control method is chemical treatment to reduce the prevalence of the pathogen in the orchard.

2.6 Chemical Control of Fire Blight

Initially attempts were made to control fire blight using chemicals used for fungal diseases, namely lead arsenate and lime sulfur. Subsequently, copper compounds were

shown to be effective for control; however they reducing produce value due to fruit russeting (Van der Zwet & Keil, 1979). Until the late 1940s, and the discovery of antibiotics there was no safe, reliable method of chemical control. Of the near 40 antibiotics tested for treatment of plant diseases in the 1950s, only streptomycin and oxytetracylcine proved to be highly effective against blossom blight (Loper and Henkels, 1991). Streptomycin is an aminoglycoside antibiotic that binds to the 30S ribosomal subunit causing miscoding, translational errors, and inhibiting protein biosynthesis (Pestka, 1971; Loper and Henkels, 1991). Streptomycin effectively controls populations of *E. amylovora* during bloom and prevention of trauma blight following severe weather (Vidaver, 2002). Streptomycin is ineffective once the bacterium enters the tissue and travels intracellularly, causing symptoms of shoot blight and canker blight (Vidaver, 2002).

Streptomycin will remain a viable treatment option for as long as there is bacterial susceptibility. There have been reports of *Erwinia* streptomycin resistance in many areas including the United States, Canada, New Zealand, Lebanon and Israel (Johnson and Stockwell, 1998; Russo et al., 2008). Additionally the European Union has banned the use of streptomycin due to concerns about resistance and development of clinical resistance to antibiotics (Rezzonico et al., 2008; Czajkowski et al., 2011). In the US, the more costly, less effective antibiotic oxytetracycline has been introduced for control of blossom blight. However, oxytetracycline resistance has already been reported in Mexico (McManus and Jones, 1994; Farkas et al., 2012). The decreased regional effectiveness of antibiotics and potential for development of resistance calls for development of new,

viable control strategies. Two alternatives that have emerged have been lytic bacteriophages and antagonistic bacteria to control *E. amylovora* orchard populations.

2.7 Biologics in Canada

Currently there are a number of biologics registered in Canada for the control of fire blight using bacteria that are antagonistic or competitive against *E. amylovora* (Svircev et al., 2006; Kabaluk et al., 2010). Two are under the commercial name BlightBan®, produced by Nufarm Agricultural Inc. BlightBan A506 is a product containing *Pseudomonas fluorescens* A506 and the second, BlightBan C9-1, contains *Pantoea agglomerans* C9-1 (Lindow and Wilson, 1993). Bloomtime Biological FD (Northwest Agricultural Products) uses *P. agglomerans* strain E325. Serenade® MAX, is a lyophilized fermentation culture of *Bacillius subtilis* QST 713, produced by AgraQuest (Kabaluk et al., 2010). The applied bacteria establish on the stigmatic surface of the blossom, preventing pathogenic organisms (particularly *E. amylovora*) from flourishing. The applied bacteria take up the available nutrient resources, thus excluding *E. amylovora* from the ecological niche on the stigma. Inadvertently the ability of the bacteria to reproduce is affected, the low population numbers results in the inability to cause disease. In addition, *P. agglomerans* suppresses growth of *E. amylovora* through the production of a diverse range of antibiotics (Ishimaru et al., 1988; Vanneste et al., 1992; Kearns and Mahanty, 1998; Jin et al., 2003; Lehman, 2008; Holtz et al., 2008). The efficacy of these bacterial antagonists is good under optimal conditions (warm, wet and humid weather), but with more variability than streptomycin (Johnson and Stockwell,

1998; Holtz et al., 2008). Another promising biological treatment for fire blight is the use of bacteriophages.

2.8 Bacteriophage Life Cycle

Bacteriophages are viruses that specifically infect bacteria (Adams, 1959; Ackermann, 2001). Viruses are non-living particles of genetic information surrounded by a protein coat. A virus, as an obligate parasite, is dependent on its host cell to reproduce as it lacks the ability to carry out any biochemical functions in and of itself. Phages inject single or double stranded RNA or DNA genetic information into the host cell, effectively taking it over and using the cellular organelles to reproduce its genetic information and associated proteins. The phage rapidly transforms the cell's metabolism, replicates the virus many times, packaging the particles into new phages. Finally phage proteins interact with the host cell membrane and compromise the cell wall causing cell lysis (Lech and Brent, 1987; Young and Wang, 2006; Anderson and Yang, 2008). Each of the new phages can interact with a new host cell continuing this cycle. When bacteriophage use only this cycle, it is considered an obligately lytic phage.

In the second type of life cycle, phages become integrated into the host genome. Temperate phages have a much more complex life cycle than obligately lytic phages. Temperate phages inject genetic material; however, the massive replication of phage particles and host cell lysis does not take place due to the rapid expression of a phage-encoded repressor. Subsequently the phage genome is incorporated into the host cell genome and is called a "prophage" and the host a "lysogen". The phage genome replicates along with the host genome and the genetic information is passed to each

daughter cell. The prophage, either as a plasmid or inserted into the host genome, may mediate rearrangements of the bacterial chromosome (Brüssow and Hendrix, 2002; Canchaya et al., 2004) or contribute to interstrain genetic variability via horizontal gene transfer (Canchaya et al., 2004).

Lysogeny typically results in the host being resistant to infection by other homologous phages because the incorporated genes provide immunity (Anderson and Yang, 2008). Mutant phages that are homologous to the temperate phage and are able to bypass the resistance and infect the lysogen are called *vir* mutants (for *vir*ulence) (Luria, 1945; Lenski and Levin, 1985; Jackson, 1989; Flaherty et al., 2001; Gill and Abedon, 2003). The advantage of *vir* mutants is that they can be used in concert with the potentially temperate phage and infects the lysogenic hosts when they appear (Gill and Abedon, 2003; Little, 2005). The lysogenic state continues until environmental factors (e.g., a strong mutagen) trigger the excision of the viral DNA and the resumption of the lytic cycle (Little, 2005). These mechanisms are poorly understood, but Sato (1983) described the phage as "abandoning ship" and resuming the lytic cycle rather than risk surviving in a dying cell. At times the excision process is not accurate and bacterial genes may be included in the phage genome, or bacteriophage DNA may become part of the host genome in a process called lysogenic conversion (Lech and Brent, 1987; Little, 2005). In this conversion it is possible for the host to acquire virulence genes from the temperate phage or *vice versa*. The presence of temperate phages or prophages is not desirable in a biocontrol program. Biopesticides should be formulated using exclusively lytic phages to reduce resistance and prevent transmission of bacterial genes.

2.9 Bacteriophages as Biopesticides

Bacteriophages are being developed as biopesticides for a variety of bacterial plant diseases (Obradovic et al., 2004; Svircev et al., 2006; Jones et al., 2007; Ravensdale et al., 2007; Balogh et al., 2008; Fujiwara et al., 2011; Czajkowski et al., 2011; Svircev et al., 2011; Nagy et al., 2012). Civerolo and Keil (1969) reported that bacteriophage treatment of *Xanthomaonus pruni* was able to reduce the incidence of infected leaves of pre-inoculated peach seedlings 6 to 8% and reduced disease 17 to 31% compared to water controls. Phages have been tested recently for the control of bacterial spot of tomato (Flaherty et al., 2000). There is evidence that that phage therapy may be effective in the control of the potato disease scabies, caused by *Streptomyces scabies*, to significantly disinfect and reduce the infection of seed tubers (McKenna et al., 2001). OmniLytics, Inc (Salt Lake City, UT) has registered a phage for the control of *X. campestis* pv. *vesicatoria* (Balogh et al., 2003). This registration should make the regulatory passage of other phage containing products easier in the future (Svircev et al., 2010). Additionally, Saccardi et al. (2005) reduced the incidence of leaf spot on peaches with a bi-weekly application of phage against *X. campestris* pv. *pruni.* The bacterial soft rot pathogen of calla lilies and potatoes *Erwinia carotovora* subsp. *carotovora* (syn. *Pectobacterium carotovorum* subsp. *carotovorum*) has been successfully controlled using bacteriophage (Ravensdale et al., 2007; Czajkowski et al., 2011). Fourteen phage isolates from greenhouse fertilizer solutions and diseased calla tubers were tested on plugs of calla tubers and were found to significantly reduce the incidence of soft rot symptoms up to 50% and bacterial numbers by up to 99% (Ravensdale et al., 2007).

Recent trials on phage based biopesticides have been effective in reducing *E. amylovora* populations (Lehman, 2008; Svircev et al., 2011; Muller et al., 2011; Boulé et al., 2011; Born et al., 2011). Lehman (2008) showed that phage applied with a non-pathogenic carrier were able to reduce pathogen levels on the blossom significantly over the water controls. In British Columbia infection of detached pear blossoms was reduced by 84 to 96% and infection on flowers of potted apple trees was reduced by 56% (Boulé et al., 2011). Muller et al. (2011) also showed a reduction of pathogen populations on blossoms using bacteriophages.

The most effective phage biocontrol for *E. amylovora* would be to use exclusively lytic phages and maintain high replicating populations of phages on the surface of blossom. Cell lysis is not guaranteed with phages that are temperate and temperate phages may transfer resistance if they excise resistance genes from the host cell genome during the resumption of the lytic cycle. Lysis ensures the destruction of the cell and death of the pathogen, preventing infection of the host plant. To maintain high populations the phage must be protected from heat, desiccation, pH extremes, copper based pesticides, or physical removal by rain, but the most significant factor in the inactivation of phage is UVA and UVB radiation (280-400nm) (Ignoffo and Garcia, 1992; 1994; Ignoffo et al., 1997; Iriarte et al., 2007; Li and Dennehy, 2011; Fisher et al., 2011; Misstear and Gill, 2012). This protection could be accomplished by inoculating *Pantoea agglomerans* with phages and using it as a carrier (Lehman, 2008). *P. agglomerans* is a common, non-pathogenic epiphyte that naturally competes with *E. amylovora* and inhibits its growth through the production of acidic metabolites and antibiotics. Some researchers (Wiggins and Alexander, 1985; Payne and Jansen, 2001)

have reported that a proliferation threshold (minimal bacterial density of ~1 x 10^4 CFU/ml) is required to have sufficient numbers of bacteria for the phage to replicate. The use of a non-pathogenic carrier could increase the density of susceptible host above the threshold. Lehman (2008) showed that using *P. agglomerans* Eh21-5 as a phage carrier was effective at maintaining phage populations, additionally, the phage preferentially infected the pathogenic host over the carrier. If viable phages are present on the blossom when *E. amylovora* arrives, the phage could effectively lyse and kill sufficient numbers of bacteria to maintain the density of pathogenic bacteria below the disease threshold to cause disease. (Lehman, 2008; Nagy et al., 2012). This carrier could protect the phage from harm and provide a reservoir of competent phage particles (Cairns and Payne, 2008).

A daunting hurdle in the process of commercializing any product is gaining regulatory approval through the Pest Management Regulatory Agency in Canada. One of the most important steps in the approval of a treatment is the value and efficacy (Kabaluk et al., 2010). The potential new treatment must be shown to be as efficacious as the conventional control methods from greenhouse or field data. Included in these data would be information on the overall efficacy and evidence to prove or disprove resistance (Kabaluk et al., 2010). Biological treatments have been plagued with a reputation of inconsistent control from season to season for many years (Vidaver, 1976; Jones et al., 2007). To ensure that bacterial host resistance does not adversely impact phage biological control process, research has been designed to study the resistance mechanisms for bacteria-phage interactions.

2.10 Bacteriophage-Host Resistance

Bacteria use several mechanisms to prevent phages from successfully multiplying (Labrie et al., 2010). The mechanisms include the prevention of phage adsorption; inhibition of phage DNA injection, transcription, and replication; and interference with phage protein synthesis, virion assembly, and bacterial cell lysis (Lenski, 1988; Bohannan and Lenski, 2000; Labrie et al., 2010).

Bacteria may become resistant to phage attack through modification of the phage surface receptors. To avoid this, a cocktail of bacteriophages would be used. The use of two (Coffey et al., 2011), three (Hooton et al., 2011; Fujiwara et al., 2011; Turki et al., 2012), four, (Flaherty et al., 2000; Flaherty et al., 2001; Balogh et al., 2003; Ravensdale et al., 2007; Balogh et al., 2008) and five or more (Harbaugh et al., 1998; Leverentz et al., 2003; Boulé et al., 2011) phage mixtures results in increased efficacy and reduces chances of developing host resistance. Different phages, utilizing different receptor proteins would limit the probability that the bacterium could acquire mutations to become resistant. To further improve phage efficiencies, phage biopesticides will be integrated into existing IPM programs (Jones et al., 2007). Chemical activators that stimulate systematic acquired resistance (SAR) have been tested with applications of phages in the control of *Xanthomonas axonopodis* pv. *allii* (Lang et al., 2007) and bacterial blight of tomato (Balogh et al., 2003) with increased treatment efficacy (Obradovic et al., 2004; Obradovic et al., 2005). Acibenzolar-S-methyl (ASM) mimics salicylic acid in apples and pears to stimulate the SAR pathway and provides additional protection to the plant from *E. amylovora* (Ward et al., 1991; Maxson and Jones. A.L., 2002). There may be other

potential adjuvants that could be used to maximize the efficacy of bacteriophages in the treatment of fire blight.

2.11 Bacterial EPS and Bacteriophages

The bacterial host has the ability to modify the components of the cell surface to become resistant to phage attack (Labrie et al., 2010). In Gram positive bacteria, phage receptors are usually cell-surface carbohydrates such as galactose or rhamnose (Forde and Fitzgerald, 1999; Quiberoni et al., 2004). Removal or modification of the sugars that the host cell produces would render the bacterium resistant to bacteriophage attack.

EPS have been reported to block or delay bacteriophage adsorption in several bacterial species (Forde and Fitzgerald, 1999; Looijesteijn et al., 2001; Deveau et al., 2002). In *Lactococcus* spp, phage resistance has been observed due to EPS masking the cell surface receptors and causing a decrease in phage adsorption (Forde and Fitzgerald, 1999; Looijesteijn et al., 2001). Similar observations have been made for *Escherichia*, *Staphylococcus*, *Streptococcus*, and *Rhizobacterium* species (Wu and Park, 1971; Defives et al., 1996; Scholl et al., 2005). Other species, however, have evolved EPS that make them more susceptible to adsorption and infection.

Phages binding to the EPS has been observed in a number of bacterial genus including *Escherichia, Klebsiella, Enterobacter,* and *Psuedomonas* (Park, 1956; Lindberg, 1973; Lindberg, 1977; Bayer et al., 1979; Stummeyer et al., 2006). In the dairy industry the production of EPS products is vital for the thickening of milk products (Brüssow et al., 1994). Cerning (1990) has reported bacteriophage active on EPS producing strains but the non-slime forming mutants are resistant to phage attack

(Quiberoni et al., 2004). *Streptococcus thermophilus* is used in both the cheese and yogurt industries with nonropy, non-EPS producing and ropy, EPS producing strains used in each industry respectively. Brüssow et al. (1994) found that there were different phages isolated from each of the ropy and nonropy host groups had a host range that was confined to the group from which they were isolated. It appears that the phages have evolved to specifically infect either the EPS producing or EPS free *S. thermophilus* hosts. Other researchers (Forde and Fitzgerald, 1999; Looijesteijn et al., 2001; Deveau et al., 2002; Broadbent et al., 2003; Lévesque et al., 2005; Rodríguez et al., 2008) have isolated phage that are specifically adapted to EPS-producing *S. thermophilus* and EPS-producing lactoccocal strains. EPS production, often influenced by environmental factors, may contain components that renders the *E. amylovora* susceptible to bacteriophage attack (Hughes et al., 1998a; Hughes et al., 1998b).

Differences in phage populations when grown on two different host isolates can be simply displayed by determining the efficiency of plating (EOP). The titre of the phage enriched on an experimental host isolate is divided by the phage titre when grown on a reference host. EOPs greater than one (EOP>1) signifies that the phage "prefers" and can grow to higher titres on the experimental host than the reference host. Conversely, an EOP less than one (EOP<1) indicates that the phage is more suited to reference host. Using EOPs, phage-host enrichment efficiencies can be compared between multiple phages and various hosts. In this thesis the reference isolate is the wild-type isolate of the *E. amylovora* host on which a given phage was first isolated.

The Vineland collection contains more than 50 *Erwinia amylovora* and *Pantoea agglomerans* phages that belong to the *Myo-*, *Sipho-*, and *Podoviridae* families (Gill et

al., 2003). Modification of the cell surface receptors is one process that could render a bacterium resistant to bacteriophage attack especially when the bacterium is exposed to high selection pressures that are present in the application of a phage biopesticide (Vidaver, 1976; Jones et al., 2007; Labrie et al., 2010). The EPS produced by *E. amylovora* may be an anti-phage defense mechanism or it could act as an important modulator of phage infection.

This study describes the role of *E. amylovora* EPS in the infectivity of phages from the *Myo-*, *Sipho-*, and *Podoviridae*. The role of the EPS was investigated through phage-bacterial host interactions under standard, sugar enriched and EPS deficient conditions. Additionally, EPS carbohydrate monomers were used as receptor mimetics to further characterize the role of EPS in phage adsorption.

Chapter 3
Materials and Methods

3.1 Phage Isolates

All phage isolates are listed in Table 1. High titer phage stocks were stored in 0.8% nutrient broth (NB; BD-Difco, Sparks, MD) in amber glass vials at 4°C unless otherwise stated.

3.2 Phage Growth Conditions and Media

To obtain high titer phage stocks, phages were grown in liquid culture (Gill, 2000; Lehman, 2008). The agar layer method (Adams, 1959) was used to determine phage titres. Unless indicated otherwise, the phages were enriched on the ideal host listed in Table 1.

In the agar layer method, a phage isolate was mixed with bacteria suspended in 0.01M phosphate buffer (PB; pH 6.8) and adjusted to an OD_{600} of 0.6 (~1.0×10^9 CFU/ml) using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland). One hundred microlitres of bacterial suspension was mixed with 100 µl of phage lysate in a glass test tube and incubated for 15 min. Three millilitres of top agar (cooled to 50°C) was then added to the tube and the contents immediately poured onto a 90 mm Petri dish containing 20 ml of solidified bottom (2.3%) nutrient agar (NA) and swirled to evenly distribute the top agar. The plates were allowed to cool then were inverted and incubated 15 to 20 h at 27°C. Following incubation, 3 ml of NB was added

to the plate and allowed to sit at room temperature for 15 min. The nutrient broth and the top agar were scraped into a 30 ml chloroform resistant FEP centrifuge tube (fluorinated ethylene propylene, Oakridge) and placed on a gyrorotary shaker for 25 min. The top agar and the cell debris were pelleted by centrifugation at 8000 x *g* for 25 min. The supernatant was removed and syringe filtered (0.2 μm; Nalgene, Rochester, NY) into an amber glass vial (Wheaton, Millville, N.J.) and stored at 4°C.

Liquid cultures were prepared by using 50 ml of sterile 8 g/l NB (BD-Difco) in a capped 250 ml flask, inoculated with ~1 x 10^9 CFU of the bacterial host. The culture was then placed in an orbital shaker at 27°C at 120 rpm. After one hour, 100 μl of phage suspension was added to the flask and returned to the orbital shaker for 15 to 20 h. To obtain high titer phage cultures, 1 ml of the overnight culture was inoculated into fresh flask containing actively growing, mid-log phase host bacteria and incubated 15 h in the orbital shaker. Chloroform was added to each flask (1 to 2% v/v) and returned to the shaker for 25 min. The crude lysate was transferred to a chloroform resistant centrifuge tube leaving the chloroform behind and centrifuged at 8000 x *g* for 25 min. The supernatant was syringe filtered into amber glass vials and stored at 4°C.

