Erwinia amylovora Bacteriophage Resistance

by

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Abstract

It has been proposed that phages can be used commercially as a biopesticide for the control of fire blight caused by the phytopathogen *Erwinia amylovora*. The aim of these studies was to investigate two common bacterial resistance mechanisms, lysogeny and exopolysaccharide production and their influence on phage pathogenesis. A multiplex real-time PCR protocol was designed to monitor and quantify *Podoviridae* and *Myoviridae* phages. This protocol is compatible with known *E. amylovora* and *Pantoea agglomerans* rtPCR primers/probes which allowed simultaneous study of both phage and bacterial targets. Using *in vitro* positive phage selection, bacteriophage insensitive derivatives were isolated within sensitive populations of *E. amylovora*. Prophage screening with real-time PCR and mitomycin C induction determined that the insensitive derivatives harboured the temperate *Podoviridae* phage ΦEaT100. Lysogenic conversion resulted in resistance to secondary homologous phage infections. Prophage screening of environmental samples of *E. amylovora* and *P. agglomerans* collected from various locations in Canada, United States and Europe did not demonstrate lysogeny. Therefore, lysogeny is rare or absent while these bacterial species reside on the plant. Recombineering was used to construct exopolysaccharide deficient *E. amylovora* mutants. The EPS amylovoran mutants became resistant to *Podoviridae* and certain *Siphoviridae* phages. Increasing amylovoran production increased phage population growth, presumably by increasing the total number of bacterial cell surface receptors which promoted increased phage infections. In contrast, amylovoran did not play a role in *Myoviridae* infections, nor did production of the EPS levan for any phage pathogenesis.

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Abbreviations

Abi – abortive infection

Am – ampicillin

AMP – antimicrobial peptide

ASM – acibenzolar-S-methyl

asm – amylovoran synthesis genes

BID - bacteriophage insensitive derivative

BLAST – basic local alignment tool

CFU - colony forming units

Cm – chloramphenicol

CPC - cetylpyridinium chloride

C_t – threshold cycle

dsDNA – double stranded DNA

EOP- efficiency of plating

EPS - exopolysaccharide

HEP - high exopolysaccharide producing

HGT – horizontal gene transfer

hrp – hypersensitivity response and pathogenicity

Km - kanamycin

LEP – low exopolysaccharide producing

LOD – limits of detection

lsc – levansucrase gene

MALDI-TOF – matrix-assisted laser desorption- time of flight

MCA – melt curve analysis

MMC – mitomycin C

MOI - multiplicity of infection

MPCA – microbial pest control agent

NTC – no template control

OD_{600} - optical density; wavelength of 600 nm

ORF - open reading frame

PAI – pathogenicity island

PB - sodium phosphate buffer

PCR - polymerase chain reaction

PFGE – pulse field gel electrophoresis

PFU - plaque forming units

PGPR – plant growth promoting rhizobacterium

qPCR- quantitative real-time PCR

R^2 – coefficient of determination

rcs – regulator of capsular synthesis gene

RFLP - restriction fragment length polymorphism

R-M – restriction modification

rtPCR – real-time PCR

SAR – systemic acquired resistance

sie - superinfection exclusion

T3SS – type III secretion system

TAE – tris acetate EDTA

TEM - transmission electron microscopy

Chapter 1

General Introduction

This work describes the continued development of a phage biopesticide for the control of fire blight caused by the phytopathogen *Erwinia amylovora* (Burrill) Winslow et al. Fire blight is a necrotic wilt disease affecting apple, pear, and other rosaceous plants (van der Zwet and Keil 1979; Bonn and van der Zwet 2000). Pathogen infections may result in wilt and eventual death of shoots, branches, limbs, trees and entire orchards (Johnson and Stockwell 1998; Bonn and van der Zwet 2000). Fire blight causes significant crop and economic losses in commercial orchards worldwide (Bonn and van der Zwet 2000). In Canada, chemical disease control relies on the antibiotic streptomycin, commercial biopesticides and orchard management (Bonn and Leuty 1993; Lightner and Van der Zwet 1993; Schnabel *et al.* 1999; Norelli *et al.* 2003; Russo *et al.* 2008). However, concerns about the emergence of streptomycin-resistant isolates (Schroth *et al.* 1979; Loper *et al.* 1991; McManus and Jones 1994; Sholberg *et al.* 2001; Thomson and Ockey 2001; Russo *et al.* 2008) along with the general trend to avoid the use of antibiotics in agriculture, have prompted efforts to find viable control measures.

Microbial biopesticides are derived from microorganisms such as bacteria, algae, fungi, viruses, protozoa, and mycloplasma or rickettsia that offer a measurable degree of pest control (referred to as microbial pest control agents or MPCAs) (Kabaluk *et al.* 2010). The benefits of biopesticides are that they are less toxic than conventional chemical pesticides by targeting only specific pathogens. Chemical bacterial pesticides tend to be broad spectrum affecting organisms as different as birds, insects, and mammals

(Schnabel *et al.* 1999; Brussow 2005; Sulakvelidze 2005; Skurnik and Strauch 2006; Jones *et al.* 2007; Gill *et al.* 2007). Biopesticides often decompose quickly thereby reducing exposure times to the grower and the environment (Jones *et al.* 2007; Parisien *et al.* 2008; Kutateladze and Adamia 2008; Garcia *et al.* 2008).

Commercial biopesticides registered for control of fire blight include Bloomtime Biological FD™ (Northwest Agricultural Products, WA), BlightBan C9-1 and A506 (Nufarm Agriculture Inc., AB) and Serenade® Max (AgraQuest, CA) (Kabaluk *et al.* 2010). These biopesticides act as pathogen antagonists capable of suppressing both the establishment and growth of *E. amylovora* on stigmatic surfaces by competitive exclusion (Johnson and Stockwell 1998, Pusey 2002; Rezzonico *et al.* 2009; Kabaluk *et al.* 2010). This may reduce the probability of blossom infection and dispersal of the pathogen to other blossoms, providing control efficacies between 40-70% which is moderate to good when compared to over 90% for streptomycin (Johnson and Stockwell 2000; Stockwell *et al.* 2002; Pusey *et al.* 2008). No other control measures that provide high levels of control are registered for use, therefore new fire blight control measures still need to be developed, registered and become commercially available to growers.

Bacteriophages (phages) are capable of targeting and killing specific bacterial hosts which may allow them to be used as biopesticides (Summers 2005; Lehman 2007; Balogh *et al.* 2008; Jones *et al.* 2007; Svircev *et al.* 2010). An important benefit of phage biopesticides is that phages can be much more specific than more chemical antimicrobials. They can not only be chosen to be harmless to humans, animals or plants, but harmless to other beneficial environmental bacteria. Phages are natural inhabitants of the environment so they present no residual issues. Because phages are self-replicating in the presence of host bacteria, a single or smaller dosage may be sufficient to achieve

disease control.

After initial discovery and characterization of *Erwinia* spp. phages by Gill *et al.* (2003), Lehman (2007) constructed a biopesticide by combining *Erwinia* spp. phage with the non-pathogenic bacterium *P. agglomerans* for blossom spray treatments. Inclusion of *P. agglomerans* in part protects the phages from inactivation from environmental stresses such as UV light as well as increases phage populations after their arrival in the blossom (Lehman 2007; Svircev *et al.* 2010). The host bacterium acts as a carrier by providing safe haven for phages that are currently in their lytic replication cycle within bacterial cells during biopesticide application. The applied uninfected bacterial cells provide susceptible hosts which promote new phage replication. This leads to increases in phage populations prior to pathogen arrival. The phage-carrier system has resulted in disease incidence reductions by 50% in apple and 33% in pear blossoms during spring field trials. These reductions were comparable to that of the control standard streptomycin during the same trials (Lehman 2007; Svircev *et al.* 2010).

Phage biopesticides are contingent on their pathogenic hosts maintaining sensitivity to phage induced lysis. However, bacteria either possess or can acquire a multitude of phage resistance mechanisms (Lenski 1988; Allison and Klaenhammer 1998; Bohannan and Lenski 2000; Labrie *et al.* 2010) which can be problematic for long term biopesticide efficacy (Okabe and Goto 1963; Vidaver 1976; Goodridge 2004; Jones *et al.* 2007). Lysogenic conversion is one such phage resistance mechanism that allows lysogenized cells to become immune to superinfecting homologous lytic phages (phenomenon called superinfection immunity) and remain viable (Abedon 2006; Deho and Ghisotti 2006). Previous studies on *E. amylovora* and their phages have shown that not all bacterial isolates are susceptible to phage attack (Erskine 1973b; Schnabel and

Jones 2001; Gill *et al.* 2003). One possible explanation is that these isolates are lysogens and therefore superinfection immune to further phage infection. Several studies have been conducted on *Erwinia* spp. phages (Erskine 1973b; Ritchie 1978; Ritchie and Klos 1979; Schnabel and Jones 2001; Gill *et al.* 2003; Lehman 2007) but lysogeny in *E. amylovora* has not been reported. Applying current molecular diagnostic techniques such as real-time PCR (rtPCR) can offer a cultivation-independent approach to assess the incidence of lysogeny within natural bacterial populations. Demonstrating the frequency of lysogeny may be used to predict the implications of lysogenic conversion for a phage biopesticide.