Erwinia amylovora isolates were grown overnight on nutrient agar (NA; 23g/l, BD-Difco) at 27°C. For long term storage, overnight cultures of bacteria were suspended in 0.5 ml of cryogenic medium (0.8% NB, 0.25% yeast extract (Difco) and 0.5% sucrose, 0.25% K_2HPO_4, 0.05% KH_2PO_4,0.025% $MgSO_4$ and 50% (v/v) glycerol) and stored at

TABLE 1. Bacteria and bacteriophages used in the study

Isolates and plasmids	Description or relevant genotype or phenotype[a,b]	Reference or source
Erwinia amylovora isolates		
Ea110R[c]	Wild type (HEP)	Ritchie and Klos, 1977
Ea110RΔ*rcsB*	*Km*^{r},*rcsB*$^-$	Roach, 2011
Ea110RΔ*lsc*	*Cm*^{r}, *lsc*$^-$	Roach, 2011
Ea29-7	Wild type (HEP)	Gill et al., 2003
Ea29-7Δ*rcsB*	*Km*^{r},*rcsB*$^-$	Roach, 2011
Ea29-7Δ*lsc*	*Cm*^{r}, *lsc*$^-$	Roach, 2011
EaD-7	Wild type (HEP)	Jeng et al., 2001
EaD-7Δ*rcsB*	*Km*^{r}, *rcsB*$^-$	this study
EaD-7Δ*lsc*	*Cm*^{r}, *lsc*$^-$	this study
Ea6-4	Wild type (LEP)	Jeng et al., 2001
Ea6-4Δ*rcsB*	*Km*^{r}, *rcsB*$^-$	Roach, 2011
Ea6-4Δ*lsc*	*Cm*^{r}, *lsc*$^-$	Roach, 2011
Ea6-4Δ*rcsB*Δ*lsc*	*Km*^{r}, *Cm*^{r}, *rcsB*$^-$, *lsc*$^-$	Roach, 2011
Ea17-1-1	Wild type (LEP)	Jeng et al., 2001
Ea17-1-1Δ*rcsB*	*Km*^{r},*rcsB*$^-$	Roach, 2011
Ea17-1-1Δ*lsc*	*Cm*^{r}, *lsc*$^-$	this study
Ea17-1-1Δ*rcsB* Δ*lsc*	*Km*^{r}, *Cm*^{r}, *rcsB*$^-$, *lsc*$^-$	this study
EaG-5	Wild type (LEP)	Jeng et al., 2001
Ea G-5Δ*rcsB*	*Km*^{r},*rcsB*$^-$	this study
EaG-5Δ*lsc*	*Cm*^{r}, *lsc*$^-$	Roach, 2011
Ea G-5Δ*rcsB* Δ*lsc*	*Km*^{r}, *Cm*^{r}, *rcsB*$^-$, *lsc*$^-$	this study

Bacteriophage isolates		
ΦEa10-1	*Myoviridae*	Gill et al., 2003
ΦEa21-2	*Myoviridae*	Gill et al., 2003
ΦEa21-4	*Myoviridae*	Lehman et al., 2009
ΦEa10-16	*Siphoviridae*	Gill et al., 2003
ΦEa35-4	*Siphoviridae*	Gill et al., 2003
ΦEa35-7	*Siphoviridae*	Gill et al., 2003
ΦEa1(h)	*Podoviridae*	Schnabel and Jones, 2001
ΦEa10-6	*Podoviridae*	Gill et al., 2003
ΦEa31-3	*Podoviridae*	Gill et al., 2003
ΦEa35-3	*Podoviridae*	Gill et al., 2003
ΦEa45-1B	*Podoviridae*	Gill et al., 2003
ΦEa46-1A2	*Podoviridae*	Gill et al., 2003
ΦEa50-3	*Podoviridae*	Gill et al., 2003
ΦEa51-7	*Podoviridae*	Gill et al., 2003
Plasmids		
pKD13	Km^r, FRT *kan* FRT PS1 PS2 oriR6 K rgbN	Datsenko and Wanner, 2000
pKD32	Cm^r, FRT *cat* FRT PS1 PS2 oriR6 K rgbN	Datsenko and Wanner, 2000
pKD46	Ap^r, P_{BAD} *γ β exo* pSC101 oriTS	Datsenko and Wanner, 2000

[a] Km^r: kanamycin resistant; Cm^r: chloramphenicol resistant; Ap^r: ampicillin resistant; *rcsB*$^-$: amylovoran deficient; *lsc*$^-$: levan deficient

[b] HEP: High exopolysaccharide producing; LEP: Low exopolysaccharide producing from Roach (2011).

[c] Rifampicin resistant isolate of *E. amylovora* 110.

-80°C. Alternatively, overnight cultures were suspended in Microbank cryogenic vials (Pro-Lab Diagnostics, Richmond Hill, ON) and stored at -80°C. Bacterial isolates were cultured from the freezer stocks onto 2.3% NA and incubated at 27°C for 40 to 48 h. Single colonies were obtained from these NA cultures incubated on NA at 27°C for 16 to 20 h and used for all phage experiments.

To induce higher levels of bacterial EPS nutrient growth medium was supplemented with 0.5% sucrose and 1% sorbitol (NAS) (Wang et al., 2011). The plasmids used in this study are described in Table 1. To select for successful bacterial transformants, NAS was amended with 100 μg/ml of ampicillin, 20 μg/ml of kanamycin or 20 μg/ml of chloramphenicol.

3.3 Phage Titre Determination

Phage lysate stock titres were determined using the agar layer method (Adams, 1959). Ten fold serial dilutions of the lysate stocks (grown in NB on the isolation host) were made in 0.01M PB and 100 μl of each dilution was added to 100 μl of bacterial suspension (OD_{600} = 0.6) in a 10 ml glass test tube. Following incubation for 10 min, 3 ml of molten top agar, cooled to 50°C, was added to each test tube and the contents of the contents immediately poured onto a 90 mm Petri dish containing 20 ml of solidified NA. The molten agar was swirled to an even distribution over the NA. The plates were allowed to cool then were inverted and incubated 15 to 20 h at 27°C. Following incubation, the phage titre of the stock solution was calculated multiplying the number of plaques by the dilution factor and expressing the titre as plaque forming units per

milliliter (PFU/ml). As a general rule only dilutions that contained 50 to 400 plaques were counted.

Spot lysis technique was used for a quick titre determination. To a bacterial suspension (100 µl of 10^8 CFU/ml), 3 ml of molten top agar was added and immediately poured onto a 90 mm Petri dish containing 20 ml of solid NA. Once the top agar solidified, 10 µl of phage solution was dropped onto the top agar, allowed to dry and incubated overnight at 27°C. The spots containing discreet plaques were used to estimate the phage titre by calculating plaque forming units (PFU) per millilitre.

3.4 Generation of Bacterial Exopolysaccharide Deficient Mutants

Bacterial mutants were generated using a modified one-step PCR deletion method (Datsenko and Wanner, 2000; Zhao et al., 2009). In this method, levan and amylovoran deficient mutants were generated by constructing gene knockouts for the levansucrase (*lsc*) gene and the regulator of capsular synthesis B (*rcsB*) gene. A complete list of plasmids (obtained from the Coli Genetics Stock Center, Yale University) and primers are given in Table 1 and 2, respectively. Each PCR contained final concentrations of 200 nM each primer, 1X polymerase buffer, 2.5 U *Taq* polymerase (NEB, Ipswich, MA), 300 µM of each dNTP (NEB), 3 mM $MgCl_2$ and 2 µl of DNA template. Reactions were run in a Eppendorf Vapo.protect thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: 95°C for 5 min; and 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s (Roach, 2011). Amplification products were purified using a gel-purification kit (Norgen Biotek, St. Catharines, ON). For mutant production, linear recombination constructs consisting of either a kanamycin (Km^r) or chloramphenicol

(*Cm*^{r}) resistance gene flanked by 50-nucleotide (nt) homology arms targeting either the levansucrase gene or regulation of capsule synthesis B gene were created by PCR. The *Km*^{r} gene was amplified from pKD13 with the *rcsB*-Km primers and the *Cm*^{r} gene from pKD32 with the *lsc*-Cm primers. *E. amylovora* cells were made electrocompetent by washing three times in 35 ml ice-cold, sterile H_2O and stored in 10% glycerol at -80°C. This process also removed the EPS from around the cells. Electrocompetent cells were thawed on ice then transformed with plasmid pKD46 by electroporation followed by selection for ampicillin resistance. The transformants harboring pKD46 were grown overnight on NAS containing 100 μg/ml of ampicillin at 27°C, re-inoculated in NA broth containing 0.1% arabinose, grown to exponential phase (OD_{600} = 0.8) and made electrocompetent again. Cells were then transformed with a recombination construct by electroporation and recovered in one millilitre of SOC (20 g/l tryptone, 5 g/l yeast extract, 8.56mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 20mM glucose) medium (Sambrook and Russel, 2001) for 1 to 4 h at 27°C before being plated on Luria-Bertani agar (LB, 10 g/l, BD-Difco, Sparks, MD) or NAS with appropriate selective antibiotic. Electroporation was performed at 2.5 kV and 25 uF (Gene pulser, BIO-RAD, Hercules, CA) with the pulse controller set at 200 Ω. Electroporation creates holes in the cell membrance of the bacterium allowing PCR product to enter the cell. Following entry the pKD46 gene products incorporate the antibiotic resistance cassette into the bacterial genome. Recombinants were selected on NAS containing kanamycin or chloramphenicol. In the resulting mutants, the *Cm*^{r} or *Km*^{r} insert replaced part of the coding region of the target gene by site-specific recombination in the flanking region (Fig. 1) (Datsenko and Wanner, 2000; Zhao et al., 2009). In an attempt to recover the double knockouts of the

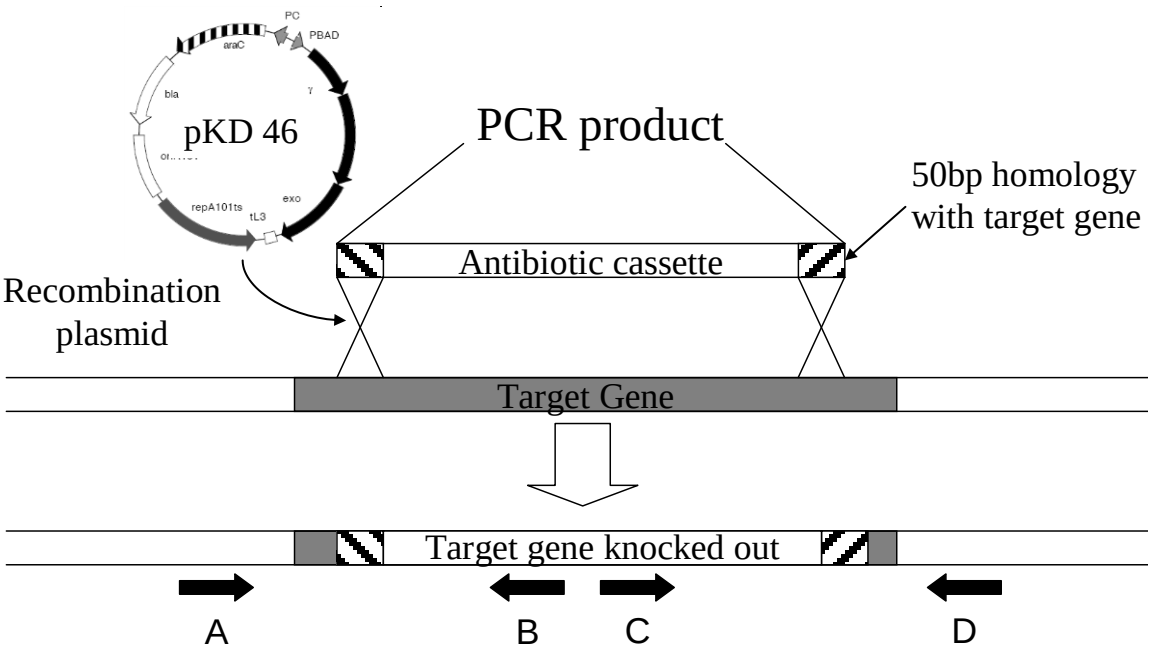

FIG. 1. Schematic of protocol used to generate gene deletions by homologous recombination with an antibiotic resistance gene in *E. amylovora* [adapted from Roach (2011)].

high EPS producing (HEP) *E. amylovora,* a number of modifications were made to the knockout protocol to maintain metabolic and solute pressures that would not be lethal to the double knockout HEP mutant. Initially the SOC was substituted for NB amended with 0.5% sucrose and 1% sorbitol to more appropriately meet the needs of *E. amylovora*. SOC medium was designed for growth of *E. coli* which has different growth requirements than *E. amylovora.* In addition, the antibiotic containing medium was also supplemented with 0.5% sucrose and 1% sorbitol or 0.5% fructose, 1% galactose, and 0.25% glucuronic acid. This modification allowed the occasional appearance of a white bacterial colony after 48 to 72 h of incubation at 27°C, however subcultures of that colony would not persist on osmotically stabilized antibiotic or antibiotic free medium. To attempt to eliminate the desiccation pressures of growing on solid medium, 100 µl of recovered, electorporated cell suspention was added to 50 ml of NB or NB amended with sucrose, sorbitol, fructose, galactose or glucuronic acid as listed above. The cultures were placed on an orbital shaker at 120 rpm overnight and assessed for turbidity the next day. Finally, concentrations of antibiotics in the nutrient agar were reduced and transformed cells were plated on the reduced antibiotic. Bacterial colonies that appeared after 48 h were subcultured to plates containing a higher concentration of antibiotic.

3.4.1 Amylovoran Production Measurement

E. amylovora amylovoran production was measured using a spectrophotometer at 600 nm. A 24 h liquid culture of *E. amylovora* was centrifuged at 5000 x *g* for 5 min and the cell pellet discarded. To 1 ml of supernatant, 50 µl of cetylpyridinium chloride (CPC, 50 mg/ml) was added. After 10 min incubation at room temperature, the precipitated the

amylovoran was quantified using a spectrophotometer by measuring the turbidity at 600 nm (Bellemann et al., 1994).

3.5 *In planta* Bacterial Virulence Assay

Pear blossom bioassay was used to evaluate the virulence of the wild type (wt) and EPS deficient bacterial isolates. Dormant pear shoots (Bartlett) were harvested in January and February, the budwood was bundled, loosely wrapped in plastic and stored at 4°C. Shoots bearing 5 to 15 flower buds were chosen and 5 cm was cut off the bottom of each shoot. The shoots were placed them in a plastic 20 liter pails with 10 to 15 cm of tap water and stored at room temperature for 8 days. Daily the branches were inspected, the bottom 2 to 5 cm trimmed and the water changed to prevent bacterial growth. Sterile glass scintillation vials were filled with water and a cap with a hole drilled in the center was loosely threaded. Newly opened blossoms, with pink anthers, were individually collected and placed into vials so that the flower pedicel extended through cap of each vial and into the water. Vials were held in plastic racks and loosely sealed into large plastic bins flooded with 1 l of tap water to maintain high levels of humidity. The newly open blossoms were stored at 4°C until a sufficient number of blossoms had been collected (Fig. 2).

Blossoms and vials were stored at 4°C for a period up to 1 to 4 days. Bacterial suspensions were prepared to a concentration of 1 x 10^9 CFU/ml (OD_{600} = 0.6) in phosphate buffer from overnight cultures plated on NA. Fully opened blossoms were inoculated with 10 µl of each of the bacterial isolates, both wild type and mutant strains. Two controls completed with each 10 µl of PB and 10 µl of sterile reverse osmosis water. Each treatment block consisted of 5 blossoms. The experiment was replicated three times

TABLE 2. Primers used in the study for the generation of PCR fragments used in production of EPS deficient mutants and the confirmation of EPS deficient mutants.

Primer	Sequence (5'-3')[a]	Function
lsc-CmF	ttatgtcagattataattataaaccaacgctgtggactcgtgcc gatgca**GTGTAGGCTGGAGCTGCTTC**	Generation of recombination fragment for *lsc* knockout
lsc-CmR	actcactgcctgacaggtctttggccacagccagaccaacac agcctgcc**ATTCCGGGGATCCGTCGACC**	
rcsB-KmF	tgaataatctgaatgtcattattgccgacgaccatcctattgttc tgttc**GCGATTGTGTAGGCTGGAGCT**	Generation of recombination fragment for *rscB* knockout
rcsB-KmR	atctaccggcgtcatgcttactgatgacaggtagttaagcaa gcaatat**ATTCCGGGGATCCGTCGACC**	
lsc-ChF	CTGCAGCGATCATGGTTATT	Confirmation of *lsc* deletion
lsc-ChR	ACCGCCAATGCGATAG	
rcsB-ChF	CGGCAAGCAGTTATGTG	Confirmation of *rcsB* deletion
rcsB-ChR	TAAGAAAGAGCCGGGAAGCGCTAA	
Cm-F	TTATACGCAAGGCGACAAGG	Confirmation of Cm^r insertion
Cm-R	GATCTTCCGTCACAGGTAGG	
Km-F	CAGTCATAGCCGAATAGCCT	Confirmation of Km^r insertion
Km-R	CGGTGCCCTGAATGAACTGC	

[a] Bold, uppercase: sequence homologous to antibiotic resistance gene, lowercase: sequence homologous to gene of interest

with independently prepared bacterial cultures. The inoculated blossoms were held in bins in a warm room (22 to 26°C) for 4 days and the virulence of each isolate was evaluated using a disease severity index rating (0 = 0%, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, 5 = 100%) based on the total progression of necrosis from the hypanthium to the ovary and pedicel (Fig. 3) (Gill, 2000; Lehman, 2008).

3.5.1 Statistical Analysis

Results were analyzed in SAS (Statistical Analysis Systems 9.2; SAS Institute Inc., Cary, NC) using the general linear model (PROC GLM). The disease severity of each blossom was reported as 0 to 100% disease and the difference of disease severity between the treatments was analyzed using a one-sided Dunnett's t-test for comparison to the PB control (Dunnett, 1955; Lehman, 2008).

3.6 Bacteriophage Pre-incubation with Sugars

To determine the specific effects of the sugars that constitute levan and amylovoran, the phage was preincubated with sugars prior to the addition of the host bacteria. One hundred microlitres of a phage of interest was added to a test tube containing a predetermined concentration of fructose, arabinose or a 4 to 1 ratio of galactose to glucuronic acid. The phage-sugar mixture was incubated at room temperature from 10 min, followed by addition of 100 µl of host bacteria at OD_{600}=0.6 and incubated at room temperature for 10 min. Three millilitres of molten top agar was cooled to 50°C and added to the contents of the tube. The solution was immediately poured onto a 90 mm Petri dish containing 20 ml of solidified NA. The top agar was

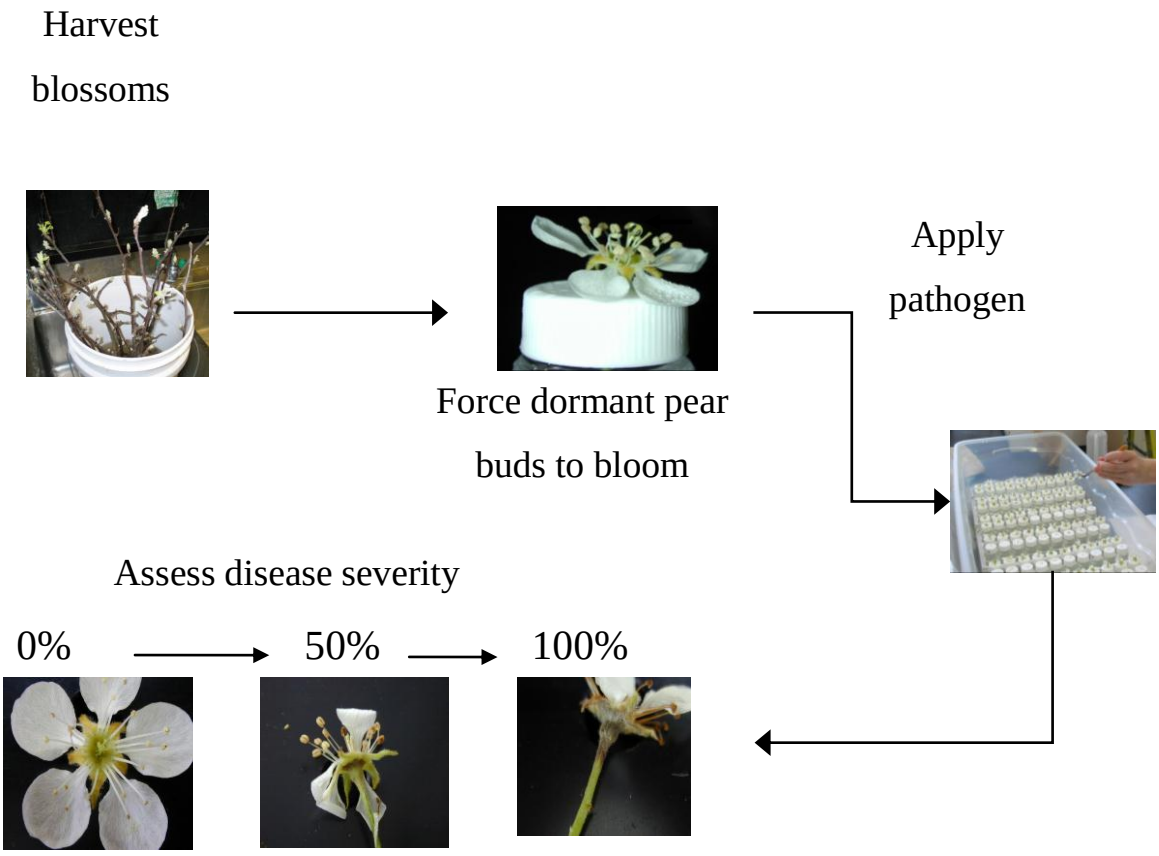

FIG. 2. Blossom assay protocol for the pear blossom bioassay. Bartlett pear blossoms, harvested in late winter, were forced to bloom in water placed in pails, as the blossoms opened they were individually removed and placed in a vial filled with water. 10µl of pathogen was added to each blossom and disease severity assessed at 96 h [adapted from Lehman (2008)].

swirled to evenly distribute the agar, allowed to cool and incubated inverted at 27°C for 15 h. The following day PFU/ml was determined for each treatment.

FIG. 3. Disease severity index for infection of pear blossoms by *Erwinia amylovora*. Integer values are based on the vertical progression of necrosis through the blossom head to the peduncle. The half-scale ratings reflect furthest vertical progress of necrosis, minus half a point if the necrosis only extends half way around the nectary [Adapted from (Pusey, 1997)].

Chapter 4
Results

4.1 Recovery of EPS deficient mutants

Amylovoran (Δ*rcsB*) and levan (Δ*lsc*) deficient mutants were obtained for *E. amylovora* isolates Ea110R, Ea29-7, EaD-7, Ea6-4, Ea17-1-1, and EaG-5. After numerous attempts only amylovoran-levan double knockouts (Δ*rcsB*Δ*lsc*) of the low EPS producing (LEP) *E. amylovora* isolates Ea6-4, Ea17-1-1, and EaG-5 could be recovered. Modifications to the knockout protocol did not produce a persistent double knockout for any of the HEP *E. amylovora* isolates Ea110R, Ea29-7, and EaD-7.

4.1.1 *E. amylovora* EPS production

The production of amylovoran was measured for *E. amylovora* under supplemented and unsupplemented medium conditions. *E. amylovora* isolates Ea110R, Ea29-7, and EaD-7 produced high levels of amylovoran compared to Ea6-4, Ea17-1-1, and EaG-5 (Fig. 4). NA with 0.5% sucrose and 1% sorbitol increased amylovoran production for all *E. amylovora* isolates except Ea6-4. All *E. amylovora* Δ*rcsB* and Δ*rcsB*Δ*lsc* mutants did not produce amylovoran.The amylovoran production Δ*lsc* mutants of Ea110R, Ea6-4, Ea17-1-1, and EaG-5 was increased over the wild-type. From these data Ea110R, Ea29-7, and EaD-7 were grouped

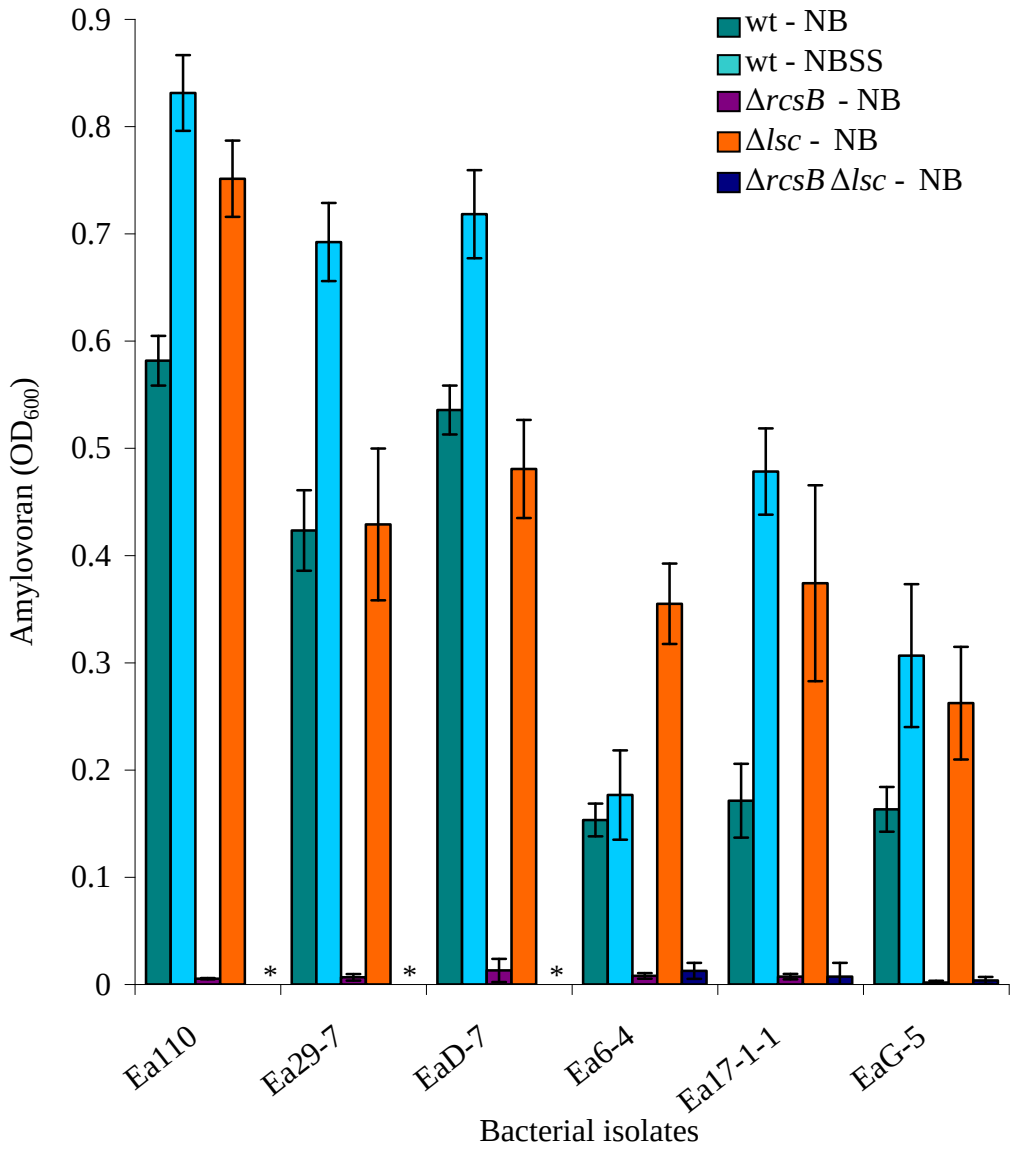

FIG. 4. Amylovoran synthesis measured by OD_{600} after adding cetylpyridinium chloride to culture supernatant of *Erwinia amylovora* wild type and deletion mutants. The amylovoran levels were assessed for six wild-type isolates grown in NB alone and NB with sucrose and sorbitol. The Δ*rcsB,* Δ*lsc*, and Δ*rcsB*Δ*lsc* mutants were grown only in NB. The error bars represent the standard deviation of triplicate experiments.

* denotes knockout strains not available for testing.

as being high EPS producing (HEP) and Ea6-4, Ea17-1-1, and EaG-5 as low EPS producing (LEP) isolates.

4.1.2 Virulence and Pathogenicity of Erwinia amylovora and the Influence of Exopolysaccarides

An *in planta* blossom assay evaluated the pathogenicity of the *E. amylovora* wild type (control) and amylovoran, levan or double knockout mutants (Fig. 5). All six of the wild type *E. amylovora* isolates and all of the Δ*lsc* mutant strains caused necrosis in blossoms. Differences in disease severity (virulence) were apparent in the different bacterial isolates. The LEP *E. amylovora* isolates Ea17-1-1 and EaG-5 caused less severe necrotic symptoms than the HEP *E. amylovora* and Ea6-4. The levan deficient strains of Ea6-4 and EaG-5 had noticeable reductions in disease compared to the wild type isolates. All amylovoran deficient strains (Δ*rcsB* and Δ*rcsB*Δ*lsc* mutant) had statistically significant reductions in disease symptoms ($P<0.05$) compared to both the wild type and levan deficient strains. The disease severity of the amylovoran deficient mutants was not significantly different from the buffer control ($P>0.05$).

4.2 Effect of Increased EPS Production on Phage Proliferation

Phage representatives from the *Myo-*, *Podo-*, and *Siphoviridae* families were grown on their *E. amylovora* hosts. *Myoviridae* phage ΦEa21-4 (Fig. 6) grew equally well on the HEP and LEP hosts on both NA and NAS media. As an exception however, growth on Ea110R was reduced 10-fold on both the NA and NAS compared to growth on the other hosts. *E. amylovora* grown on NAS increased phage titres two to three times on

LEP hosts, but resulted in insignificant changes on the HEP hosts.

Siphoviridae phage ΦEa35-7 (Fig. 7) produced high titres on HEP hosts grown on NA with a 10- to 100-fold reduction on the LEP hosts. When the bacterial growth medium was supplemented, titres increased in four of five hosts. Bacterial isolate Ea17-1-1 was the exception with no change occurring on the non- and supplemented medium. The HEP hosts had a 10-fold increase in phage titre when grown on sugar supplemented medium. Supplemented with sugar, the titre of ΦEa35-7 increased 100-fold, when grown on LEP hosts Ea6-4 and EaG-5. This is consistent with the observation that this phage prefers hosts that produce large amounts of EPS.

Podoviridae phage ΦEa1(h) had a low titre on all hosts when compared to the phages ΦEa21-4 and ΦEa35-7 (Fig. 8). When grown on NA, the phage proliferated to the highest titre on Ea110R and there was a 10- to 100-fold reduction in the titre on the other *E. amylovora* hosts (Fig. 8). The addition of sucrose and sorbitol to the growth medium had a very strong effect and increased phage titre on all host with the greatest increase with HEP *E. amylovora* Ea29-7 and the LEP *E. amylovora*.

Host modification of a phage could affect growth characteristics and phage titres in different, subsequent hosts. To ensure that previous enrichment of a phage was not affecting current results, four phages were grown in each Ea110R (HEP grouping) and Ea6-4 (LEP grouping) to determine if plaque morphology would change by host modification. Plaque appearance produced by progeny was examined on Ea110R, Ea29-7 EaD-7, Ea6-4, Ea17-1-1, and Ea G-5. The plaque morphologies on either host type were

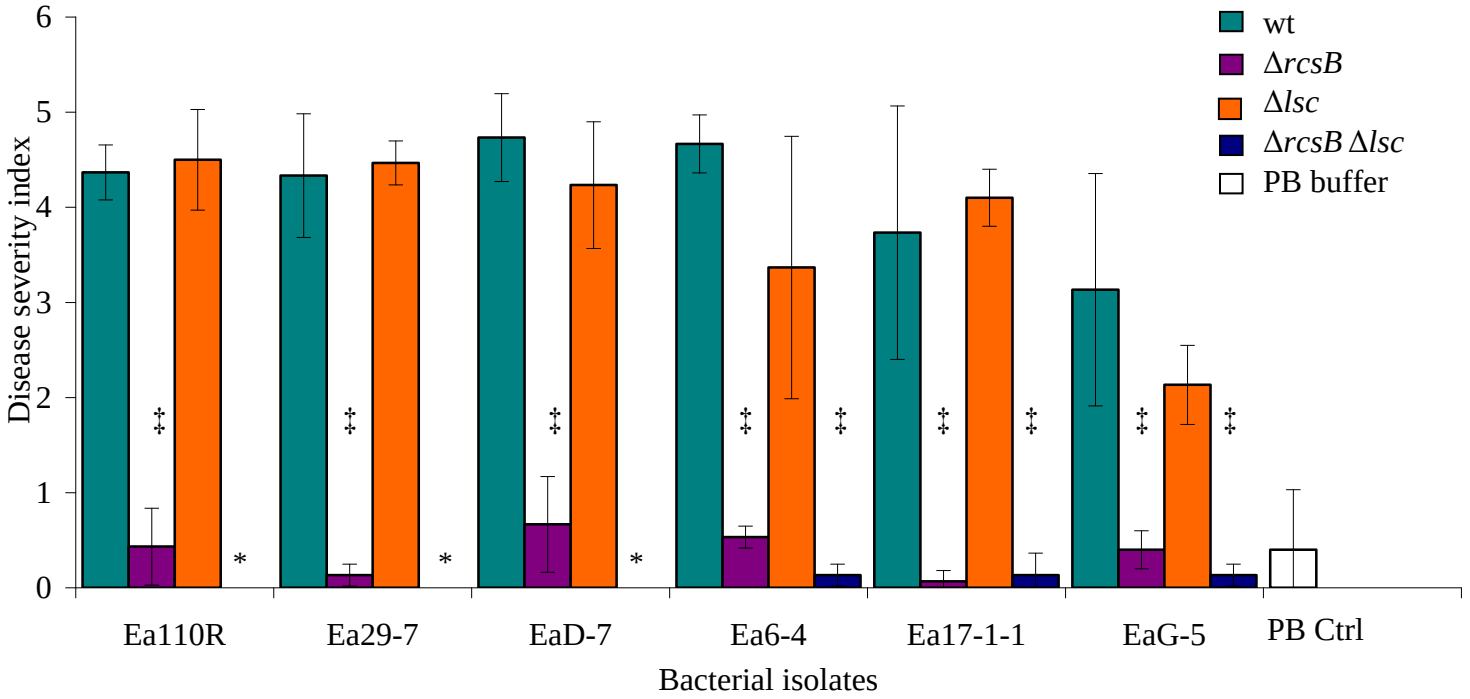

FIG. 5. Disease severity in pear blossoms caused by *Erwinia amylovora* wild type, Δ*rcsB*, Δ*lsc* and Δ*rcsB*Δ*lsc* deletion mutants. Integer values of disease severity are based on the vertical progression of necrosis through the infected blossom to the peduncle. "0" means no visible necrosis, "5" means complete necrosis of blossom extending into the peduncle. The error bars represent the standard deviation of each test complete in triplicate of 10 pear blossoms. ‡ significantly different from wild-type (P>0.05) * denotes knockout strains not available for testing.

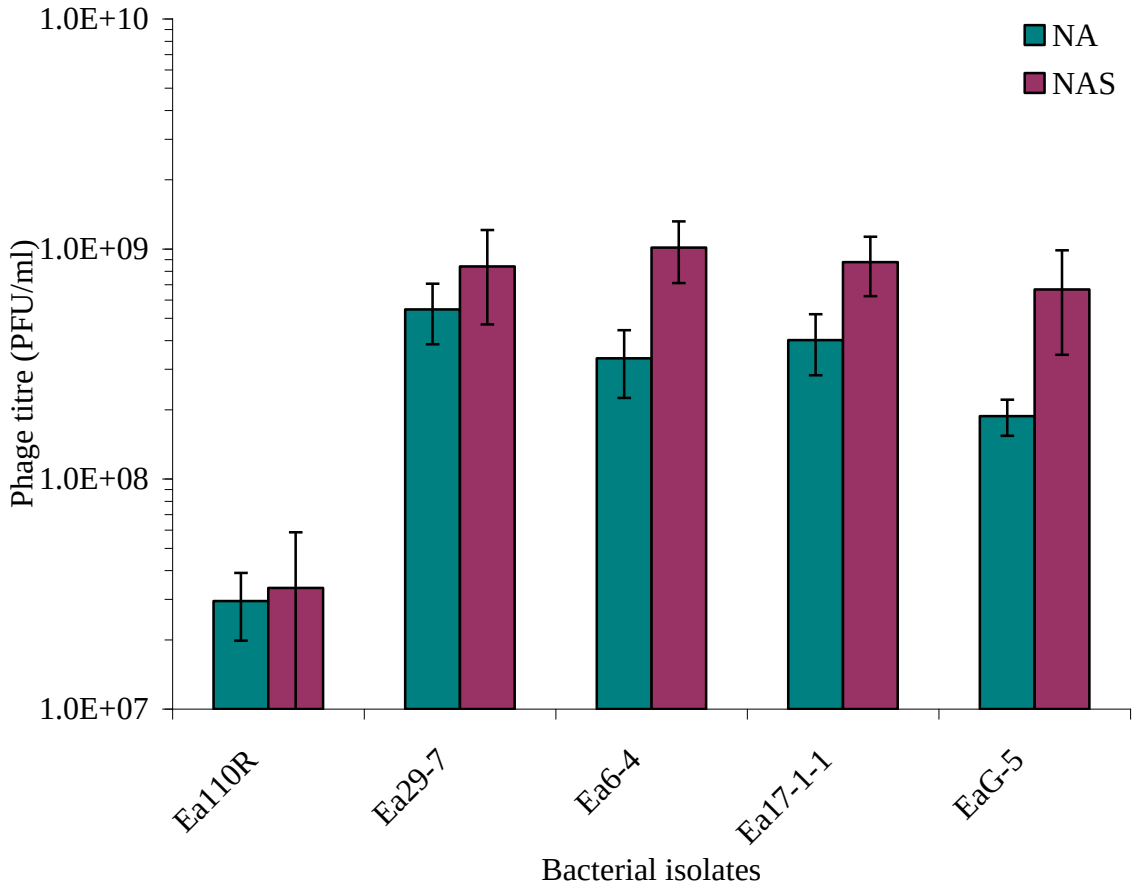

FIG. 6. Bacteriophage titres (PFU) of *Myoviridae* phage ΦEa21-4, grown on six *E. amylovora* hosts using NA and NAS media. Error bars represent standard deviation of triplicate experiments.

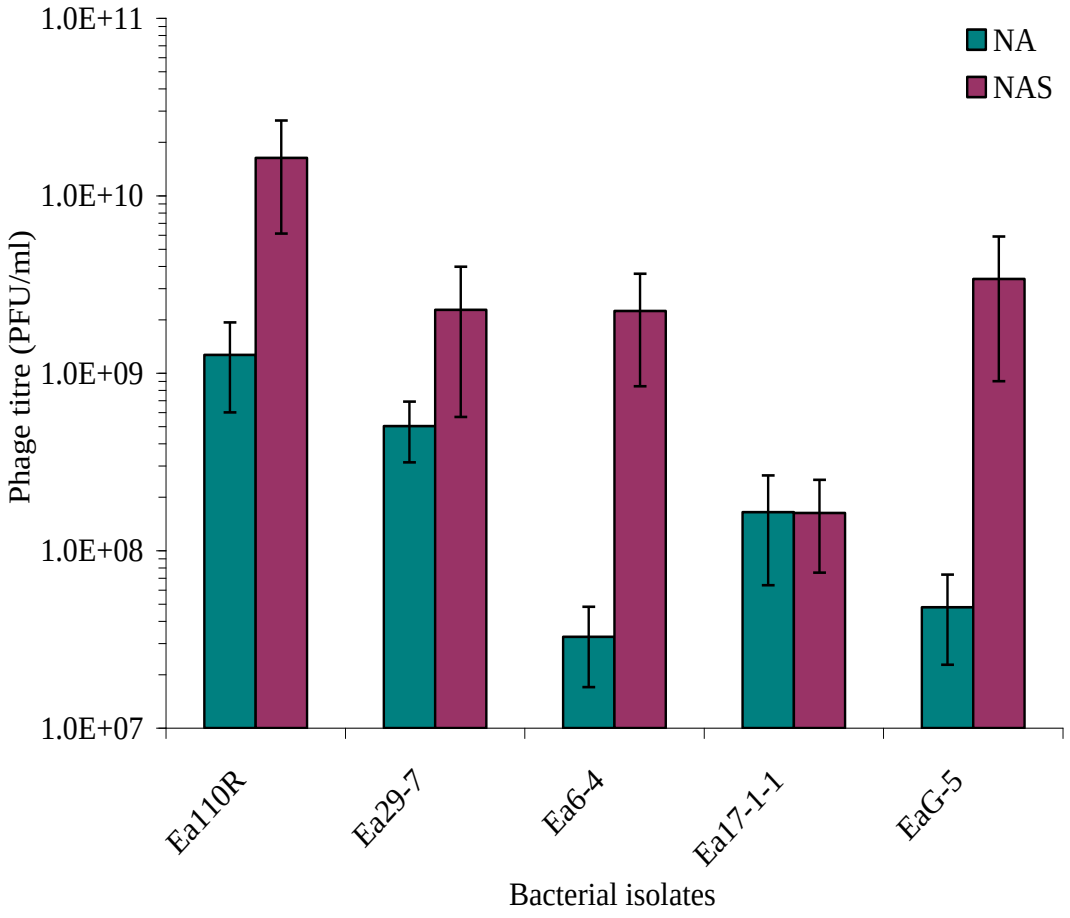

FIG. 7. Bacteriophage titres (PFU) of *Siphoviridae* phage ΦEa35-7, grown on six *E. amylovora* hosts using NA and NAS media. Error bars represent standard deviation of triplicate experiments.

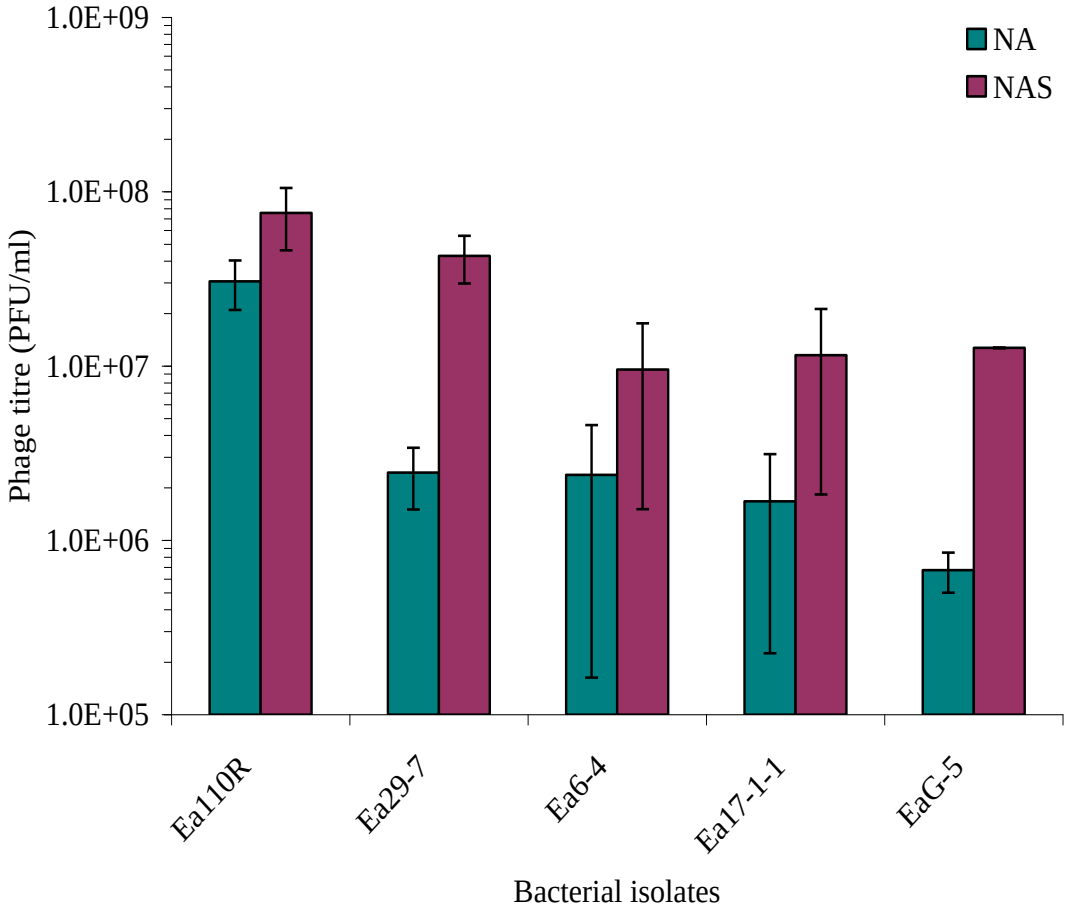

FIG. 8. Bacteriophage titres (PFU) of *Podoviridae* phage ΦEa1(h), grown on six *E. amylovora* hosts using NA and NAS media. Error bars represent standard deviation of triplicate experiments.

not altered by the passage through a host for any the four phages; they remained the same (Gill, 2000; Gill et al., 2003; Lehman, 2008; Roach, 2011) (data not shown).