Another phage resistance mechanism possessed by bacteria is the ability to prevent phage adsorption (Lenski 1988; Allison and Klaenhammer 1998; Bohannan and Lenski 2000; Coffey and Ross 2002; Labrie *et al.* 2010). The production of exopolysaccharides (EPS) has been shown to prevent adsorption, presumably by providing a physical barrier to cell surface phage receptors (Bernheimer and Tiraby 1976; Wilkinson and Holmes 1979; van der Ley *et al.* 1986; Valyasevi *et al.* 1990; Cerning 1990; Looijesteijn *et al.* 2001; Deveau *et al.* 2002; Scholl *et al.* 2005). *E. amylovora* produces long-chain EPS which form a well-delimited loosely attached capsule made up of the levan (Gross *et al.* 1992; Geier and Geider 1993; Zhang and Geider 1997; Du and Geider 2002) and amylovoran (Bellemann *et al.* 1994; Bereswill and Geider 1997). Therefore production of EPS may play a role in phage adsorption but it is unknown whether it contributes to phage resistance. Possession of either of these phage resistance mechanisms is a major concern when phages are used in agriculture (Okabe and Goto 1963; Vidaver 1976; Goodridge 2004; Jones *et al.* 2007) since it would most likely drastically reduce a phage biopesticides efficacy.

1.1 Goals and objectives

The aim of this research was to provide an understanding of the interactions between phages and their *E. amylovora* hosts by investigating two common bacterial resistance mechanisms, lysogeny and exopolysaccharide production. The objectives were:

(i) To design and validate a genomic based phage diagnostic and quantitative technique utilizing real-time PCR technology.

(ii) To use real-time PCR as a cultivation-independent approach to assess the incidence of lysogeny within natural populations of *E. amylovora* and *P. agglomerans*.

(iii) To characterize lysogeny in *E. amylovora* by isolating superinfection immune cells.

(iv) To construct amylovoran and levan EPS deficient *E. amylovora* mutants.

(v) Determine whether the presence and type of *E. amylovora* EPS either inhibit or promote phage adsorption.

Chapter 2

Background

2.1 The pathogen, *Erwinia amylovora*

Erwinia amylovora (Burrill) Winslow et al. is a Gram negative bacterium that belongs to the *Enterobacteriaceae*. The plant pathogen has a broad host range of some 200 species in 40 *Rosaceous* genera (Momol and Aldwinckle 2000; Bonn and van der Zwet 2000). *E. amylovora* has both respiratory and fermentative metabolism but is unable to reduce nitrate (Vanneste 2000). Despite being a chemoautotroph, the bacterium is incapable of degrading plant tissue since it does not produce cellulolytic or pectinolytic enzymes (Seemuller and Beer 1976). Several studies have indicated that strains of *E. amylovora* are relatively homogeneous in their biochemical and protein electrophoretic characteristics despite their different geographical and host origins (Vantomme *et al.* 1982; Paulin 2000).

Essential for *E. amylovora* pathogenicity on *Rosaceous* hosts are the hypersensitive response and pathogenicity (*hrp*) genes and amylovoran biosynthesis (Steinberger and Beer 1988; Bellemann and Geider 1992; Geider *et al.* 1993; Bernhard F. *et al.* 1996; Oh and Beer 2005; Zhao *et al.* 2009). The *hrp* genes encode for three protein secretion/translocation pathways. The type three secretion system (T3SS) delivers effector proteins into the host plant (Wei and Beer 1995; Oh *et al.* 2005; Smits *et al.* 2010). The mechanisms by which these secreted effector proteins lead to disease development remains unclear (Barny 1995; Oh and Beer 2005). Effector proteins such as DspA/E encoded by the *dsp* genes have been shown to be required for pathogenicity (Wei

et al. 1992; Gaudriault *et al.* 1997; Oh and Beer 2005).

Another major pathogenicity factor by *E. amylovora* is the production of the exopolysaccharide (EPS) amylovoran. *E. amylovora* is capable of metabolizing the major transport and storage carbohydrates, sorbitol and sucrose, found in *Roasaceous* plant flowers (Grant and Rees 1981; Bogs and Geider 2000). Sorbitol metabolism is particularly important since it is required for the efficient production of amylovoran (Bennett and Billing 1980; Bellemann *et al.* 1994). Amylovoran negatively affects plants by plugging the vascular tissues which induces the wilting of host tissues (Bellemann and Geider 1992). Amylovoran deficient mutants lack pathogenicity (Geider *et al.* 1993; Koczan *et al.* 2009).