4.3 Decreased EPS production and Phage Efficiency of Plating (EOP)

A representative phage from each family was also tested on all wild type *E. amylovora* isolates and EPS deficient strains. *Myoviridae* phage ΦEa21-4 (Fig. 9) had equal titres on all hosts and knockouts with two exceptions. Phage titre was reduced 10-fold on all Ea110R strains and on the levan deficient mutant of Ea6-4. *Siphoviridae* phage ΦEa35-7 (Fig. 10) could not infect any of the amylovoran deficient bacterial mutants. Generally, the phage titre on the levan mutant was equal to that of the wild-type with the exception of Ea6-4 Δ*lsc* which had a 150-fold increase in phage titre. *Podoviridae* phage ΦEa1(h) (Fig. 11) did not infect any of the amylovoran deficient mutants. Levan deficiency in the host cells did not appear to affect the phage titre compared to the growth on the wild-type hosts with the exception of Ea 6-4 Δ*lsc* which had a 10-fold increase in phage titre.

To further investigate the trends seen with the representative phages additional phages were enriched on their ideal hosts; the wild type, Δ*lsc* and Δ*rcsB* strains. The ideal host for each phage was determined by Roach (2011) as the host isolate which supported the best proliferation of phage to the highest titre. Phage titre was substantially reduced for most phages enriched on both the Δ*rcsB* and Δ*lsc* strains compared to growth on their non-mutant progenitor (Table 3). The *Podoviridae* phages yielded drastically fewer progeny on the Δ*rcsB* strains (EOP≈0) and variable titres of phage on the Δ*lsc*

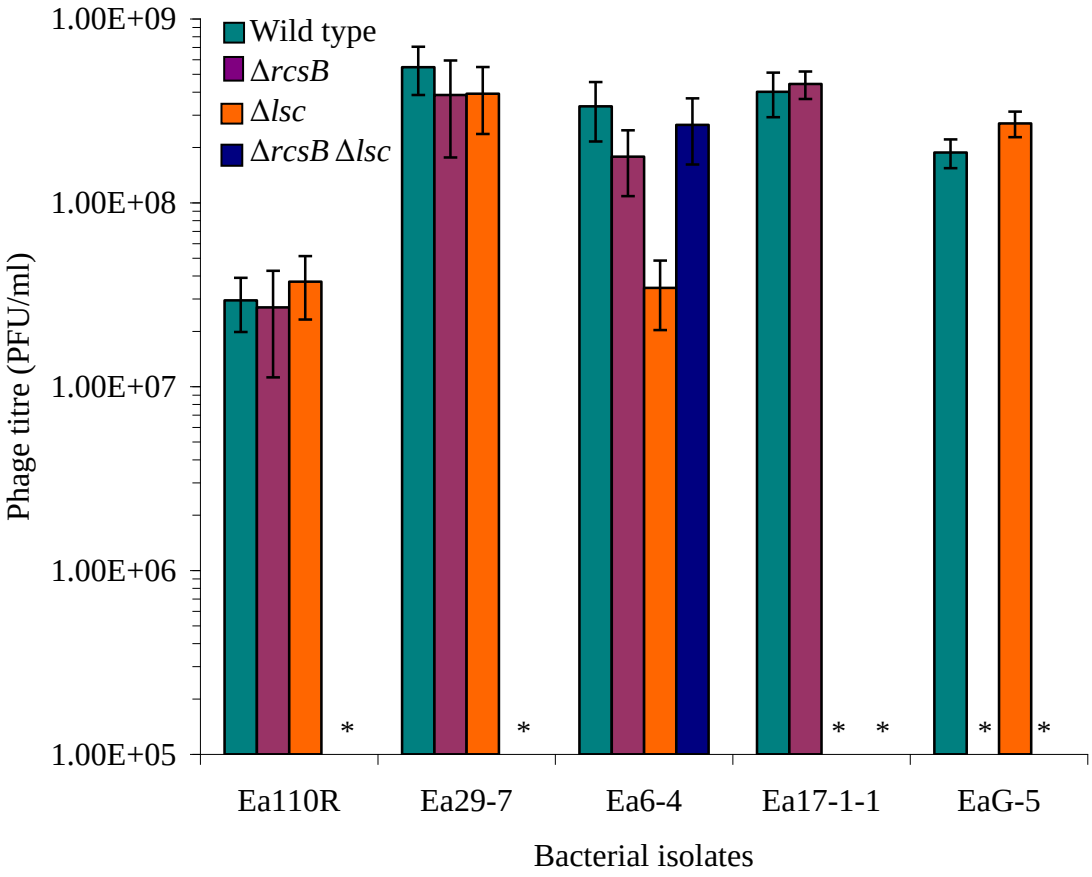

FIG. 9. Bacteriophage titres (PFU) of *Myoviridae* phage ΦEa21-4 grown on the wild type, Δ*rcsB*, Δ*lsc*, and Δ*rcsB*Δ*lsc* mutants of *E. amylovora*. Error bars represent the standard deviation of triplicate experiments. * denotes knockout strains not available for testing.

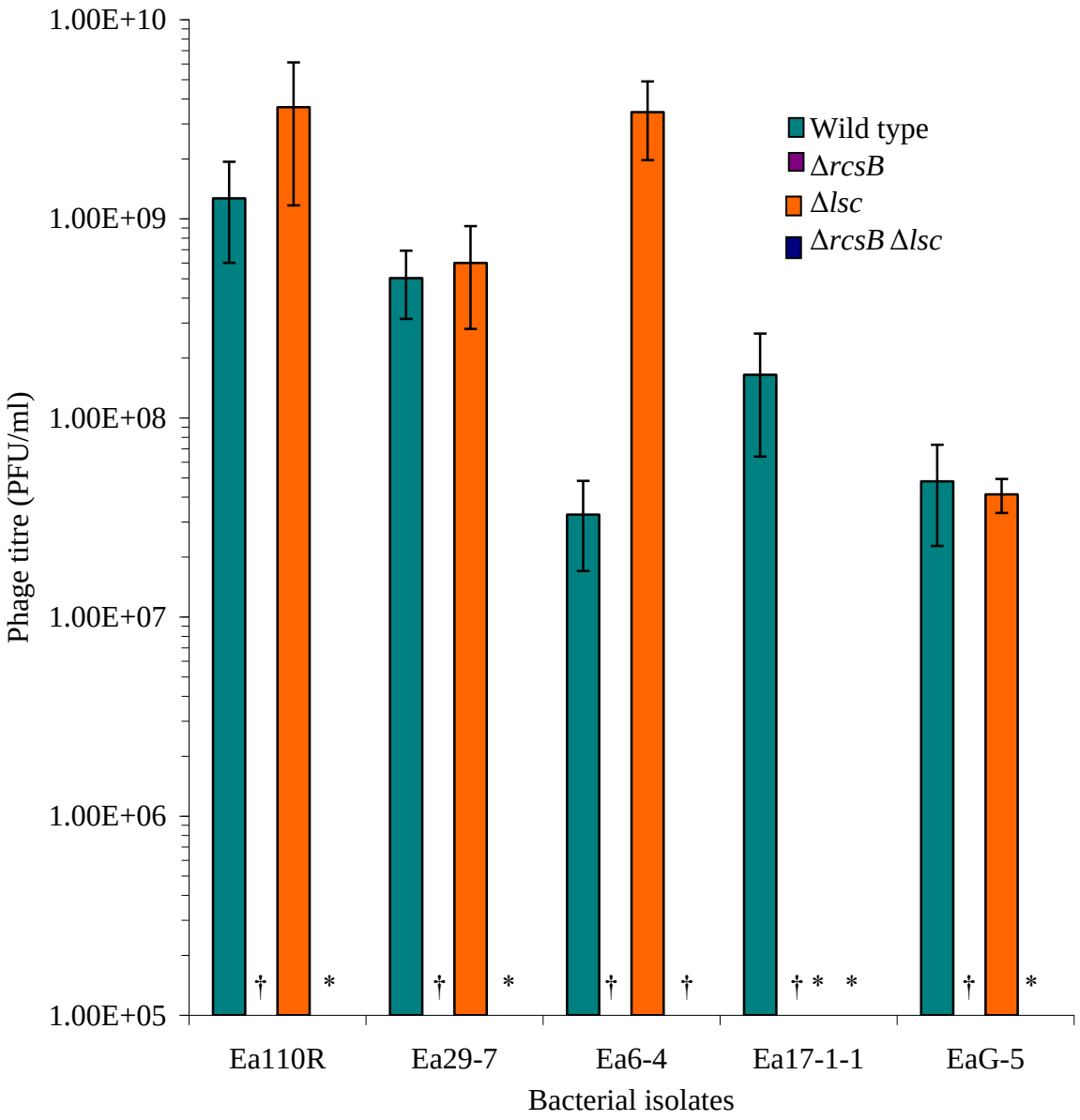

FIG. 10. Bacteriophage titres (PFU) of *Siphoviridae* phage ΦEa35-7 grown on the wild type, Δ*rcsB,* Δ*lsc,* and Δ*rcsB*Δ*lsc* mutants of *E. amylovora.* Error bars represent the standard deviation of triplicate experiments. * denotes knockout isolate not available for testing, † denotes no phage proliferation.

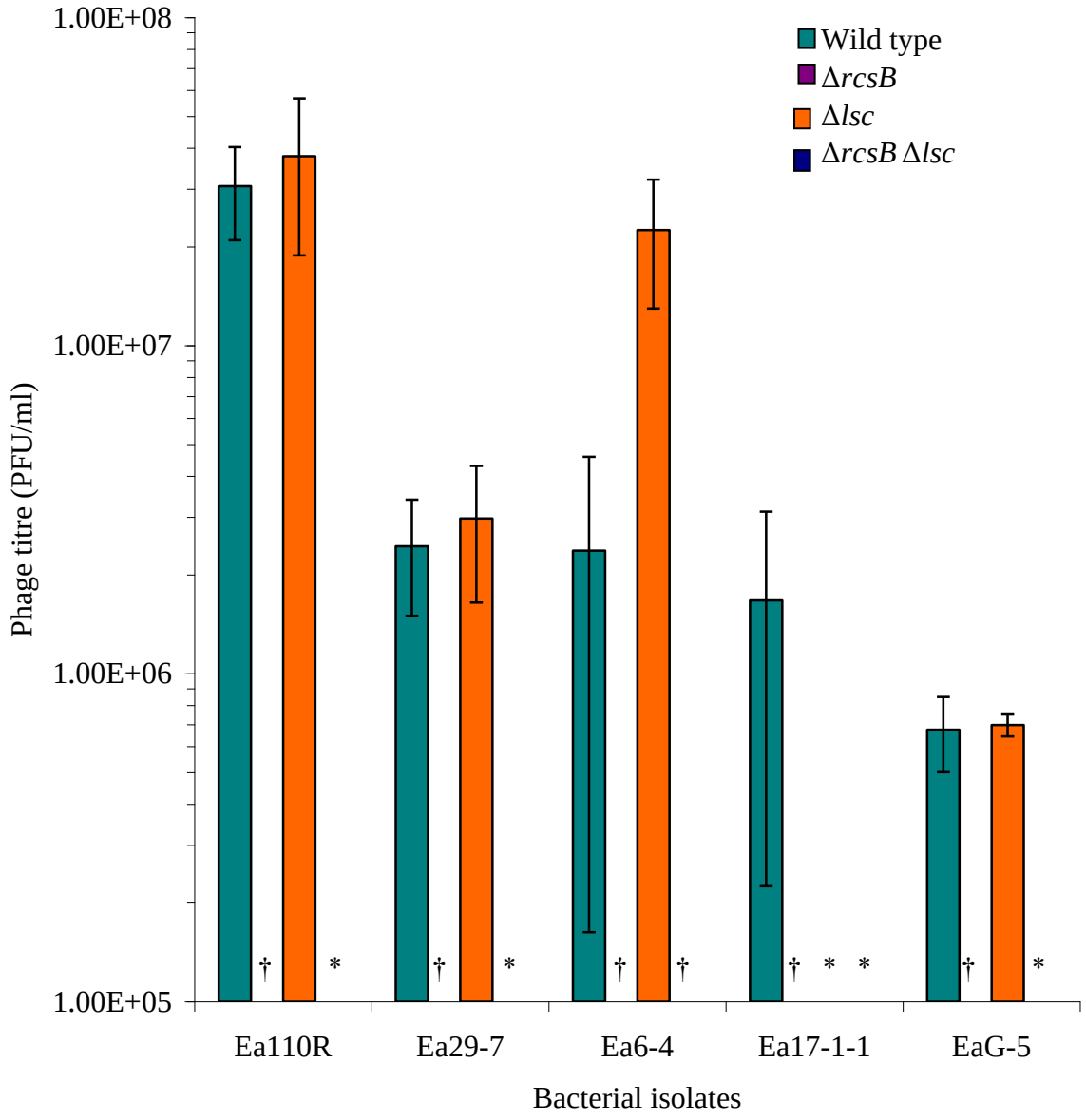

FIG. 11. Bacteriophage titres (PFU) of *Podoviridae* phage ΦEa1(h) grown on the wild type, Δ*rcsB,* Δ*lsc,* and Δ*rcsB*Δ*lsc* mutants of *E. amylovora.* Error bars represent the standard deviation of triplicate experiments. * denotes knockout isolate not available for testing, † denotes no phage proliferation.

mutants. The proliferation of *Podoviridae* phages ΦEa35-3, ΦEa31-3, ΦEa46-1A2 appeared to be restricted on the Δ*lsc* strains (EOP=0.23-0.34). In contrast, ΦEa45-1B, ΦEa50-3, and ΦEa51-7 showed good growth on the same knockout (EOP=1.35-1.84). The production of the *Siphoviridae* phages was highly variable on both the Δ*rcsB* and the Δ*lsc* strains. Phage ΦEa10-16 showed reduced growth on Δ*rcsB* and Δ*lsc* strains compared to the wild type *E. amylovora* (EOP=0.64 and 0.05 respectively). Growth of ΦEa35-4 was five times greater than the wild type growth in the absence of amylovoran (EOP=5.34), but the Δ*lsc* strain had EOP of 0.26 compared to growth on the wild-type. Tests on the *Myoviridae* phages resulted in variable but higher phage titres on the Δ*rcsB* strains and low titres on the Δ*lsc* mutants. Phage ΦEa10-1 showed decreased growth on Δ*rcsB* and the Δ*lsc* strains, with levan apparently playing a larger role in infection than amylovoran. Phages ΦEa21-2 and ΦEa21-4 showed moderate increases in proliferation (EOP=1.11 and 1.38 respectively) in the Δ*rcsB* mutants with a significant decrease in phage titre on the Δ*lsc* mutant (EOP=0.12 and 0.02 respectively).

4.4 Quantity of EPS production and Phage Population Growth

The *Podoviridae* phage ΦEa1(h) was enriched on *E. amylovora* hosts including the wild type, Δ*rcsB*, Δ*lsc*, and Δ*rcsB*Δ*lsc* strains (Fig. 12). Both the HEP and the LEP hosts show an exponential relationship between EPS production and the efficiency of plating

TABLE 3. Bacteriophage efficiency of plating from each of *Myo-*, *Sipho-*, and *Podoviridae* phages enriched on *E. amylovora* wild type, Δ*rcsB* and Δ*lsc* deletion mutants of the ideal host. The levels of growth were calculated as efficiency of plating defined as phage titre on the mutant strain divided by titre on the wild type isolate.

		Efficiency of plating	
Family Phage Isolate	Host Isolate	Δ*rcsB*	Δ*lsc*
Myoviridae			
ΦEa10-1	Ea17-1-1	0.25	0.06
ΦEa21-2	Ea17-1-1	1.11	0.12
ΦEa21-4	Ea 6-4	1.38	0.02
Siphoviridae			
ΦEa10-16	Ea17-1-1	0.64	0.05
ΦEa35-4	Ea110R	5.34	0.26
Podoviridae			
ΦEa10-6	Ea17-1-1	<0.0001	0.98
ΦEa31-3	Ea29-7	<0.0001	0.34
ΦEa35-3	Ea110R	0.0005	0.23
ΦEa45-1B	Ea29-7	0.0007	1.84
ΦEa46-1A2	Ea110R	0.0003	0.34
ΦEa50-3	Ea29-7	0.0012	1.35
ΦEa51-7	Ea29-7	0.0026	1.44

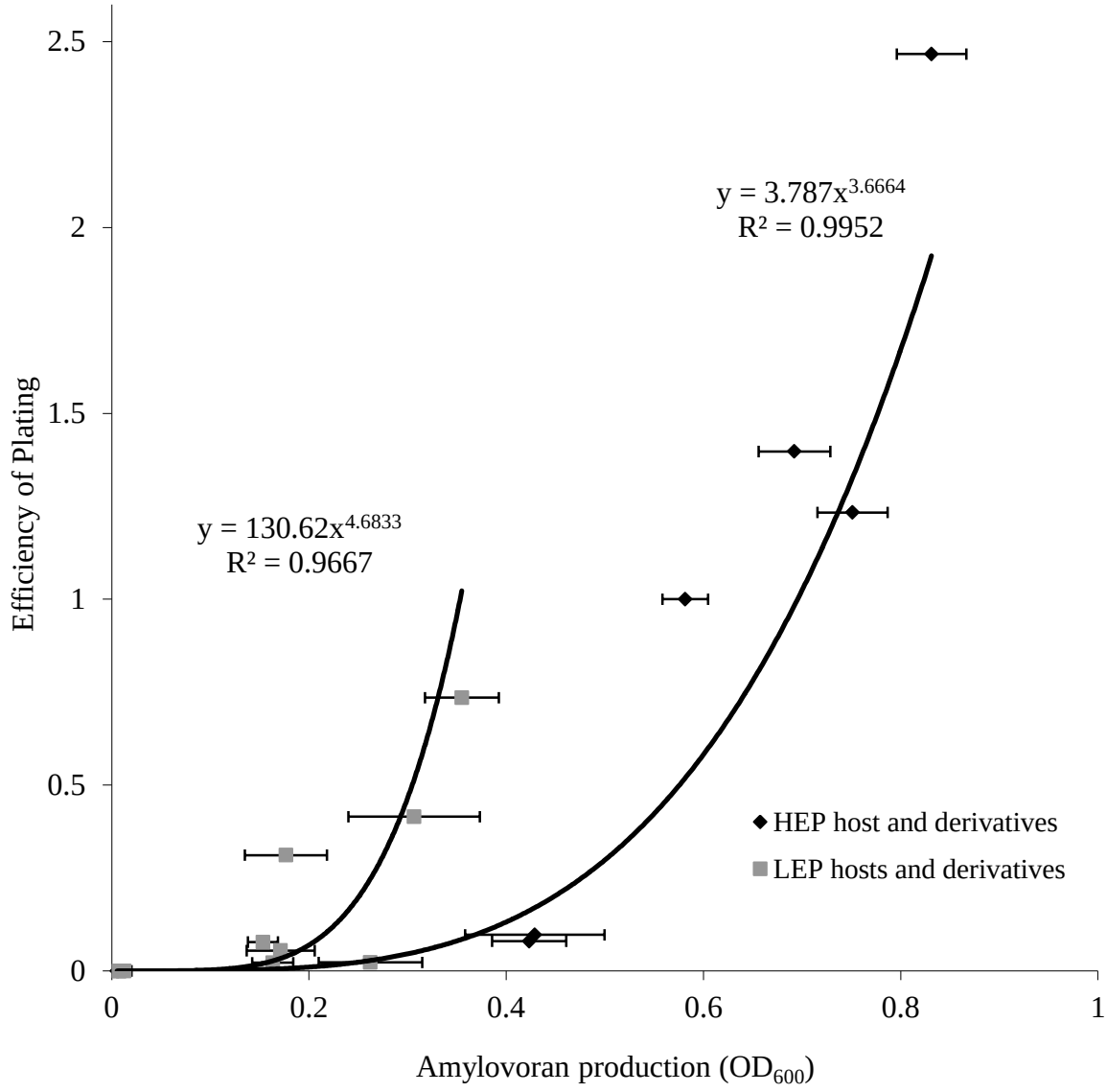

FIG. 12. Relation between amylovoran production by wild type isolates, Δ*rcsB*, Δ*lsc,* and Δ*rcsB*Δ*lsc* mutants and phage efficiency of plating when ΦEa1(h) is grown on wild type isolates, Δ*rcsB*, Δ*lsc,* and Δ*rcsB*Δ*lsc* mutants of six *E. amylovora* host isolates. Efficiency of plating is defined as phage titre on the mutant strain divided by titre on the wild type isolation host. Error bars represent the standard deviation of amylovoran production in triplicate experiments.

(EOP, compared to phage titre on wild-type *E. amylovora* Ea110R isolate). The LEP hosts, in comparison to the HEP hosts, require less amylovoran production to achieve the same phage populations. However, the HEP hosts, with the capability of producing copious amounts of EPS, can result in much higher phage titres.

4.5 Effect of Supplementing Media with EPS Mimetic

To determine the specific effect of the sugars that constitute levan, ΦEa21-4 was grown with fructose in the culture medium. Supplementing the culture with fructose did not have a significant effect on the growth of phage ΦEa21-4 titre, when the phage was pretreated with 2% fructose, compared to growth on NA (Fig. 13). This is different than the effect produced adding fructose to the culture when the phage was pre-incubated with arabinose or water. In the arabinose and water pretreatment controls, adding fructose to the medium resulted in an increase in phage titre. However, pre-treating the phage with fructose resulted in an 81% decrease in phage titre compared to phage that had been pretreated with arabinose or water. A similar effect was seen both on NA and NA amended with 2% fructose. Neither pretreatment nor amended medium altered the phage titres of phage growth on the Δ*lsc* host.

4.6 Effect of Pretreatment with EPS Mimetic

Pre-treatment of the *Myoviridae* phage ΦEa21-4 with fructose reduced the phage titre by as much as 76% compared to the arabinose control (Fig. 14). The fructose pre-

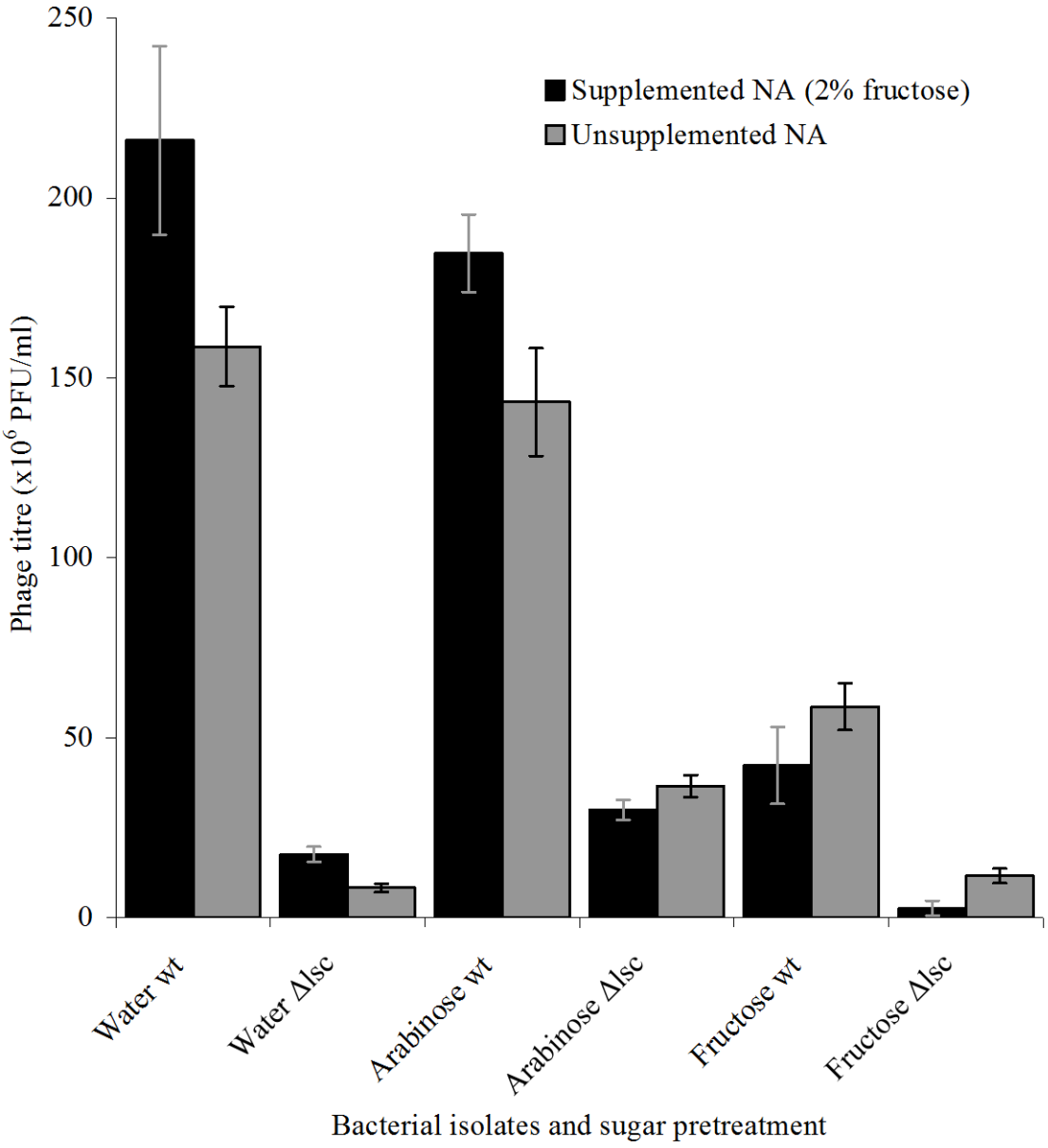

FIG. 13. Bacteriophage titre after pre-treating phage ΦEa21-4 with fructose, arabinose, or water and adding fructose to the growth medium using the wild-type and Δ*lsc* deletion mutant of *E. amylovora* isolate Ea6-4. Error bars represent the standard deviation of triplicate experiments.

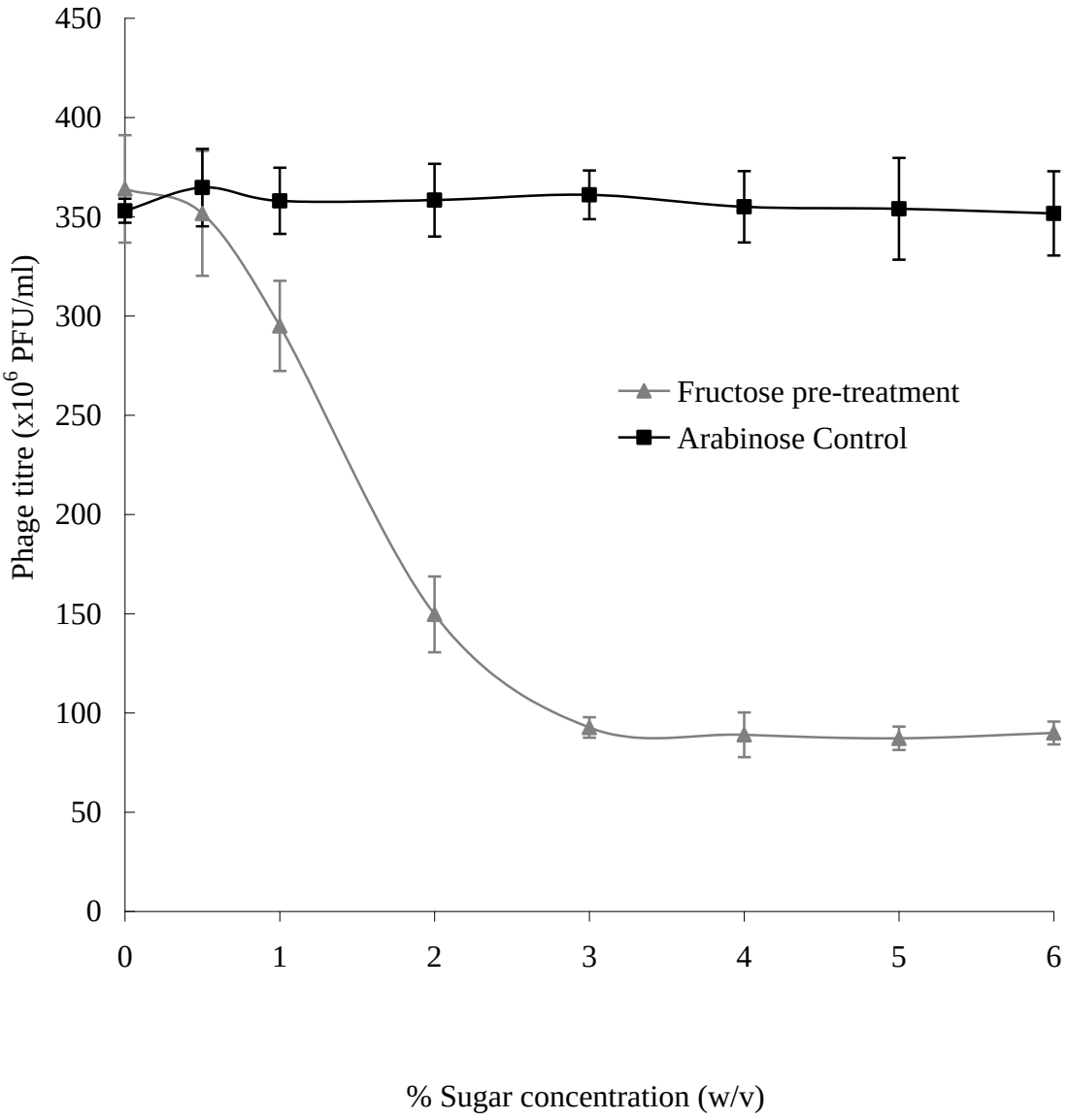

FIG. 14. Bacteriophage titre after pre-treating *Myoviridae* phage ΦEa21-4 with fructose and arabinose at concentrations of 0-6%; grown on *E. amylovora* isolate Ea6-4. Error bars represent the standard deviation of triplicate experiments.

incubation had little effect at the concentration of 0.5%, however the phage titres began to decrease more with every increase in fructose concentration. There was a maximal effect of the fructose at about 3% (w/v) fructose pretreatment. The *Podoviridae* phage ΦEa31-3 was not significantly affected by pretreatment with fructose or arabinose (Fig. 15) however, pretreatment with a 4:1 galactose:glucuronic acid solution reduced the phage titre by up to 50%. The time of a 2% fructose (proposed as a levan EPS mimetic) exposure to the phage before host introduction did not affect the magnitude of the effective phage titre reduction (Fig. 16).

As determined by visual observation, galactose appeared to inhibit growth of the bacterial lawn of the Ea110RΔ*lsc* isolate. There was a graded effect, on the medium that did not contain any galactose, the Δ*lsc* grew as well as the wild-type and the Δ*rcsB*. With 0.5% and 1% galactose there was less growth in comparison to the control and substantially less bacterial growth in on the plate that contained the 2% galactose pretreatment compared to the wild-type and rcsB knockout (data not shown).

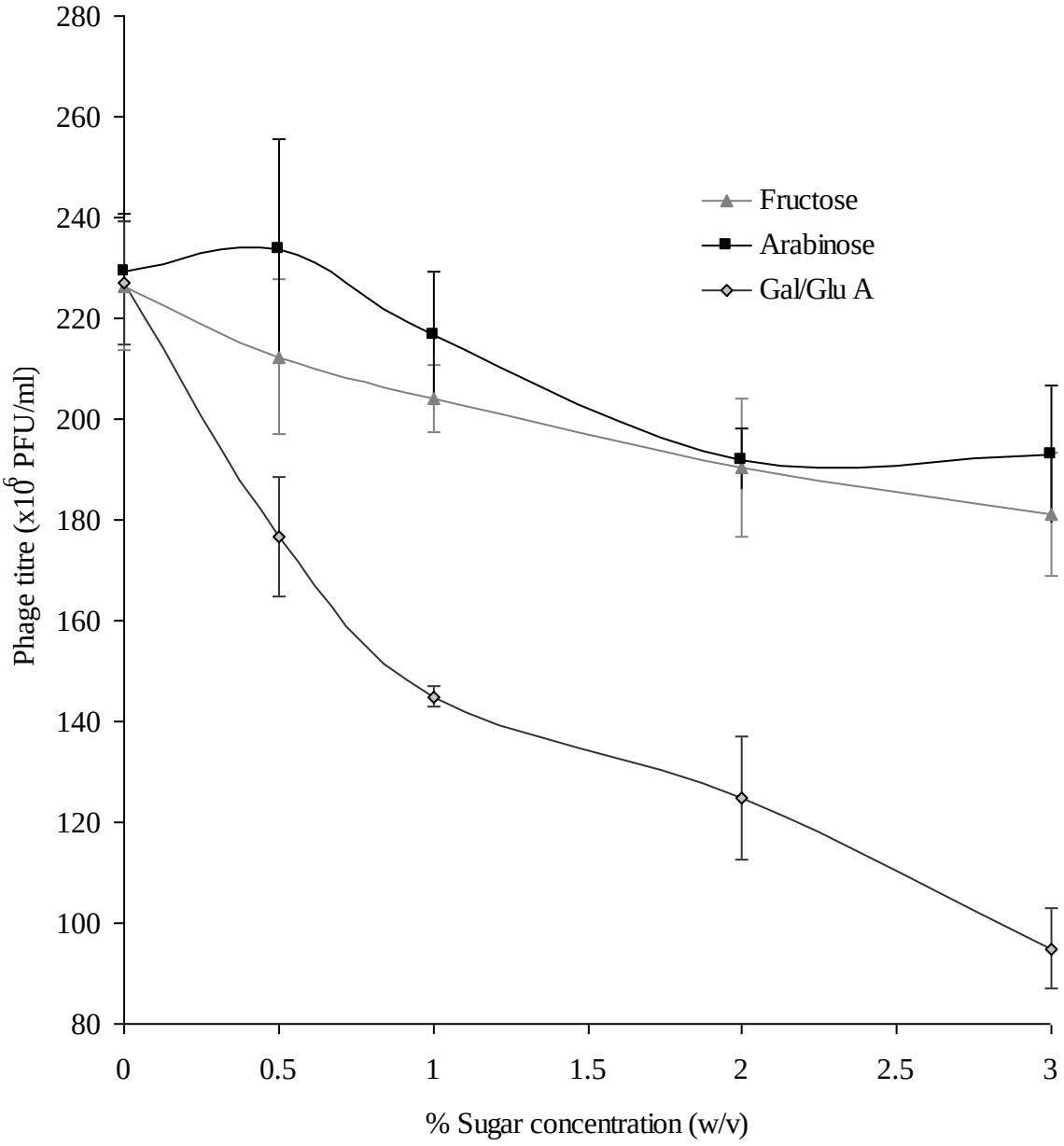

FIG. 15. Bacteriophage titre after pre-treating *Podoviridae* phage ΦEa31-3 with fructose (triangles), galactose/glucuronic acid (4:1)(circles) and arabinose (squares) prior to the infection of wild-type *E. amylovora* isolate Ea110R. Error bars represent the standard deviation of triplicate experiments.

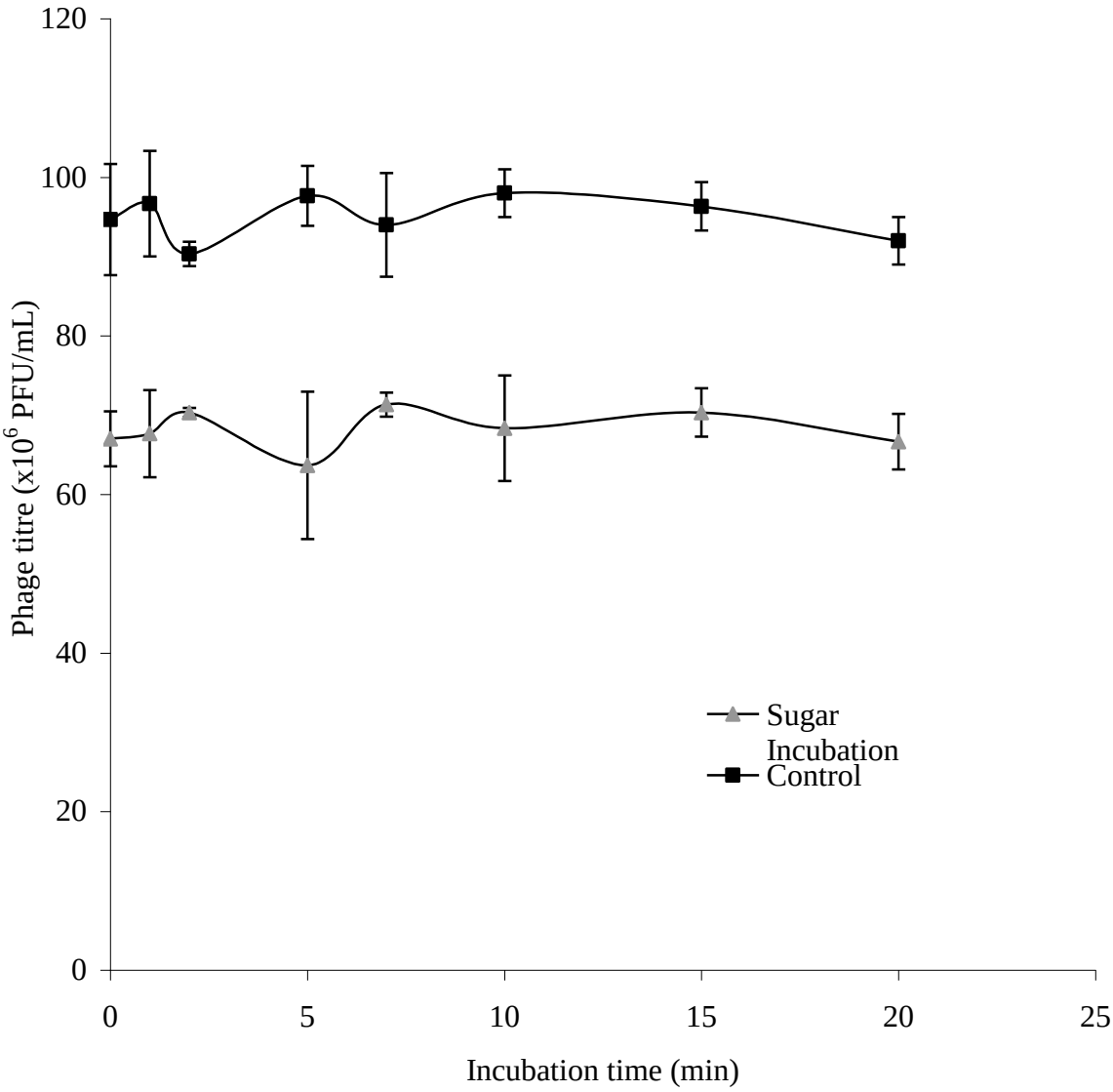

FIG. 16. Bacteriophage titre after pre-treating *Myoviridae* phage ΦEa21-4 with 2% fructose for 0 to 20 min prior to infection of *E. amylovora* isolate Ea6-4. Error bars represent the standard deviation of triplicate experiments.

Chapter 5
Discussion

Fire blight causes significant global economic losses of pome fruits, particularly apple and pear. Research to control the causative bacterium, *E. amylovora*, recently focused on the use of biological control agents. Phages have been proposed as agents for this purpose. The registration process for phage biopesticides will require that incidence of phage-host resistance development is well documented in some phage-pathogen combinations. Extended use of phage with a bacterial population has the potential to result in bacterial resistance to phage infection (Goodridge, 2004; Jones et al., 2007). Bacterial species commonly create resistance by modifying cell-surface receptors that phages require for adsorption and infection (Labrie et al., 2010). This study investigates the involvement of the *E. amylovora* EPS and EPS sugars in the bacteriophage infection efficacy and plaque appearance through the effect of loss or modification of these components on bacterial host susceptibility.

E. amylovora phages have been observed to produce clear plaques on some host isolates and turbid plaques on other isolates (Billing, 1960; Bennett and Billing, 1978; Bayer et al., 1979; Roach, 2011). Also plaque turbidity has been well documented in *E. coli* and other enterobacteria (Park, 1956; Lindberg, 1977; Bayer et al., 1979; Pelkonen et al., 1992). A common conclusion from the production of turbid plaques is that a portion of the population has undergone lysogeny, resulting in resistance to homologous phages (Schnabel and Jones, 2001; 2008; Hyman and Abedon, 2010). However, none of the *E. amylovora* hosts contained detectable prophage DNA by real time PCR screening and/or

produced virions through induction (Roach, 2011). A more reasonable explanation for differences in plaque appearance is host preference. The grouping of the AAFC Vineland *E. amylovora* isolates into high- and low EPS producing hosts (HEP and LEP respectively) has further defined the observations of clear and turbid plaques (Roach, 2011). Examination of plaque morphology on HEP and LEP hosts has lead to the observation that the *Myoviridae* phages produce clear plaques on the LEP hosts and turbid on the HEP hosts (Roach, 2011). On the other hand, *Podoviridae* phages produce clear plaques on the HEP and turbid plaques on the LEP hosts (Roach, 2011). This observation suggests that the EPS of *E. amylovora* plays a role in the phage-host interaction. *E. amylovora* mutants, deficient in EPS production, were generated and the interaction between EPS and phage was further characterized.

The flower stigma is a nutrient rich environment that provides the initial critical environment for the phage-pathogen interaction. To investigate the role of carbohydrates on the process of EPS production and phage proliferation artificial growth media was amended. Wild type *E. amylovora* isolates Ea110R, Ea29-7, and EaD-7 produced higher levels of amylovoran on NA than isolates Ea6-4, Ea17-1-1, and EaG-5. Amendment of the growth medium with 0.5% sucrose and 1% sorbitol resulted in increased amylovoran production. From these results Ea110R, Ea29-7, and EaD-7 were grouped as high EPS producers (HEP) and Ea6-4, Ea17-1-1, and EaG-5 as low EPS producers (LEP). Additionally, amylovoran deficient mutants with deletions of *rcsB* gene were recovered for isolates Ea110R, Ea29-7, EaD-7, Ea6-4, Ea17-1-1, and EaG-5. Amylovoran production was negligible in Δ*rcsB* strains. The Ea6-4, Ea17-1-1, and EaG-5 Δ*rcsB*Δ*lsc* double mutant produced extremely small amounts amylovoran.

Mutant strains deficient in the production of EPS in *E. amylovora* were generated using recombineering (Datsenko and Wanner, 2000). The regions of the genome that coded for the generation of amylovoran and levan were replaced with antibiotic resistance cassettes to disrupt the production of EPS. The current research indicates that amylovoran and levan synthesis are loosely co-regulated (Wang et al., 2011). The biosynthesis of amylovoran is regulated by the *ams* operon which contains the *rcsA* and *rcsB* genes (Belleman and Geider, 1992; Bugert and Geider, 1995; Wang et al., 2011). Over-expression of amylovoran results in a reduction of levansucrase activity, however mutations that prevent the expression of amylovoran have been reported to have variable effects on the production of levan (Geider et al., 1993; Roach, 2011). Additionally, mutation in the levansucrase gene was reported to not effect the production of amylovoran in Geier and Geider (1993), but this work reports an increase of 29 to 119% in amylovoran levels (Fig. 4) in levansucrase deficient strains. While it has not been possible to determine the exact mechanism of co-regulation between the production of amylovoran and levan, it is evident that the bacterium attempts to maintain some minimal level of EPS.