There are two published complete genome sequences for *E. amylovora,* the European strain CFBP 1430 (Smits *et al.* 2010) and the American strain Ea273 (Sebaihia *et al.* 2010). CFBP 1430 was originally isolated in 1972 from a *Crataegus* sp. and has a genome sequence EMBL accession number of FN434113. Ea273 was originally isolated from a *Malus* sp. and has an EMBL accession number of FN666575. The genomes of CFBP 1430 and Ea273 have lengths of 3.8 Mb and have sequences that are nearly identical, >99.99% (Smits *et al.* 2010). This low number of nucleotide changes reinforces prior indications that there is a low diversity amongst isolates from geographically distinct areas. Minimal evolutionary changes are apparent in the globally distributed *E. amylovora* populations (Zhang and Geider 1997; Jock *et al.* 2002; Smits *et al.* 2010).

Sequence comparison with three other *Erwinia* spp. showed that *E. amylovora* CFBP 1430 has approximately 95% sequence identity to *E. pyrifoliae* DSM 12163T (Smits *et al.* 2010) and approximately 90% sequence identity to *E. tasmaniens*is Et1/99 (Kube *et al.* 2008). There have been only a few large-scale chromosomal reorganizations

since the divergence of these three species (Kube *et al.* 2008; Smits *et al.* 2010). Smits *et al.* (2010) reported that between these species there are a number of virulence gene similarities but a number of added genes are specific to *E. amylovora.* This may contribute to *E. amylovora* broad host range and severe-disease phenotype compared to the other *Erwinia* spp. *E. amylovora* also has a relatively short genome of 3.8 Mb compared to other *Enterobacteriaceae.* Most free-living plant pathogens have genomes lengths greater than 4.5 Mb (Sebaihia *et al.* 2010). This suggests that *E. amylovora* has disposed of its non-essential metabolic and virulence genes.

2.1.1 Levan and amylovoran

EPSs are long-chain polysaccharides that are secreted by several bacterial species during growth (Bellemann *et al.* 1994; Ruas-Madiedo *et al.* 2002; Kim *et al.* 2002; Durlu-Ozkaya *et al.* 2007). EPSs are not permanently attached to the cell surface. Instead they can either form a well-defined capsule, be loosely attached or diffusely exuded into the cell's medium (Cerning 1990; Kumar *et al.* 2007). EPS production is responsible for the slime-forming or mucoid phenotypes of bacterial colonies. *E. amylovora* produces a large (50 to 150 MDa) loose capsular EPS throughout its entire life cycle (Bellemann *et al.* 1994). EPS production prevents water loss and protection of the bacterial cells against recognition by plant defense mechanisms (Leigh and Coplin 1992). EPS also contributes to the virulence of *E. amylovora* and is the main component of the bacterial ooze seen during fire blight infections (Bennett and Billing 1978; 1980; Bellemann and Geider 1992).

Two distinct types of EPS are produced by *E. amylovora*: levan, a homopolysaccharide composed of sugar polymers containing residues of a single type of monosaccharide; and amylovoran, a heteropolysaccharide composed of residues of different types of monosaccharides (Bennett and Billing 1980; Bellemann *et al.* 1994). Levan is a common constituent of many Gram negative and Gram positive bacteria (Poetter and Coplin 1991; Gross *et al.* 1992; Hettwer *et al.* 1998). Structurally levan is a polyfructan (β-2, 6-D-fructofuranan) synthesized by levansucrase (Rairakhwadal *et al.* 2010). This enzyme cleaves sucrose into glucose and fructose and then polymerizes fructose to levan (Gross *et al.* 1992; Geier and Geider 1993; Zhang and Geider 1997; Du and Geider 2002). The enzyme is encoded by the gene *lsc*, which is expressed independently of sucrose concentration (Geier and Geider 1993; Zhang and Geider 1999; Du and Geider 2002; Du *et al.* 2004). Mutants deficient in levansucrase synthesis still induce fire blight symptoms on pear shoots, but necrosis is slowed when compared to wild type isolates (Geier and Geider 1993).

Amylovoran is an acidic heteropolymer which contains one glucuronic acid and four galactose residues as a repeating unit decorated with pyruvate and acetate groups (Nimtz *et al.* 1996; Jumel *et al.* 1997). Biosynthesis of amylovoran is encoded by the large chromosomal gene cluster *ams* (Bugert and Geider 1995). The *ams* region covers functions for transfer of nucleotide-activated sugars, transport of the repeating units through the cell membranes and their polymerization to high molecular weight EPS. Regulators of EPS synthesis in *E. amylovora* are the products from the *rcsA* and *rcsB* genes, which are similar to genes described as activators of colanic acid synthesis in *Escherichia coli* (Stout 1996). RcsA encoded by *rcs*A aids in the biosynthesis of amylovoran (Bernhard *et al.* 1990), whereas RcsB produced by *rcsB*, is an important

activator of amylovoran biosynthesis (Bereswill and Geider 1997). Mutants of either *rcs*A or *rcs*B show strong reductions or a complete lack of amylovoran biosynthesis (Bernhard *et al.* 1990; Bereswill and Geider 1997). Amylovoran-deficient mutants were found to be non-pathogenic on susceptible host tissue (Steinberger and Beer 1988; Bellemann and Geider 1992; Geider *et al.* 1993). It is unknown if RcsA or RcsB are involved in *lsc* gene expression since *rcs*-mutants produce the same amount of levan as the non-mutant strain. Overexpression of amylovoran does decreased the amount of levansucrase secreted by the cell (Bereswill *et al.* 1997), suggesting there is some level of co-regulation.

2.2 Fire blight, the disease

Fire blight is a devastating necrotic wilt disease of *Rosaceae* caused by the highly virulent bacterium *E. amylovora* (Bonn and van der Zwet 2000). Diseased tissues take on a wilted and blackened appearance as if scorched by fire. This has led to the usage of the common term fire blight to describe the disease. Fire blight symptoms may include flower necrosis, shoot recurvature (also known as shepherd's crook), bacterial ooze, and cankers on woody tissues (Bonn and van der Zwet 2000). *E. amylovora* while indigenous to North America has spread to most parts of the globe, where it has become a serious problem for commercial apple (*Malus* spp.) and pear (*Pyrus* spp.) growers. Other rosaceous hosts include crabapple, hawthorn, cotoneaster, quince, mountain ash, raspberry, and blackberry (Bonn and van der Zwet 2000).

The disease cycle for *E. amylovora* is outlined in Fig. 2-1. The pathogen overwinter in woody tissues at the edge of cankers formed in the previous growing

season. Spring temperatures above 18°C result in an increase in bacterial populations inside the canker. Under high relative humidity and optimal temperatures of 24-28°C bacteria become active and multiply within the canker resulting in ooze formation on the outer surface of the canker. During this period, insects and to a lesser extent wind and rain, disseminate the pathogen throughout the orchard (Thomson 2000). During bloom, pollinating insects (primarily bees) transfer the pathogen to blossoms where the cells colonize flower stigmas. Rain, dew and pesticide sprays wash colonized bacterial cells down to the hypanthium where they multiply to very high titres within the nectar. The pathogen eventually gains entry into the natural openings (nectaries) on the hypanthium. These open nectarthodes allow direct access for the pathogen to the underlying tissues, where infection becomes established (Johnson and Stockwell 1998; Thomson 2000). The intercellular bacteria induce plant cell membrane dysfunction by promoting ion and nutrient leakage which eventually leads to cell collapse and tissue decompartmentalization. Following primary infection in the blossoms, under optimum temperatures and high relative humidity bacteria may move into succulent tissues (1-4 yr old tissues) (Thomson 2000).

Throughout the growing season secondary infection may establish in newly growing shoot tissue. Infected blossoms clusters, green shoots, as well as oozing cankers provide inoculum sources which allow cells to be dispersed via rain, wind, and feeding insects to different parts of the tree. Wounds caused by chewing insects, wind-whipping, and hail allow dispersed *E. amylovora* access to the plant interior which would otherwise be inaccessible due to mature harden tissues. *E. amylovora* secondary infections continue throughout the late spring as long as the temperatures remain at 24-28°C, high relative humidity or rain, actively growing shoot tissue and the presence of bacterial ooze (Bonn

and van der Zwet 2000; Thomson 2000). As the growing season progresses succulent tissues mature and become woody. This stage is less susceptible to infection by the pathogen. Pathogen growth stops in infected tissues at temperatures above 30°C. The pathogen may overwinter in the cankers until warm spring conditions revitalize cell multiplication and bacterial ooze leaks from margins of the canker (Thomson 2000).