This work also indicates that some production of EPS is essential for the viability of the HEP *E. amylovora* isolates. Recovery of all the single (Δ*rcsB* and Δ*lsc*) mutants was relatively simple as was the creation of the double (Δ*rcsB*Δ*lsc*) mutants of the LEP isolates. Additionally, EPS production mutants deficient in amylovoran have been recovered in natural environments (Bennett and Billing, 1978; Geider et al., 1993). This work demonstrates that recovering a stable strain of the double mutant HEP isolates was not possible. Researchers have hypothesized that the EPS may provide a source of

energy, protect the cell from desiccation and mediate the external osmotic pressures (Goodman et al., 1974; Weiner et al., 1995; Hughes et al., 1998a). Mutations in both the levan and the amylovoran synthesis may have resulted in excessive, deleterious osmotic pressure or bacterial desiccation. The double mutant bacterium could be expending copious amounts of cellular resources to up-regulate the production of ineffective capsular synthesis genes. It seems evident that the bacterium requires at least a rudimentary production of EPS. The regulation and importance of the EPS could also be seen in the inhibitory effects of galactose on the levan production mutant of Ea110R. This work indicates that galactose (amylovoran subunits) may down-regulate the production of amylovoran as proved by Hignett (1988). This could make the bacterial cells grow more slowly as they approached a Δ*rcsB*Δ*lsc* phenotype (Hignett, 1988). On the other hand, the EPS may play an even more vital role in the basic processes of the cells. Recovery of HEP double knockouts was not possible even using various amended media that could have reduced some environmental osmotic and desiccation pressures.

The role of the EPS in the virulence, or severity of disease, of *E. amylovora* has been well characterized in the literature (Geider et al., 1993). Comparison of the EPS deficient mutants produced in this study to mutant strains of other isolates required an *in planta* pathogenicity test. EPS mutants that were generated in this study could have been pathogenic, that is, had the ability to cause disease even when the production of amylovoran or levan was effectively abolished. All the amylovoran deficient strains (Δ*rcsB*), as well as the strains deficient in both levan and amylovoran (Δ*rcsB*Δ*lsc*), did not cause necrosis above baseline in the blossom assay (Fig. 5). This is in agreement with other studies that demonstrate that amylovoran is essential for pathogenicity (Bennett and

Billing, 1978; Ayers et al., 1979; Steinberger and Beer, 1988; Bernhard et al., 1990; Belleman and Geider, 1992; Geider et al., 1993) Geier and Geider (1993) showed that *E. amylovora* levansucrase mutants had retarded development of disease symptoms. The levan deficient LEP strains of Ea6-4 and EaG-5 may have showed some reduction in virulence after four days (Fig. 5), however HEP isolates of *E. amylovora* did not differ in disease severity between the wild type isolates and the Δ*lsc* mutant strains. This difference could be explained by isolate specific differences between LEP and HEP isolates. After four days the levels of necrosis in the HEP mutant strains had reached the same level as the wild-type isolates, however the LEP levan deficient mutants may have been delayed more than the HEP isolates. In other words, perhaps if observation of the blossom assay was made two instead of four days after inoculation there would be a more significant difference in disease severity between the levan deficient (Δ*lsc*) HEP and the LEP hosts.

Level of EPS production in the wild type isolates did not appear to significantly correlate with the degree of disease that the isolate causes. These results conflicts with some other research that points to a increase in virulence with highly capsulated bacterial isolates (Ayers et al., 1979; Billing, 1984; Hignett, 1988; Muller et al., 2011). EaD-7 and Ea6-4 caused the highest rates of necrosis in the blossoms, marginally higher than Ea110R and Ea29-7 even though Ea6-4 produces the least EPS of the six isolates. LEP isolates Ea17-1-1 and EaG-5 appeared to cause less severe disease symptoms, but with more variability. This lack of correlation between the disease severity and level of EPS production could be explained by the presence of other pathogenicity factors, such as hrp proteins, that augment the disease severity caused by the bacteria (Barny et al., 1990; Oh

and Beer, 2005; Lalli et al., 2008; Smits et al., 2011). This *in planta* test confirmed that amylovoran is a required pathogenicity factor in *E. amylovora* and the deletion mechanism used to abolish amylovoran production is effective.

E. amylovora EPS biosynthesis has been well characterized because of its role in pathogenicity and bacterial virulence (Gross et al., 1992; Geier and Geider, 1993; Geider, 2006). The amounts of EPS produced by the bacterium are influenced by nutrient availability (Bellemann et al., 1994). The addition of sucrose and sorbitol (primary carbohydrates produced by *Prunus* and *Malus* species) results in higher yields of EPS (Fig 4) (Loescher, 1987; Moriguchi et al., 1992; Geider et al., 1993; Pusey et al., 2008). Alteration of the EPS production, either through supplemented media or EPS deficient mutants, indicates that bacteriophage titres are associated with host production of EPS. The data (Fig. 5) are consistent with plaque morphology data (Roach, 2011) in showing that ΦEa21-4 infects both HEP and LEP hosts equally well. Growth on NAS did not significantly increase the titres of ΦEa21-4 with HEP hosts but there was a slight increase when LEP hosts were supplemented.

Decreased levan synthesis had variable effects on the proliferation of the bacteriophage. The *Myoviridae* group had the most radical change in growth on the levan deficient bacteria, reducing phage titres as much as 98%. When *Myoviridae* phages ΦEa10-1, ΦEa21-1, and ΦEa21-4 were enriched on the levan deficient mutant (Table 3), the phage titre was reduced quite significantly. The EOP of those three phages grown on Ea6-4Δ*lsc* strain compared to the wild-type Ea6-4 was 0.06, 0.12, and 0.02 respectively. This indicated that levan may be involved in the infection process of the *Myoviridae* phages. Levan may act as a primary receptor for the *Myoviridae* phage, stabilizing the

phage and keeping it closer to the cell surface, increasing the probability that the phage's tail fibers will come into contact with the secondary receptors on the surface of the bacterium. This work shows that, in the absence of amylovoran, the *Myoviridae* phages had higher EOPs (ΦEa21-2, ΦEa21-4; EOP=1.11 and 1.38 respectively), indicating for the first time the minor role that amylovoran may play in masking the phage receptors on the bacterial cell surface. Bacterial EPS has been proposed as a resistance method for some enterobacteria (Forde and Fitzgerald, 1999; Looijesteijn et al., 2001); consistent with results of the *Myoviridae* interaction with *E. amylovora* isolates producing high amounts of amylovoran EPS.

Siphoviridae phage infection of wild type and mutant strains of *E. amylovora* suggested that these phages use different receptors than the *Myo*- and *Podoviridae* when adsorbing to the bacterium. The variable results would concur with the observation that many of the phage isolates in this group have different plaque morphologies and host ranges than the phages in the *Myo*- and *Podoviridae* families (Gill, 2000; Gill et al., 2003; Lehman, 2008; Roach, 2011). When *Siphoviridae* phage ΦEa35-7 was grown on NAS, the phage titre increased for all *E. amylovora* isolates except Ea17-1-1 (Fig. 7). Amylovoran deficiency prevented the phage from infecting the host, but levan deficiency did not appear to change the phage titre (Fig. 9). ΦEa35-4 had titres five times higher on Ea110RΔ*rcsB* (Table 3) but titres were reduced 74% on Ea110RΔ*lsc*.

It appears, from observation of turbid plaque appearance, that the *Siphoviridae* phages in the Vineland collection do not infect the *E. amylovora* isolates with a high degree of efficiency. In fact, the Vineland collection is the only in the world to report having any *Siphoviridae* bacteriophage of *E. amylovora*. Additionally, there are no

published sequences of *Siphoviridae* that infect *E. amylovora* in the gene library. This is curious because all known tailed phages (Order *Caudovirales*) constitute the largest (96%) and most widespread group of bacteriophage, and the family of *Siphoviridae* accounts for approximately 61% of the group (59% of all identified phages in the world) (Ackermann, 2001). The absence of this family from the collections of *E. amylovora* phage in the world and turbid plaque appearance of the *Siphoviridae* phages in the Vineland collection could indicate that a lysogenic *E. amylovora* host carries a *Siphoviridae* prophage or the receptor that the phage bound to has been lost due to mutational loss or the alteration of the receptor. It is possible that the *Siphoviridae* in the Vineland collection are able to infect due to an acquired compensating adaptation. The latter hypothesis is supported by the host range that is seen in this family. The *Siphoviridae* phages in the collection achieve the highest titres when enriched on HEP host isolates even without any depolymerase activity indicated by the absence of a halo around the plaque (Gill, 2000; Gill et al., 2003; Roach, 2011). Additionally, phage ΦEa35-4 has increased infectivity in the absence of amylovoran indicating the *Siphoviridae* phages do not prefer the HEP hosts because of the EPS, but rather the EPS is a blocking factor and the phage binds to other sites on the bacterium.

Podoviridae phage ΦEa1(h) had an increase in titre on all *E. amylovora* hosts when grown on NAS (Fig. 8). When grown on Δ*rcsB* mutants, there was a complete inhibition of phage infection (Fig. 11). Levan deficiency did not appear to effect titres of ΦEa1(h). Amylovoran was virtually essential for growth of all *Podoviridae* phages (Table 3). Phage production dropped to an EOP<1 in the Δ*rcsB* mutant compared to the wild-type progenitor for all *Podoviridae* phage tested. In agreement with this work,

Müller et al. (2011) found that *Podoviridae* phages of *E. amylovora* were not able to infect the bacteria without the EPS capsule, and phages identified as *Myoviridae* were not dependent on the capsule. Additionally, there was a general correlation between *Podoviridae* infection efficiency and the amount of EPS produced by the host (Muller et al., 2011). The *Podoviridae* phages may use the bacterial EPS as a receptor in the same way that the *Myoviridae* use levan, but in this case the amylovoran is essential for infection. *Podoviridae* tail spike binding of amylovoran could activate the activity of EPS depolymerase or cause essential protein modification to expose other binding sites on the phage tail.

To date, the specific roles of amylovoran and levan in adsorption have not been described in literature. This is the first work to investigate the specific roles that levan and amylovoran play in the susceptibility of *E. amylovora* to bacteriophage attack. It is may be possible that these polymers serve as phage binding sites. Phages have evolved to recognize specific sites in the polysaccharide capsule, however there are only a few specific examples (Dandekar and Modi, 1978; Cerning, 1990; Brüssow et al., 1994; Quiberoni et al., 2004; Stummeyer et al., 2005; Rodríguez et al., 2008). *Podoviridae* phage M-1 has the capacity to recognize specific carbohydrates in the EPS of *Rhizobium japonicum* and use the EPS as a receptor for infection and adsorption was inhibited by EDTA and some monosaccharides. The EPS of *R. japonicum* is similar to *E. amylovora* in that the composition is primary galactose, glucuronic acid and glucose. Phage M-1 also produced an EPS degrading enzyme that was released into the culture medium (Dandekar and Modi, 1978). Capsular-producing strains of *Streptomyces thermophilus* have also been found to be more sensitive to phage attack than noncapsular-producing strains

(Lévesque et al., 2005; Rodríguez et al., 2008). Additionally, Rodríguez et al. (2008) found that the *S. thermophilus* strain CRL1190, which was the most sensitive strain to phage attach, had EPS composed of galactose and glucose. The process of adsorption and infection for many of these phages is not well characterized and to better understand how EPS may act as a receptor reference to other systems is required. For *E. coli*, some phages only bind and infect the bacterium when a capsular EPS with a serotype-specific surface antigen, removal of the capsule rendered the host resistant to bacteriophage attack (Wilson et al., 1970; Scholl et al., 2005; Pusey et al., 2008).

E. coli phage T4 has six asymmetrically spaced tail fibers that have the ability to reversibly bind to the bacterial LPS (Wilson et al., 1970). This reversible binding suggests that the tail fiber-LPS interaction is weak and would allow the phage to wander along the surface of the bacterium until a suitable irreversible receptor is located. The phage would move over the cell membrane as the tail fibers randomly attach and detach from the LPS, preventing the phage from rising off the cell surface and increasing the probability that the phage interacts with a secondary, irreversible receptor (Henning and Jann, 1979; Goldberg et al., 1994). When the tail fiber of the T4 phage recognizes an appropriate area of the host cell, it must signal this recognition to other tail structures. Upon sufficient binding of enough tail fibers to the LPS, the fiber proteins change their orientation in order to bring the phage baseplate down to the cell surface, resulting in irreversible binding and DNA ejection (Goldberg et al., 1994; Eiserling and Black, 1994).

The bacterial capsule most often functions to create a physical barrier that mask cellular attachment sites or prevent the bacteriophage from moving close enough to the

membrane to attach to the site (Wilkinson and Holmes, 1979; Defives et al., 1996; Forde and Fitzgerald, 1999; Labrie et al., 2010). It appears that the HEP *E. amylovora* isolates have achieved some level of resistance to the *Myoviridae* phages through the production of EPS leading to turbid plaques being produced. It is not known how these phages bypass the EPS barrier because they do not produce depolymerase (Lehman, 2008) in culture and do appear to lack the gene for it. For this reason it is logical that these phages prefer the LEP isolates due to an increased infectivity.

Pretreatment of *Myoviridae* phage ΦEa21-4 with an EPS mimetic fructose resulted in the reduction of phage titre. Levan appears to act as a receptor for the phages of the *Myoviridae* family as phage titre when grown on Ea6-4Δ*lsc* was reduced 88-98% compared to growth on the wild-type isolate. Infection of Ea 6-4Δ*lsc* with the phage ΦEa21-4 resulted in a 98% phage titre reduction. Pretreatment of the ΦEa21-4 with levan mimetic reduced the phage titre up to 78% when enriched on the wild-type isolate. This may indicate that the monosaccharide fructose bound to the phage to block binding to the polysaccharide levan, preventing primary attachment. In the absence of this transient, primary binding, permanent adsorption to the cell surface and infection may have been prevented. The binding of the fructose to the phage appears to occur very quickly, as the time of pretreatment did not alter the magnitude of the phage inhibition. Levan is produced by levansucrase in close proximity to the cell in solid media. Extracellular levan could be a stabilizing factor that holds the phage proximal to the cell wall, increasing the probability of coming into contact with a secondary receptor that serves as an entry point into the cell.

Amylovoran was virtually essential for the proliferation of some of the *Siphoviridae* and all of the *Podoviridae*. The proliferation of these phages dropped below 1% in the Δ*rcsB* mutant compared to the wild-type. When the phage was pretreated with galactose and glucuronic acid the phage titre was reduced about 50% (Fig. 15). This observation supported the hypothesis that amylovoran is involved in the adsorption process. However, the binding of phage protein lectins to carbohydrates may be a complex three dimensional process and likely requires amylovoran in its full branched, polymerized form in order to effectively bind to the phage receptor and activate it. Monomeric galactose and glucuronic acid likely do not bind the phage as effectively as the EPS to completely prevent phage infection.

Studying the phage-host interaction contributes significantly to the development of a successful biocontrol agent. In this study phages and hosts from two geographical area were used. *E. amylovora* host Ea110R and *Podoviridae* phage ΦEa1(h) were isolated from orchards in Michigan State and all the other *E. amylovora* hosts and phages were isolated in Southern Ontario. Fig. 5 shows the titres of *Myoviridae* phage ΦEa21-4 grown on four hosts (Ea29-7, Ea6-4, Ea17-1-1, and EaG-5) that were isolated in Niagara and Michigan's Ea110R. The results show a significant decrease in phage titre when grown on Ea110R and close to equal titre on the other four hosts. The inverse relationship is seen when the *Podoviridae* phage ΦEa1(h) was enriched on the same five hosts. ΦEa1(h) reached a high titre on the hosts that was isolated in the same geographical area (Ea110R) but a significant reduction in titre on the other four hosts from the Vineland collection. This host range specificity indicated that *E. amylovora* phages may have more effective infection rates on *E. amylovora* hosts from the same geographical area. These

results agree with data from British Columbia that indicated that foreign phages showed little or no lytic activity on *E. amylovora* isolates from BC (Boulé et al., 2011). To successfully utilize lytic phages as biocontrol agents for *E. amylovora* the phage-host interactions need to be satisfactorily understood and assessed for the future work which will involve the development of phage cocktails or mixtures of phage. Mixtures are required to prevent the development of resistance (Cairns and Payne, 2008). The same mixture of phage, isolated from one geographical area, may not have the same efficacy when applied in a different region.

The potential advent of bacteriophage resistance has long been one of the most detrimental criticisms of bacteriophage control (Vidaver, 1976; Carlton, 1999; Balogh et al., 2003; Labrie et al., 2010; Loc-Carrillo and Abedon, 2011). In order for bacteria to gain resistance to an obligately lytic bacteriophage it must alter the sites to which the phage binds (Lenski, 1988; Guttman et al., 2005). This resistance is known to have a detrimental effect on the fitness of the bacteria, so often the bacteria will "tolerate" some level of phage (Lenski and Levin, 1985; Smith et al., 1987; Bohannan and Lenski, 2000; Jensen et al., 2006; Zahid et al., 2008). Antibiotic and phage resistance is beneficial to the pathogen for obvious reasons, however in the absence of the antibiotic or phage, the resistant bacteria show reduced virulence and fitness compared to their susceptible counterparts. Antibiotics and bacteriophages often target important structures in or on the bacterial cell wall. Producing resistance often comes at a metabolic (other substances that need to be produced, e.g. β-lactamase) or structural cost. This fitness cost is especially

pronounced in bacterial strains that are resistant to phage infection (Smith et al., 1987; Scott et al., 2007; Zahid et al., 2008; Capparelli et al., 2010).

This presents an exciting prospect for the development of a fire blight biocontrol. If the receptor for the *E. amylovora* phages is amylovoran and amylovoran is an essential pathogenicity factor, any bacterium that modifies its EPS to become resistant to bacteriophage attack via amylovoran will inevitably become avirulent. This would result in the loss of an ecological niche for that strain of *E. amylovora*, it would not cause disease and due to environmental pressures, the populations of the mutant bacteria would decline. When used in concert with phages from other families that use other infection modalities, a phage biocontrol could be developed that includes different phage isolate that utilize various infection processes, reducing the incidence of phage resistance.

Chapter 6
Future Considerations

This work represents a continuation in the study of phage-host interactions (Gill, 2000; Lehman, 2008; Roach, 2011). The nature of the tail fiber lectins that bind to the EPS need to be further characterized and a better understanding of the nature of the EPS receptor is required. Additionally, there are a number of other potential resistance mechanisms that need to be further explored. Roach (2011) investigated the prevalence and incidence of lysogeny in *E. amylovora*, but this story is incomplete since the work did not screen for *Siphoviridae* prophages. For this reason and to investigate the curious absence of *Siphoviridae* isolates in the global collections of *E. amylovora* phages, a complete sequence and characterization of at least one phage from that family needs to be completed. Real-time PCR primers and probes can then be designed and optimized with the current screening programs. These primers can then be used to screen for *Siphoviridae* prophages and allow the *Siphoviridae* phages to be tracked in field trials using real-time PCR. Less urgent is the investigation into the presence and significance of clustered, regulated interspaced short palindromic repeat (CRISPR) sequences, another potential resistance mechanism in *E. amylovora* (Mills et al., 2009; Smits et al., 2010; Rezzonico et al., 2011). Finally, an evaluation needs to be completed to determine if abortive infection systems exist within the *E. amylovora* host-phage interactions.

After these considerations are identified and taken into account the continued development of the bacteriophage biocontrol can continue. The development of this biocontrol hinges on the Yes/No decision of whether or not the issue of phage resistance

is great enough in *E. amylovora* to prevent the successful implementation of treatment option. This work has contributed to the understanding of the interaction of EPS, HEP and LEP host isolates, and the binding sites for the *E. amylovora* bacteriophages. The understanding of these interactions is not complete. Additional studies need to be completed to choose which *Myo-*, *Sipho-*, and *Podoviridae* phages will be added to a biological control cocktail for optimal efficacy.

Chapter 7
Literature Cited

Abedon, S. T. 2003. Bacteriophages could fight plant pathogens. Ind. Bioproc. **25**:7.

Abedon, S. T. 2008. Bacteriophage ecology. Population growth, evolution, and impact of bacterial viruses. Cambridge University Press, Cambridge, UK.

Ackermann, H.-W. 2001. Frequency of morphological phage descriptions in the year 2000. Arch. Virol. **146**:843-857.

Adams, M. H. Bacteriophages. 1959. Interscience Publishers, New York.

Aksyuk, A. A. and M. G. Rossmann. 2011. Bacteriophage assembly. Viruses **3**:172-203.

Alexandrova, M., B. Cimini, C. Bazzi, E. Carpana, S. Massi, and A. G. Sabatini. 2002. The role of honeybees in spreading *Erwinia amylovora*. Acta Hort. **590**:55-60.

Anderson, L. M. and H. Yang. 2008. DNA looping can enhance lysogenic CI transcription in phage lambda. Proc. Natl. Acad. Sci USA **105**:5827-5832.

Ark, P. A. and H. E. Thomas. 1936. Persistence of *Erwinia amylovora* in certain insects. Phytopathology **26**:375-381.

Arthur, J. C. 1885. Proof that bacteria are the direct cause of the disease in trees known as pear blight. Bot. Gaz. **10**:343-345.

Ayers, A. R., S. B. Ayers, and R. N. Goodman. 1979. Extracellular polysaccharide of *Erwinia amylovora*: a correlation with virulence. Appl. Environ. Microbiol. **38**:659-666.

Azegami, K., T. Matsuura, Y. Inoue, A. Sasaki, and T. Shimane. 2008. Anthers act as "tinder" and "reservoir" for *Erwinia amylovora* to infect pear blossoms - Observation of *Erwinia amylovora* tagged with bioluminescence genes on pear surfaces. Acta Hort. **793**:95-98.

Balogh, B., B. I. Canteros, R. E. Stall, and J. B. Jones. 2008. Control of citrus canker and citrus bacterial spot with bacteriophages. Plant Dis. **92**:1048-1052.

Balogh, B., J. B. Jones, M. T. Momol, S. M. Olson, A. Obradovic, P. King, and L. E. Jackson. 2003. Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. Plant Dis. **87**:949-954.

Barny, M. A., M. H. Guinebretiére, B. Marçais, E. Coissac, J. P. Paulin, and J. Laurent. 1990. Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. Mol. Microbiol. **4**:777-786.

Bayer, M. E., H. Thurow, and M. H. Bayer. 1979. Penetration of the polysaccharide capsule of *Escherichia coli* (Bi161/42) by bacteriophage K29. Virology **94**(1): 95-118.

Beer, S. V. and J. L. Norelli. 1977. Fire blight epidemiology: factors affecting release of *Erwinia amylovora* by cankers. Phytopathology **67**:1119-1125.

Belleman, P. and K. Geider. 1992. Localization of transposon insertions in pathogenic mutants of *Erwinia amylovora* and their biochemical characterization. J. Gen.

Microbiol. **138**:931-940.

Bellemann, P., S. Bereswill, S. Berger, and K. Geider. 1994. Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. Int. J. Biol. Macromol. **16**:290-296.

Bennett, R. A. 1980. Evidence for two virulence determinants in the fireblight pathogen *Erwinia amylovora*. J. Gen. Microbiol. **116**:351-356.

Bennett, R. A. and E. Billing. 1978. Capsulation and virulence in *Erwinia amylovora*. Ann. Appl. Biol. **89**:41-45.

Bereswill, S. and K. Geider. 1997. Characterization of the *rcsB* gene from *Erwinia amylovora* and its influence on exopolysaccharide synthesis and virulence of the fire blight pathogen. J. Bacteriol. **179**:1354-1361.

Bernhard, F., K. Poetter, K. Geider, and D. L. Coplin. 1990. The *rcsA* gene from *Erwinia amylovora*: Identification, nucleotide sequence, and regulation of exopolysaccharide biosynthesis. Mol. Plant-Microbe Interact. **3**:429-437.

Biggs, A. R. 1994. Characteristics of fire blight cankers following shoot inoculations of three apple cultivars. HortScience **29**:795-797.

Billing, E. 1960. An association between capsulation and phage sensitivity in *Erwinia amylovora*. Nature **186**:819-820.

Billing, E. 1984. Studies on avirulent strains of *Erwinia amylovora*. Acta Hort. **151**:249-254.

Billing, E. 2007. Challenges in adaptation of plant disease warning systems to new locations: re-appraisal of Billing's integrated system for predicting fire blight in a warm dry environment. Phytopathology **97**:1036-1039.

Billing, E. 2008. Testing theories on over-wintering twig blight, pre-bloom disease, shoot blight and stem invasion. Acta Hort. **793**:105-115.

Billing, E. 2011. Fire blight. Why do views on host invasion by *Erwinia amylovora* differ? Plant Pathol. **60**:178-189.

Blachinsky, D., D. Shtienberg, E. Zamski, D. Weinthal, and S. Manulis. 2006. Effects of pear tree physiology on fire blight progression in perennial branches and on expression of pathogenicity genes in *Erwinia amylovora*. Eur. J. Plant. Pathol. **116**:315-324.

Boerlin, P. 2010. Implications of antimicrobial agents as therapeutics and growth promoters in food animal production. ASM Press, Washington, D.C.

Bogs, J., I. Bruchmüller, C. Erbar, and K. Geider. 1998. Colonization of host plants by the fire blight pathogen *Erwinia amylovora* marked with genes for bioluminescence and fluorescence. Phytopathology **88**:416-421.

Bohannan, B. J. M. and R. E. Lenski. 2000. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. Ecol. Lett. **3**:362-377.

Bonn, W. G. and T. van der Zwet. 2000. Distribution and economic importance of fire blight, *In* J. L. Vanneste (ed.), Fire blight: the disease and its causative agent, *Erwinia amylovora.* CABI Publishing, Wallingford, United Kingdom.

Born, Y., L. Fieseler, J. Marazzi, R. Lurz, B. Duffy, and M. J. Loessner. 2011. Novel virulent and broad-host-range *Erwinia amylovora* bacteriophages reveal a high degree of mosaicism and a relationship to *Enterobacteriaceae* phages. Appl. Environ. Microbiol. **77**:5945-5954.

Boulé, J., P. L. Sholberg, S. M. Lehman, D. T. O'gorman, and A. M. Svircev. 2011. Isolation and characterization of eight bacteriophages infecting *Erwinia amylovora* and their potential as biological control agents in British Columbia, Canada. Can. J. Plant Path. **33**:308-317.

Broadbent, J. R., D. J. McMahon, D. L. Welker, C. J. Oberg, and S. Moineau. 2003. Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: a review. J. Dairy Sci. **86**:407-423.

Brüssow, Harald and Hendrix, Roger W. 2002. Phage genomics: small is beautiful. Cell **108**(1):13-16.

Brüssow, H., M. Fremont, A. Bruttin, J. Sidoti, A. Constable, and V. Fryder. 1994. Detection and classification of *Streptococcus thermophilus* bacteriophages isolated from industrial milk fermentation. Appl. Environ. Microbiol. **60**:4537-4543.

Bugert, P., S. Bereswill, K. Geider, and W. G. Bonn. 1996. Regulation and structure of the ams-region involved in exopolysaccharide synthesis of *Erwinia amylovora.* Acta Hort. **411**:275-280.

Bugert, P. and K. Geider. 1995. Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora.* Mol. Microbiol. **15**:917-933.

Burrill, T. J. 1883. Botany. New species of *Mycrococcus* (bacteria). Am. Nat. **17**:319.

Cairns, B. J. and R. J. H. Payne. 2008. Bacteriophage therapy and the mutant selection window. Antimicrob. Agent. Chemo. **52**:4344-4350.

Calenge, F., D. Drouet, C. Denancé, W. E. Van de Weg, M.-N. Brisset, J.-P. Paulin, and C.-E. Durel. 2005. Identification of a major QTL together with several minor additive or epistatic QTLs for resistance to fire blight in apple in two related progenies. Theor. Appl. Genet. **111**:128-135.

Canchaya, C., Fournous, G., and Brüssow, H. 2004. The impact of prophages on bacterial chromosomes. Mol. Microbiol. **53**(1):9-18.

Capparelli, R., N. Nocerino, R. Lanzetta, A. Silipo, A. Amoresano, C. Giangrande, K. Becker, G. Blaiotta, A. Evidente, A. Cimmino, M. Iannaccone, M. Parlato, C. Medaglia, S. Roperto, F. Roperto, L. Ramunno, and D. Iannelli. 2010. Bacteriophage resistant *Staphylococcus aureus* mutant confers broad immunity against staphylococcal infection in mice. PLoS One **5**:e11720.

Carlton, R. M. 1999. Phage therapy: past history and future prospects. Arch. Immunol. Ther. Ex. **47**:267-274.

Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Rev. **87**:113-130.

Chanishvili, N., T. Chanishvili, M. Tediashvili, and P. A. Barrow. 2001. Phages and their application against drug-resistant bacteria. J. Chem. Technol. Biotech. **76**:689-699.

Civerolo, E. L. and H. L. Keil. 1969. Inhibition of bacterial spot of peach foliage by *Xanthomonas pruni* bacteriophage. Phytopathology **59**:1966-1967.

Clark, J. R. and J. B. March. 2006. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. Trends Biotech. **24**:212-218.

Coffey, B., L. Rivas, G. Duffy, A. Coffey, R. P. Ross, and O. McAuliffe. 2011. Assessment of *Escherichia coli* O157:H7-specific bacteriophages e11/2 and e4/1c in model broth and hide environments. Int. J. Food. Microbiol. **147**:188-194.

Costa, G., C. Andreotti, F. Bucchi, E. Sabatini, C. Bazzi, S. Malaguti, and W. Rademacher. 2001. Prohexadione-Ca (Apogee®): Growth regulation and reduced fire blight incidence in pear. HortScience **36**:931-933.

Crosse, J. E., M. Bennett, and C. M. E. Garrett. 1958. Fire-blight of pear in England. Nature **182**:1530.

Czajkowski, R., M. C. M. Perombelon, J. A. Van Veen, and J. M. Van der Wolf. 2011. Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. Plant Path. **60**:999-1013.

d'Herelle, F. 1917. Sur un microbe invisible antagoniste des bacilles dysenteriques. Comptes Rendus Acad. Sci. **165**:373-375.

Dandekar, A. M. and V. V. Modi. 1978. Interaction between *Rhizobium japonicum* phage M-1 and its receptor. Can. J. Microbiol. **24**:685-688.

Datsenko, K. A. and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA

97:6640-6645.

Davies, R. H. and M. Breslin. 2003. Persistence of *Salmonella enteritidis* phage type 4 in the environment and arthropod vectors on an empty free-range chicken farm. Environ. Microbiol. **5**:79-84.

Davison, W. C. 1922. Observations on the nature of bacteriolysants (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.). J. Bacteriol. **7**:491-504.

Defives, C., M. Werquin, P. Mary, and J. P. Hornez. 1996. Roles of exopolysaccharides and lipopolysaccharides in the adsorption of the *Siphovirus* phage NM8 to *Rhizobium meliloti* M11S cells. Curr. Microbiol. **33**:371-376.

Denning, W. 1794. On the decay of apple trees. NY Soc. Promot. Agric. Arts Man. Trans. **2**:219-222.

Deveau, H., M.-R. Van Calsteren, and S. Moineau. 2002. Effect of exopolysaccharides on phage-host interactions in *Lactococcus lactis*. Appl. Eviron. Microbiol. **68**:4364-4369.

Duckworth, D. H. 1976. "Who discovered bacteriophage?". Bacteriol. Rev. **40**:793-802.

Duckworth, D. H. 1987. History and basic properties of bacterial viruses, p. 320. *In* S. M. Goyal, C. P. Gerba, and G. Bitton (ed.), Phage ecology. John Wiley & Sons, New York.

Dunnett, C.W. 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Soc. **50**(120): 1096-1121.

Eiserling, F. A. and L. W. Black. 1994. Pathways in T4 morphogenesis, p. 209-212. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. ASM Press, Washington, D.C.

Emmett, B. J. 1971. Insect visitors to pear blossom. Plant Path. **20**:36-40.

Emmett, B. J. and L. A. E. Baker. 1971. Insect transmission of fireblight. Plant Path. **20**:41-45.

European and Mediterranean Plant Protection Organization. 1983. *Erwinia amylovora* (Burrill 1882) Winslow et al. 1920. EPPO **13**:1E-7E.

Farkas, A., E. Mihalik, L. Dorgai, and T. Bubán. 2012. Floral traits affecting fire blight infection and management. Trees **26**:1-20.

Fisher, M. B., D. C. Love, R. Schuech, and K. L. Nelson. 2011. Simulated sunlight action spectra for inactivation of MS2 and PRD1 bacteriophages in clear water. Environ. Sci. Tech. **45**:9249-9255.

Flaherty, J. E., J. B. Jones, B. K. Harbaugh, G. C. Somodi, and L. E. Jackson. 2000. Control of bacterial spot on tomato in the greenhouse and field with h-mutant bacteriophages. HortScience **35**:882-884.

Flaherty, J. E., B. K. Harbaugh, J. B. Jones, G. C. Somodi, and L. E. Jackson. 2001. H-mutant bacteriophages as a potential biocontrol of bacterial blight of geranium. HortScience **36**:98-100.

Flemming, A. 1929. On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. Brit. J. Exp. Pathol. **10**:226-

236.

Forde, A. and G. F. Fitzgerald. 1999. Analysis of exopolysaccharide (EPS) production mediated by the bacteriophage adsorption blocking plasmid, pCI658, isolated from *Lactococcus lactis* ssp. *cremoris* HO2. Int. Dairy J. **9**:465-472.

Fujiwara, A., M. Fujisawa, R. Hamasaki, T. Kawasaki, M. Fujie, and T. Yamada. 2011. Biocontrol of *Ralstonia solanacearum* by treatment with lytic bacteriophages. Appl. Envrion. Microbiol. **77**:4155-4162.

Geider, K. 2000. Exopolysaccharides of *Erwinia amylovora*: structure, biosynthesis, regulation, role in pathogenicity of amylovoran and levan, p. 117-140. *In* J. L. Vanneste (ed.), Fire blight: the disease and its causative agent, *Erwinia amylovora*. CABI Publishing, Wallingford, United Kingdom.

Geider, K. 2006. Twenty years of molecular genetics with *Erwinia amylovora*: answers and new questions about EPS-synthesis and other virulence factors. Acta Hort. **704**:397-402.

Geider, K., G. Geier, P. Bellemann, F. Bernhard, P. Bugert, and M. Metzger. 1993. Exopolysaccharides in pathogenicity of *Erwinia amylovora*. Acta Hort. **338**:255-262.

Geier, G. and K. Geider. 1993. Characterization and influence on virulence of the levansucrase gene from the fire blight pathogen *Erwinia amylovora*. Physiol. Mol. Plant Pathol. **42**:387-404.

Geier, M. R., M. E. Trigg, and C. R. Merril. 1973. Fate of bacteriophage lambda in non-immune germ-free mice. Nature **246**:221-223.

Gill, J. and S. T. Abedon. 2003. Bacteriophage Ecology and Plants. APSnet Features, Online at http://www.apsnet.org/publications/apsnetfeatures/Pages/Bacteriophage Ecology.aspx.

Gill, J. J. Bacteriophages of *Erwinia amylovora* and their potential use in biological control. 2000. M.Sc. Thesis. Brock University, St. Catharines, Ontario, Canada.

Gill, J. J., A. M. Svircev, R. Smith, and A. J. Castle. 2003. Bacteriophages of *Erwinia amylovora*. Appl. Environ. Microbiol. **69**:2133-2138.

Goldberg, E., L. Grinius, and L. Leteller. 1994. Recognition, attachment, and injection, p. 347-356. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. ASM Press, Washington, D.C.

Goodman, R. N., J. S. Huang, and P.-Y. Huang. 1974. Host-specific phytotoxic polysaccharide from apple tissue infected by *Erwinia amylovora*. Science **183**:1081-1082.

Goodridge, L. D. 2004. Bacteriophage biocontrol of plant pathogens: fact or fiction? Trends Biotechnol. **22**:384-385.

Gross, M., G. Geier, K. Rudolp, and K. Geider. 1992. Levan and levansucrase synthesized by the fireblight pathogen *Erwinia amylovora*. Physiol. Mol. Plant Pathol. **40**:371-381.

Gupta, R. and Y. Prasad. 2011. Efficacy of polyvalent bacteriophage P-27/HP to control multidrug resistant *Staphylococcus aureus* associated with human infections. Curr. Microbiol. **62**:255-260.

Guttman, F. J., R. Raya, and E. Kutter. 2005. Basic phage biology, p. 29-66. *In* E. Kutter and A. Sulakvelidze (ed.), Bacteriophages: biology and applications. CRC Press, Boca Raton, Fla.

Harbaugh, B. K., J. B. Jones, J. E. Flaherty, and L. E. Jackson. 1998. Beneficial viruses-new allies in the fight against bacterial diseases. GrowerTalks **10**:84-87.

Henning, U. and K. Jann. 1979. Two-component nature of bacteriophage T4 receptor activity in *Escherichia coli* K-12. J. Bacteriol. **137**:664-666.

Hignett, R. C. 1988. Effects of growth conditions on the surface structures and extracellular products of virulent and avirulent forms of *Erwinia amylovora*. Physiol. Mol. Plant Pathol. **32**(3):387-394.

Hildebrand, E. M. 1939. Studies of fire blight ooze. Phytopathology **29**:142-156.

Hildebrand, E. M. and A. J. Heinicke. 1937. Incidence of fire blight in young apple trees in relation to orchard practices. NY Agri. St. Memo **203**:1-12.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. Holt, J. G., Krieg, N. R., Sneath, P. H. A, Staley, J. T., and Williams, S. T. 1994. Bergey's manual of determinative bacteriology. Williams and Wilkins, Baltimore.

Holtz, B. A., T. Martin-Duvall, J. E. Adaskaveg, and S. E. Lindow. 2008. Efficacy of bactericides and biological antagonists for the control of fire blight of apple in the San Joaquin Valley of California. Acta Hort. **793**:445-450.

Hooton, S. P. T., R. J. Atterbury, and I. F. Connerton. 2011. Application of a bacteriophage cocktail to reduce *Salmonella typhimurium* U288 contamination on

pig skin. Int. J. Food. Microbiol. **151**:157-163.

Hughes, K. A., I. W. Sutherland, J. Clark, and M. V. Jones. 1998a. Bacteriophage and associated polysaccharide depolymerases - novel tools for study of bacterial biofilms. J. Appl. Microbiol. **85**:583-590.

Hughes, K. A., I. W. Sutherland, and M. V. Jones. 1998b. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbiology **144**:3039-3047.

Hyman, P. and S. T. Abedon. 2010. Bacteriophage host range and bacterial resistance. Adv. Appl. Microbiol. **70**:217-248.

Ignoffo, C. M. and C. Garcia. 1992. Combinations of environmental factors and simulated sunlight affecting activity of inclusion bodies of the Heliothis (*Lepidoptera: Noctuidae*) ucleopolyhedrosis virus. Environ. Entomol. **21**:210-213.

Ignoffo, C. M. and C. Garcia. 1994. Antioxidant and oxidative enzyme effects on the inactivation of inclusion bodies of the Heliothis baculovirus by simulated sunlight-UV. Environ. Entomol. **23**:1025-1029.

Ignoffo, C. M., C. Garcia, and S. G. Saathoff. 1997. Sunlight stability and rain-fastness of formulations of baculovirus Heliothis. *Environ. Entomol.* **26**:1470-1474.

Inchley, C. J. 1969. The activity of mouse kupffer cells following intravenous injection of T4 bacteriophage. Clin. Exp. Immun. **5**:173-187.

Iriarte, F. B., B. Balogh, M. T. Momol, L. M. Smith, M. Wilson, and J. B. Jones.

2007. Factors affecting survival of bacteriophage on tomato leaf surfaces. Appl. Environ. Microbiol. **73**:1704-1711.

Ishimaru, C. A., E. J. Klos, and R. R. Brubaker. 1988. Multiple antibiotic production by *Erwinia herbicola*. Phytopathology **78**:746-750.

Jackson, L. E. May 1989. U.S. patent 4,828,999.

Jensen, M. A., S. M. Faruque, J. J. Mekalanos, and B. R. Levin. 2006. Modeling the role of bacteriophage in the control of cholera outbreaks. Proc. Natl. Acad. Sci. USA **103**:4652-4657.

Jin, M., L. Liu, S. A. I. Wright, S. V. Beer, and J. Clardy. 2003. Structural and functional analysis of pantocin A: an antibiotic from *Pantoea agglomerans* discovered by heterologous expression of cloned genes. Angew. Chem. Int. Ed. **42**:2898-2901.

Johnson, K. B., T. L. Sawyer, V. O. Stockwell, and T. N. Temple. 2008. Implications of pathogenesis by *Erwinia amylovora* on *Rosaceous* stigmas to biological control of fire blight. Phytopathology **99**:128-138.

Johnson, K. B. and V. O. Stockwell. 1998. Management of fire blight : a case study in microbial ecology. Annu. Rev. Phytopathol. **36**:227-248.

Johnson, K. B., V. O. Stockwell, and T. L. Sawyer. 2004. Adaptation of fire blight forecasting to optimize the use of biological controls. Plant Dis. **88**:41-48.

Jones, J. B., L. E. Jackson, B. Balogh, A. Obradovic, F. B. Iriarte, and M. T. Momol. 2007. Bacteriophages for plant disease control. Ann. Rev. Phytopath. **45**:245-262.

Kabaluk, J. T., V. R. Brookes, and A. M. Svircev. 2010. Canada, p. 59-73. IOBC Global, Available online through www.IOBC-Global.org.

Kazempour, M. N., E. Kamran, and B. Ali. 2006. *Erwinia amylovora* causing fire blight of pear in the Guilan province of Iran. J. Plant Pathol. **88**:113-116.

Kearns, L. P. and H. K. Mahanty. 1998. Antibiotic production by *Erwinia herbicola* Eh1087: its role in inhibition of *Erwinia amylovora* and partial characterization of antibiotic biosynthesis genes. Appl. Environ. Microbiol. **64**:1837-1844.

Khan, M. A., Y. F. Zhao, and S. S. Korban. 2012. Molecular mechanisms of pathogenesis and resistance to the bacterial pathogen *Erwinia amylovora*, causal agent of fire blight disease in *Rosaceae*. Plant Mol. Biol. Rep. **30**(2):247-260.

Koczan, J. M., B. R. Lenneman, M. J. McGrath, and G. W. Sundin. 2011. Cell surface attachment structures contribute to biofilm formation and xylem colonization of *Erwinia amylovora*. Appl. Environ. Biol. **77**:7031-7039.

Koczan, J. M., M. J. McGrath, Y. Zhao, and G. W. Sundin. 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. Phytopathology **99**:1237-1244.

Kong, X. and D. Li. 2011. Hydrogen peroxide is not involved in HrpN from *Erwinia amylovora*-induced hypersensitive cell death in maize leaves. Plant Cell Rep. **30**:1273-1279.

Labrie, S. J., J. E. Samson, and S. Moineau. 2010. Bacteriophage resistance mechanisms. Nat. Rev. **8**:317-327.

Lagonenko, A. L., V. S. Komardina, Y. A. Nikolaichik, and A. N. Evtushenkov. 2008. First report of *Erwinia amylovora* fire blight in Belarus. J. Phytopathol. **156**:638-640.

Lalli, D. A., T. S. Artlip, M. E. Wisniewski, J. L. Norelli, and R. E. Farrell. 2008. Transgenic expression of *Erwinia amylovora* effectors *eop1* and *hopCEa* in apple. Acta Hort. **793**:241-245.

Lang, J. M., D. H. Gent, and H. F. Schwartz. 2007. Management of *Xanthomonas* leaf blight of onion with bacteriophages and a plant activator. Plant Dis. **91**:871-878.

Langlotz, C., M. Schollmeyer, D. L. Coplin, M. Nimtz, and K. Geider. 2011. Biosynthesis of the repeating units of the exopolysaccharides amylovoran from *Erwinia amylovora* and stewartan from *Pantoea stewartii*. Physiol. Mol. Plant Pathol. **75**:163-169.

Lech, K. and R. Brent. 1987. Introduction to lambda phages, current protocols in molecular iology. John Wiley & Sons, Inc. New York.