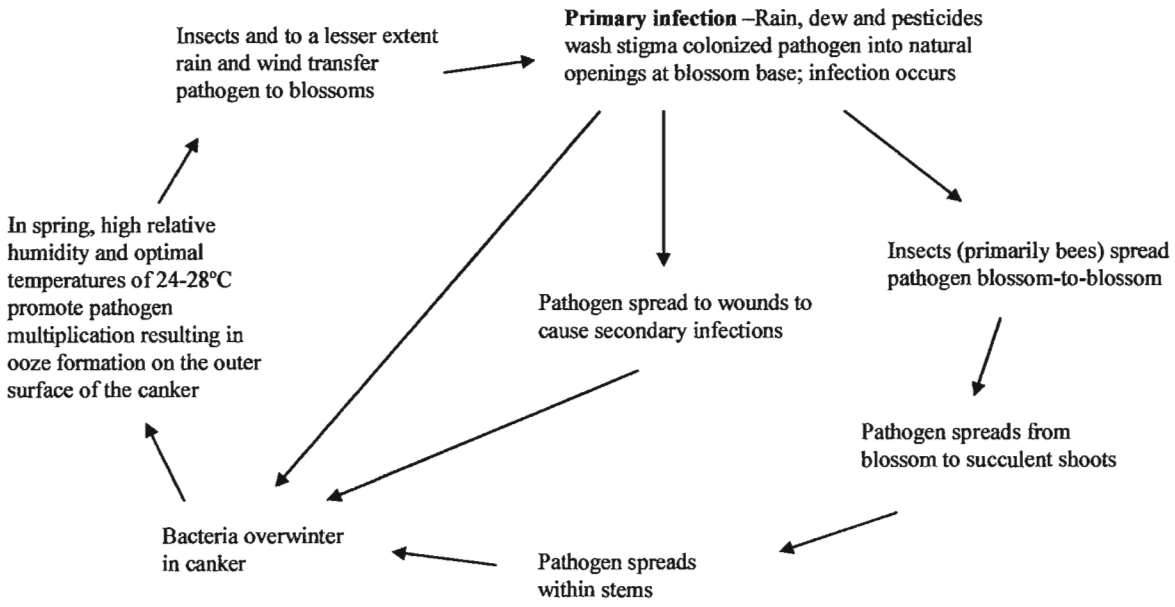

Figure 2-1. *Erwinia amylovora* disease cycle.

2.2.1 Fire blight management

Fire blight is best controlled with an integrated pest management approach. Horticultural practices that minimize host susceptibility and reduce pathogen inoculum and spread in the orchard are combined with predictive models to determine the potential for blossom infections and subsequent well-timed applications of bactericides (Steiner 2000; Vanneste 2000). The problem is that there are very few bactericides registered for use in Canada - antibiotics, biopesticides and growth regulators.

The ideal horticultural practice would be the use of resistant cultivars and rootstocks. Unfortunately, all commercially desirable apple cultivars are moderately to highly susceptible to the pathogen. In addition, the culturally desirable M9 dwarfing rootstock is highly susceptible to fire blight. For example, apple variety Gala on M.9 rootstock is a highly susceptible combination while apple variety Red Delicious on M.7 rootstock would be an ideally disease resistant combination. The consumer dictates which apple varieties are desirable and currently there is a greater consumer demand for Gala versus Red Delicious. All pear varieties grown worldwide are moderately to highly susceptible to fire blight.

Controlling plant vigor may reduce the susceptible new succulent tissues and the incidence and/or severity of disease (Southwick *et al.* 2004; Greene 2007). Cultural practices that employ growth-retardant compounds such as prohexidione-calcium, produced commercially as Apogee® (BASF, Mississauga, ON), can be used to control vegetative growth on apple. Prohexidione-calcium reduces shoot growth by inhibiting the plant hormones gibberellin, responsible for shoot elongation in apple (Rademacker, 2000). Another practice is to reduce the amount of inoculum in the orchard. Pruning

infected shoots and limbs that exhibit early symptoms, and dormant pruning to remove infection helps reduce the sources of inoculum in the spring. Controlling insects with piercing and sucking mouthparts (aphids, leafhoppers, pear psylla) can be important to slow the spread of secondary infections (Steiner 2000; Hildebrand *et al.* 2000).

Streptomycin, an antibiotic, provides the most effective and consistent control. Streptomycin is an aminoglycoside that inhibits protein synthesis, disrupting normal bacterial cell function that eventually leads to cell death (Psallidas and Tsiantos 2000; Norelli *et al.* 2003). Applied during spring bloom, streptomycin sprays are highly effective in preventing primary infection of blossoms. But the overuse has led to the development of streptomycin-resistant *E. amylovora* isolates in areas such as the Okanagan Valley in British Columbia, as well as throughout parts of the United States (Schroth *et al.* 1979; Loper *et al.* 1991; McManus and Jones 1994; Sholberg *et al.* 2001; Thomson and Ockey 2001). The concern with antibiotic resistance combined with the general trend to limit antibiotic use in agriculture has restricted streptomycin use in Canada to only three applications per bloom period.