Lee, S. A., H. K. Ngugi, N. O. Halbrendt, G. O'Keefe, B. Lehman, J. W. Travis, J. P. Sinn, and T. W. McNellis. 2010. Virulence characteristics accounting for fire blight disease severity in apple trees and seedlings. Phytopathology **100**:539-550.

Lehman, S. M. Development of a bacteriophage-based biopesticide for fire blight. 2008. Ph.D. Thesis. Brock University, St. Catharines, Ontario, Canada.

Leigh, J. A. and D. L. Coplin. 1992. Exopolysaccharides in plant-bacterial interactions. Ann. Rev. Microbiol. **46**:307-346.

Lenski, R. E. 1988. Dynamics of interactions between bacteria and virulent bacteriophage. Adv. Microb. Ecol. **10**:1-44.

Lenski, R. E. and B. R. Levin. 1985. Constraints on the coevolution of bacteria and virulent phage: a model, some experiments, and predictions for natural communities. Am. Nat. **125**:585-602.

Leverentz, B., W. S. Conway, M. J. Camp, W. J. Janisiewicz, T. Abuladze, M. Yang, R. Saftner, and A. Sulakvelidze. 2003. Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. Appl. Environ. Microbiol. **69**:4519-4526.

Li, J. and J. J. Dennehy. 2011. Differential bacteriophage mortality on exposure to copper. Appl. Environ. Microbiol. **77**:6878-6883.

Lindberg, A. 1973. Bacteriophage receptors. Ann. Rev. Microbiol. **27**:205-241.

Lindberg, A. A. 1977. Bacterial surface carbohydrates and bacteriophage adsorption, *In* I. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, London.

Lindow, S. and M. Wilson. 1993. Population dynamics of *Pseudomonas fluorescens* strain A506 in pear flowers following inoculation in relation to strategies of biological control of fire blight and frost injury. Acta Hort. **338**:331-332.

Little, J. W. 2005. Lysogeny, prophage induction, and lysogenic conversion, p. 37-54. *In* M. K. Waldor, D. I. Friedman, and S. L. Adhya (ed.), Phages: their role in bacterial pathogenesis and biotechnology. ASM Press, Washington, D.C.

Loc-Carrillo, C. and S. T. Abedon. 2011. Pros and cons of phage therapy. Bacteriophage **1**:111-114.

Loescher, W. H. 1987. Physiology and metabolism of sugar alcohols in higher plants. Physiol. Plantarum **70**:553-557.

Looijesteijn, P. J., L. Trapet, E. de Vries, T. Abee, and J. Hugenholtz. 2001. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. Int. J. Food Microbiol. **64**:71-80.

Loper, J. E. and M. D. Henkels. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington State. Plant Dis. **75**:287-290.

Luria, S. E. 1945. Mutations of bacterial viruses affecting their host range. Genetics **30:**84-99.

Luria, S. E. and M. Delbrück. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491-511.

Lévesque, C., M. Duplessis, J. Labonté, S. Labrie, C. Fremaux, D. Tremblay, and S. Moineau. 2005. Genomic organization and molecular analysis of virulent bacteriophage 2972 infecting an exopolysaccharide-producing *Streptococcus thermophilus* strain. Appl. Environ. Microbiol. **71**:4057-4068.

Marks, T. and R. Sharp. 2000. Bacteriophages and biotechnology: a review. J. Clin. Technol. Biotechnol. **75**:6-7.

Maxson, K. L. and Jones. A.L. 2002. Managment of fire blight with gibberellin

inhibitors and SAR inducers. Acta Hort. **590**:217-224.

McKenna, F., K. A. El-Tarabily, G. E. S. J. Hardy, and B. Dell. 2001. Novel in vivo use of polyvalent *Streptomyces* phage to disinfest *Streptomyces scabies*-infected seed potatoes. Plant Path. **50**:666-675.

McManus, P. S. and A. L. Jones. 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathology **84**:627-633.

McNally, R. R., I. K. Toth, P. J. A. Cock, L. Pritchard, P. E. Hedley, J. A. Morris, Y. Zhao, and G. W. Sundin. 2012. Genetic characterization of the HrpL regulon of the fire blight pathogen *Erwinia amylovora* reveals novel virulence factors. Mol. Plant Pathol. **13**:160-173.

Meijneke, C. A. R. 1974. The 1971 outbreak of fire blight in the Netherlands. Int. Hort. Congress **2**:373-382.

Mills, S., C. Griffin, A. Coffey, W. C. Meijer, B. Hafkamp, and R. P. Ross. 2009. CRISPR analysis of bacteriophage-insensitive mutants (BIMs) of industrial *Streptococcus thermophilus* – implications for starter design. J. App. Microbiol. **108**:945-955.

Misstear, D. B. and L. W. Gill. 2012. The inactivation of phages MS2, p174 and PR772 using UV and solar photocatalysis. J Photoch. Photobiol. **107**:1-8.

Momol, M. and Aldwinckle, H. S. 2000. Genetic diversity and host range of *Erwinia amylovora*, p. 55-72. *In* J. L.Vanneste (ed.), Fire blight: the disease and its causative agent, *Erwinia amylovora.* CABI Publishing, Wallingford, United

Kingdom.

Monsur, K. A., M. A. Rahman, F. Huq, M. N. Islam, R. S. Northrup, and N. Hirschhorn. 1970. Effect of massive doses of bacteriophage on excretion of vibrios, duration of diarrhoea and output of stools in acute cases of cholera. B. World Health Organ. **42**:723-732.

Moriguchi, Takaya, Abe, Kazuyuki, Sanada, Tetsuro, and Yamaki, Shohei. 1992. Levels and role of sucrose synthase, sucrose-phosphate synthase, and acid invertase in sucrose accumulation in fruit of Asian pear. J. Am. Soc. Hort. Sci. **117**(2):274-278.

Muller, I., R. Lurz, M. Kube, C. Quedenau, W. Jelkmann, and K. Geider. 2011. Molecular and physiological properties of bacteriophages from North America and Germany affecting the fire blight pathogen *Erwinia amylovora*. Microb. Biotech. **4**:735-745.

Nagy, J. K., L. Király, and I. Schwarczinger. 2012. Phage therapy for plant disease control with a focus on fire blight. Cent. Eur. J. Biol. **7**:1-12.

Nimtz , M., A. Mort, T. Domke, V. Wray, Y. Zhang, F. Qiu, D. Coplin, K. Geider, and Y. X. Zhang. 1996. Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. Carbohyd. Res. **287**:59-76.

Obradovic, A., J. B. Jones, M. T. Momol, B. Balogh, and S. M. Olson. 2004. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. Plant Dis. **88**:736-740.

Obradovic, A., J. B. Jones, M. T. Momol, S. M. Olson, L. E. Jackson, B. Balogh, K.

Guven, and F. B. Iriarte. 2005. Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. Plant Dis. **89**:712-716.

Oh, C.-S. and S. V. Beer. 2005. Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. Microbiol. Lett. **253**:185-192.

Okabe, N. and M. Goto. 1963. Bacteriophages of plant pathogens. Annu. Rev. Phytopathology **1**:397-418.

OMAFRA. 2011. Publication 360: Fruit production recommendations 2010 - 2011, Ontario Minstry of Agriculture, Food and Rural Affairs, Toronto, Ontario, Canada.

Park, B. H. 1956. An enzyme produced by a phage-host cell system: the properties of a *Klebsiella* phage. Virology **2**:711-718.

Payne, R. J. H. and V. A. A. Jansen. 2001. Understanding bacteriophage therapy as a density-dependent kinetic process. J. Theor. Biol. **208**:37-48.

Pelkonen, S., J. Aalto, and J. Finne. 1992. Differential activities of bacteriophage depolymerase on bacterial polysaccharide: Binding is essential but degradation is inhibitory in phage infection of K1-defective *Escherichia coli*. J. Bacteriol. **174**:7757-7761.

Pestka, S. 1971. Inhibitors of ribosome functions. Ann. Rev. **25**:487-562.

Pusey, L. P., D. R. Rudell, E. A. Curry, and J. P. Mattheis. 2008. Characterization of stigma exudates in aqueous extracts from apple and pear flowers. HortScience

43:1471-1478.

Pusey, P. L. 1997. Crab apple blossoms as a model for research on biological control of fire blight. Biol. Cont. **87**:1096-1102.

Pusey, P. L. and E. A. Curry. 2004. Temperature and pomaceous flower age related to colonization by *Erwinia amylovora* and antagonists. Phytopathology **94**:901-911.

Pusey, P. L., V. O. Stockwell, and M. Mazzola. 2009. Epiphytic bacteria and yeasts on apple blossoms and their potential as antagonists of *Erwinia amylovora*. Phytopathology **99**:571-581.

Quamme, H. A., F. Kappel, and J. W. Hall. 1990. Efficacy of early selection for fire blight resistance and the analysis of combining ability for fire blight resistance in several pear progenies. Can. J. Plant Sci. **70**: 905-913.

Quiberoni, A., D. Guglielmotti, A. Binetti, and J. Reinheimer. 2004. Characterization of three *Lactobacillus delbrueckii* subsp. *bulgaricus* phages and the physicochemical analysis of phage adsorption. J. App. Microbiol. **96**:340-351.

Ravensdale, M., T. J. Blom, J. A. Gracia-Garza, A. M. Svircev, and R. J. Smith. 2007. Bacteriophages and the control of *Erwinia carotovora* subsp. *carotovora*. Can. J. Plant Pathol. **29**:121-130.

Rezzonico, F., B. Duffy, and V. O. Stockwell. 2008. Absence of streptomycin resistance genes in antibiotic formulations. Acta Hort. **793**:415-418.

Rezzonico, F., T. H. M. Smits, and B. Duffy. 2011. Diversity, evolution, and functionality of clustered regularly interspaced short palindromic repeat

(CRISPR) regions in the fire blight pathogen *Erwinia amylovora*. Appl. Environ. Microbiol. **77**:3819-3829.

Roach, D. R. *Erwinia amylovora* bacteriophage resistance. 2011. Ph.D. Thesis. Brock University, St. Catharines, Ontario, Canada.

Rodríguez, C., R. Van Der Meulen, F. Vaningelgem, G. Font De Valdez, R. Raya, L. De Vuyst, and F. Mozzi. 2008. Sensitivity of capsular-producing *Streptococcus thermophilus* strains to bacteriophage adsorption. Lett. App. Microbiol. **46**:462-468.

Russo, N. L., T. L. Robinson, G. Fazio, and H. S. Aldwinckle. 2008. Fire blight resistance of Budagovsky 9 apple rootstock. Plant Dis. **92**:385-391.

Russo, N. L., T. L. Robinson, G. Fazio, and H. S. Aldwinckle. 2007. Field evaluation of 64 apple rootstocks for orchard performance and fire blight resistance. HortScience **42**:1517-1525.

Saccardi, A., E. Gambin, M. Zaccardelli, G. Barone, and U. Mazzucchi. 1993. *Xanthomonas campestris* pv. *pruni* control trials with phage treatments on peach in the orchard. Phytopath. Medit. **32**:206-210.

Sato, M. 1983. Phage induction from lysogenic strains of *Pseudomonas syringae* pathovar mori by the extract from mulberry leaves. Ann. Phytopathol. Soc. Japan **49**:259-261.

Schnabel, E. L. and A. L. Jones. 2001. Isolation and characterization of five *Erwinia amylovora* bacteriophages and assessment of phage resistance in strains of *Erwinia amylovora*. Appl. Environ. Microbiol. **67**:59-64.

Scholl, D., S. Adhya, and C. Merril. 2005. *Escherichia coli* K1's capsule is a barrier to bacteriophage T7. Appl. Environ. Microbiol. **71**:4872-4874.

Schollmeyer, M., C. Langlotz, A. Huber, D. L. Coplin, and K. Geider. 2012. Variations in the molecular masses of the capsular exopolysaccharides amylovoran, pyrifolan and stewartan. Int. J. Biol. Macromol. **50**:518-522.

Schroth, M. N., S. V. Thompson, D. C. Hildebrand , and W. J. Moller. 1974. Epidemiology and control of fire blight. Ann. Rev. Phytopathol. **12**:389-412.

Scott, A. E., A. R. Timms, P. L. Connerton, C. Loc Carrillo, K. Adzfa Radzum, and I. F. Connerton. 2007. Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. PLoS Path. **3**:e119.

Sinn, J. P., C. Oh, P. J. Jensen, S. C. D. Carpenter, S. V. Beer, and T. W. McNellis. 2008. The C-terminal half of the HrpN virulence protein of the fire blight pathogen *Erwinia amylovora* is essential for its secretion and for its virulence and avirulence activities. Mol. Plant Microb. Int. **21**:1387-1397.

Smith, H. W., M. B. Huggins, and K. M. Shaw. 1987. The control of experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. J. Gen. Microbiol. **133**:1111-1126.

Smits, T. H. M., F. Rezzonico, and B. Duffy. 2011. Evolutionary insights from *Erwinia amylovora* genomics: new frontiers in microbial genome research. J. Biotech. **155**:34-39.

Smits, T. H. M., F. Rezzonico, T. Kamber, J. Blom, A. Goesmann, J. E. Frey, and B. Duffy. 2010. Complete genome sequence of the fire blight pathogen *Erwinia*

amylovora CFBP 1430 and comparison to other *Erwinia spp*. Mol. Plant Microb. Int. **23**:384-393.

Spinelli, F., F. Ciampolini, M. Cresti, K. Geider, and G. Costa. 2005. Influence of stigmatic morphology on flower colonization by *Erwinia amylovora* and *Pantoea agglomerans*. Eur. J. Plant Pathol. **113**:395-405.

Spinelli, F., J. L. Vanneste, F. Ciampolini, M. Cresti, W. Rademacher, K. Geider, and G. Costa. 2007. Potential and limits of acylcyclohexanediones for the control of blossom blight in apple and pear caused by *Erwinia amylovora*. Plant Pathol. **56**:702-710.

Statistics Canada. http://www.statcan.gc.ca/pub/22-003-x/2011001/t029-eng.pdf . 2010. Feb 2012.

Steinberger, E. M. and S. V. Beer. 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. Mol. Plant Microbe Int. **1**:135-144.

Steiner, P. W. 2000. Integrated orchard and nursery management for the control of fire blight, p. 339-358. *In* J. L. Vanneste (ed.), Fire blight: the disease and its causative agent *Erwinia amylovora*. CABI Publishing, Wallingford, United Kingdom.

Stewart, A. 2001. Commercial biocontrol - reality or fantasy? Austral. Plant Pathol. **30**:127-131.

Stockwell, V. O., R. J. McLaughlin, M. D. Henkels, J. E. Loper, D. Sugar, and R. G. Roberts. 1999. Epiphytic colonization of pear stigmas and hypanthia by bacteria during primary bloom. Phytopathology **89**:1162-1168.

Stummeyer, K., A. Dickmanns, M. Muhlenhoff, R. Gerardy-Schahn, and R. Ficner. 2005. Crystal structure of the polysialic acid-degrading endosialidase of bacteriophage K1F. Nat. Struct. Mol. Biol. **12**:90-96.

Stummeyer, K., D. Schwarzer, H. Claus, U. Vogel, R. Gerardy-Schahn, and M. Muhlenhoff. 2006. Evolution of bacteriophages infecting encapsulated bacteria: Lessons from *Escherichia Coli* K1-specific phages. Mol. Microbiol. **60**:1123-1135.

Summers, W. C. Summers, W. C. 1999. Felix d'Herelle and the origins of molecular biology. Yale University Press, New Haven, Conn.

Summers, W. C. 2001. Bacteriophage therapy. Annu. Rev. Microbiol. **55**:437-451.

Sutherland, I. W. 1972. Bacterial exopolysaccharides. Ad. Microb. Physiol. **8**:143-213.

Sutherland, I. W. 1988. Bacterial surface polysaccharides: structure and function, p. 187-231. *In* K. W. Jeon and M. Friedlander (ed.), International review of cytology. Academic Press, Maryland Heights, Mo.

Svircev, A. M., A. J. Castle, and S. M. Lehman. 2010. Bacteriophages for control of phytopathogens in food production systems, p. 79-102. *In* P. M. Sabour and M. W. Griffiths (ed.), Bacteriophages in the control of food- and waterborne pathogens. ASM Press, Washington, D.C.

Svircev, A. M., S. M. Lehman, P. Sholberg, D. Roach, and A. J. Castle. 2011. Phage biopesticides and soil bacterial: multilayered and complex interactions, *In* G. Witzany (ed.), Biocommunication in soil microorganisms. Springer-Verlag, Berlin.

Svircev, A. M., P. L. Sholberg, and A. J. Castle. 2006. Bacteriophages: fighting fire with a twist. Biocontrol Files **7**:5.

Thomson, S. V. 2000. Epidemiology of fire blight, p. 9- 36. *In* J. Vanneste (ed.), Fire blight: the disease and its causative agent, *Erwinia amylovora*. CABI Publishing, Wallingford, United Kingdom.

Thomson, S. V. and S. C. Gouk. 2003. Influence of age of apple flowers on growth of *Erwinia amylovora* and biological control agents. Plant Dis. **87**:502-509.

Toselli, M., D. Malaguti, B. Marangoni, L. Cabrini, C. Bazzi, G. Sponza, and D. Scudellari. 2002. Reduction of fire blight infection by manipulation of pear tree water status. Acta Hort. **590**:193-199.

Turki, Y., H. Ouzari, I. Mehri, A. B. Ammar, and A. Hassen. 2012. Evaluation of a cocktail of three bacteriophages for the biocontrol of *Salmonella* of wastewater. Food Res. Int. **45**:1099-1105.

Van Den Bogaard, A. E. and E. E. Stobberingh. 2000. Epidemiology of resistance to antibiotics: links between animals and humans. Int. J. Antimicrob. Ag. **14**:327-335.

van der Zwet, K. H. and H. L. Keil. 1979. Fire blight. A bacterial disease of rosaceous plants. U.S. Dept. Agric. Handbk. **510**:200.

van der Zwet, P. M. J. and J. H. C. Reiber. 1994. A new approach for the quantification of complex lesion morphology: the gradient field transform; basic principles and validation results. J. Am. Coll. Cardiol. **24**:216-224.

van der Zwet, T. and S. V. Beer. 1995. Fire blight - its nature, prevention, and control: a practical guide to integrated disease management. U.S. Dep. of Agric. **631**:1-97.

Vanneste, J. L. and S. Eden-Green. 2000. Migration of *Erwinia amylovora* in host plant tissues, p. 73-83. *In* J. L. Vanneste (ed.), Fire blight: the disease and its causative agent, *Erwinia amylovora*. CABI Publishing, Wallingford, United Kingdom.

Vanneste, J. L., J. Yu, and S. V. Beer. 1992. Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora.* J. Bacteriol. **174**:2785-2796.

Vidaver, A. K. 1976. Prospects for control of phytopathogenic bacteria by bacteriophages and bacteriocins. Ann. Rev. Phytopathol. **14**:451-465.

Vidaver, A. K. 2002. Uses of antimicrobials in plant agriculture. Clin. Infec. Dis. **34**:S107-S110.

Végh, A., Zs. Némethy, L. Hajagos, and L. Palkovics. 2012. First report of *Erwinia amylovora* causing fire blight on plum (*Prunus domestica* L.) in Hungary. Plant Dis. **11**:1013.

Wang, D., M. Qi, B. Calla, S. S. Korban, S. J. Clough, P. J. A. Cock, G. W. Sundin, I. Toth, and Y. Zhao. 2012. Genome-wide identification of genes regulated by the rcs phosphorelay system in *Erwinia amylovora*. Mol. Plant Microb. Int. **25**:6-17.

Wang, D. P., S. J. Clough, Y. F. Zhao, G. W. Sundin, S. S. Korban, and I. K. Toth. 2011. Regulatory genes and environmental regulation of amylovoran biosynthesis

in *Erwinia amylovora*. Acta Hort. **896**:195-202.

Ward, E. R., S. J. Uknes, S. C. Williams, S. S. Dincher, D. L. Wiederhold, D. C. Alexander, P. Ahl-Goy, J.-P. Métraux, and J. A. Ryals. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3**:1085-1094.

Wei, Z. M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S. Y. He, A. Collmer, and S. V. Beer. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science **257**:85-88.

Weiner, R., S. Langille, and E. Quintero. 1995. Structure, function and immunochemistry of bacterial exopolysaccharides. J. Ind. Microbiol **15**:339-346.

Whitfield, C. 1988. Bacterial extracellular polysaccharides. Can. J. Microbiol. **34**:415-420.

Wiggins, B. A. and A. Alexander. 1985. Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. Appl. Environ. Microbiol. **49**:19-23.

Wilkinson, B. J. and K. M. Holmes. 1979. *Staphylococcus aureus* cell surface: capsule as a barrier to bacteriophage adsorption. Infect. Immun. **23**:549-552.

Wilson, J. H., R. B. Luftig, and W. B. Wood. 1970. Interaction of bacteriophage T4 tail fiber components with a lipopolysaccharide fraction from *Escherichia coli*. J. Mol. Biol. **51**:423-434.

Wu, T. C. M. and J. T. Park. 1971. Chemical characterization of a new surface

antigenic polysaccharide from a mutant of *Staphylococcus aureus*. J. Bacteriol. **108**:874-884.

Young, R. and I.-N. Wang. 2006. Phage lysis, p. 401-125. *In* R. Calendar (ed.), The bacteriophages, 2nd ed. Oxford University Press, New York.

Zahid, M. S. H., S. M. N. Udden, A. S. G. Faruque, S. B. Calderwood, J. J. Mekalanos, and S. M. Faruque. 2008. Effect of phage on the infectivity of *Vibrio cholerae* and emergence of genetic variants. Infect. Immun. **76**:5266-5273.

Zhao, Y., G. W. Sundin, and D. Wang. 2009. Construction and analysis of pathogenicity island deletion mutants of *Erwinia amylovora*. Can. J. Microbiol. **55**:457-464.