Biopesticides are also being employed for control of *E. amylovora* (Kabaluk *et al.* 2010). Biopesticides are derived from natural materials as animals, plants, bacteria, and certain minerals. Microbial based biopesticides are derived from microorganisms such as bacteria, algae, fungi, viruses, protozoa, and mycloplasma or rickettsia that offer a measurable degree of pest control (referred to as microbial pest control agents or MPCAs) (Kabaluk *et al.* 2010). The use of biopesticides offer several advantages. Biopesticides are less toxic targeting only specific pathogens. In contrast, chemical pesticides tend to be broad spectrum affecting non-target organisms as different as birds, insects, and mammals. Biopesticides often decompose quickly thereby reducing exposure times to the

grower and the environment (Jones *et al.* 2007; Parisien *et al.* 2008; Kutateladze and Adamia 2008; Garcia *et al.* 2008).

In 2006, commercial biopesticides were registered in Canada for the control of fire blight (Kabaluk *et al.* 2010). These include Bloomtime Biological FD™ (Northwest Agricultural Products, WA), BlightBan C9-1 and A506 (Nufarm Agriculture Inc., AB) and Serenade® Max (AgraQuest, CA). The active ingredients for Bloomtime is *P. agglomerans* E325 (Pusey *et al.* 2008) and for Serenade, *Bacillus subtilis* QST 713 (Joshi and Gardener 2006). BlightBan active ingredient is either *P. vegans* C9-1 or *Pseudomonas fluorescence* A506. Through a combination of mechanisms, these antagonists can suppress both the establishment and growth of *E. amylovora* on stigmatic surfaces (Johnson and Stockwell 1998, Pusey 2002; Rezzonico *et al.* 2009; Kabaluk *et al.* 2010), which reduces the probability of blossom infection and dispersal of the pathogen to other blossoms. For control, most stigmatic surfaces in an orchard must be colonized by the bacterial antagonists, and the population size of the antagonists on stigmas must approach the carrying capacity of population size of the tissue (10^5 to 10^6 CFU/blossom) (Ishimaru *et al.* 1988; Johnston and Stockwell 198; Pusey 2002). In this regard, competitive exclusion is the primary mode of action by which these antagonists achieve suppression of the pathogen. Efficacy trials have shown 40-70% which have moderate to good levels of control. Unlike streptomycin, antagonists are only effective if they colonize blossoms prior to pathogen arrival. Another limitation of the antagonists is that their survival in the orchard is limited which does not provide ongoing control (Lindow *et al.* 1996; Johnson and Stockwell 1998; Kearns and Mahanty 1998; Aldwinckle *et al.* 2002; Rezzonico *et al.* 2009).

Fire blight disease forecasting is used to identify the potential for high infection

periods. Two of the best known software prediction models are Maryblyt (http://www.caf.wvu.edu/kearneysville/maryblyt) and Cougarblight (http://www.ncw.wsu.edu/treefruit/fireblight/2000f.htm). These prediction models use weather and apple or pear phenology to forcase infections. The main difference between these prediction models is how disease risk values are evaluated. Maryblyt includes several algorithms to account for the different phases of fire blight (canker blight, blossom blight, shoot blight, and trauma blight) and predicts both infection events and symptom development (Bonn and Leuty 1993; Lightner and van der Zwet 1993; Lightner and Steiner 1993; Dewdney *et al.* 2007). CougarBlight developed by Tim Smith also takes into account daily mean temperature and disease history (Smith 1993). Cougarblight only predicts blossom infection and does not predict shoot blight, trauma blight infections or when symptoms may appear. Cougarblight also uses a lower base temperature than the Maryblyte model that may be more accurate for predicting blossom infection in regions with cooler spring temperatures. Growers are encouraged to utilize either model to help them make decisions on timing the application of antimicrobials.

2.3 Bacteriophages: historical perspective

In 1915, Frederick Twort discovered the viruses that infection bacteria. This discovery was also made independently, in 1917, by Félix d'Herelle (Summers 2001). Working with *Staphylococcus* cultures, Twort (1915) discovered minute glassy areas that would not grow when subcultured. Realizing these glassy areas were the result of the destruction of the bacterial cells, Twort was able to transfer the destructive element from

one *Staphylococcus* colony to another. Further experiments showed that the agent could pass through porcelain filters and that it required bacteria for growth. These observations led Twort to propose that bacteriolytic agents were responsible for cell lysis (Twort 1915). D'Herelle observed a similar unexplained cell lysis phenomenon when working with the dysentery bacteria *Shigella*. D'Herelle hypothesized that these unseen ultramicroscopic infectious agents were actually viruses that could infect bacteria. He named the viruses 'bacteriophages', the literal Greek translation means 'eaters of bacteria' (Summers 2001). Both Twort and d'Herelle are credited for the discovery of bacteriophages.

D'Herelle was the first to realize and investigate the therapeutic ability of phages. He conducted several phage therapy studies to control avian typhosis, bovine hemorrhagic septicemia, bacillary dysentery, and bubonic plague (Summers 1999; 2001; 2005). Unfortunately, the idea of using phages as therapeutic agents never really caught on in western civilizations due to the emergence of chemical antibiotics. The antibiotics are broad spectrum bactericides with high treatment efficacies (Clark and March 2006).

2.4 What are phages?

Bacteriophages or phages are viruses that only infect bacteria (Fig. 2-2). Like all viruses, they are metabolically inert in their extracellular form and rely on parasitizing a host to multiply. Phages are ubiquitous, found in all niches inhabited by bacteria, and have population sizes an order of magnitude higher than that of bacteria. Tailed phages make up the largest group of phages and belong to the order *Caudovirales* (Weinbauer

2004; Ackermann 2006). All known *E. amylovora* spp. phages belong to this order (Fig. 2-3) (Gill *et al.* 2003; Lehman 2007; Lehman *et al.* 2009). Virions in this order consist of an icosahedral symmetric head attached to a helical symmetric hollow tail. The head contains a non-segmented genome of a single linear double stranded DNA (dsDNA). Phage genomes encode only proteins. To date, encoded lipids and carbohydrates have not been reported (Ackermann *et al.* 1995; Ackermann 2006; 2007). Phage tail-type structures may be contractile or non-contractile, straight or flexible, long or short. Based on their tail morphology, phages are grouped into families: *Sipho-, Myo-*, and *Podoviridae* (Fig. 2-3). *Siphoviridae* have long non-contractile tails and constitute about 61% of tailed phages. *Myoviridae* have contractile tails consisting of a sheath and a central tube and constitute about 25% of tailed phages whereas the *Podoviridae,* which constitute about 14% of the tailed phages, have short tails. *Erwinia* spp. phages represent all three families (Gill *et al.* 2003).

2.5 Bacteriophage life cycles

Generally, the phage's life cycles can be divided into stages, commencing with adsorption, transfer of nucleic acids, expression and replication of the nucleic acids, virion assembly, and release and transmission (Fig. 2-2) (Lindberg 1977; Abedon 2006). Phage adsorption occurs in two steps, a reversible followed by an irreversible binding of the phage tail fibers to a specific cell surface structure or 'receptor'. The nature of the receptor varies for individual phages and may include carbohydrates, lipopolysaccharide, or pili (Lindberg 1977). Once the phage has bound to the cell, in some cases the phage

base plate rearranges creating a hole in the base plate. The outer sheath contracts and an internal tube goes through the outer membrane, peptidoglycan, and periplasm to inject DNA into the cell (i.e. T4). Not all phages have the structures to physically inject their DNA (i.e. T7). Instead these phages degrade the cell wall with enzymes located in the tail or capsid. This creates pores in the cell wall and allows phage DNA entry into the cell. The empty phage capsid remains outside of the bacterial cell.

Following DNA entry, the genetic material may undertake one of several different life cycles (Fig. 2-2) (Campbell 2006; Abedon 2006; Koch 2007). In lytic infections, virulent (or lytic) phage nucleic acids redirect the host metabolism towards the production of new virions which are released following cell lysis. Chronic infections differ slightly to lytic infections in that parasitized cells continue to release new virions by budding or extrusion and lack immediate lysis of the host cell (Weinbauer 2004). In lysogenic infections, the nucleic acid from temperate phages typically integrates into the host chromosome (Lwoff 1953; Campbell 2006; 2007; 2008) (See section 2.5.1 for further detail). Lysogeny is the integration of the phage genome, called a prophage, which is not transcribed to produce new virions and bacterial viability is retained (Campbell 1961; Stewart and Levin 1984). The prophage is multiplied during host replication. This dormant lysogenic state may persist indefinitely or be terminated at any time (transfer to a lytic infection) by specific environmental cues or stresses (Lwoff 1953; Campbell 1961; Stewart and Levin 1984; Campbell 2006). In pseudolysogenic infections, the phage genome coexists in an unstable state within the host bacterium, where the genome fails to replicate (Ripp and Miller 1997; Weinbauer 2004; Abedon 2008). Unlike lysogeny, the phage genome does not integrate into the host chromosome during pseudolysogeny (See section 2.5.2 for further detail).

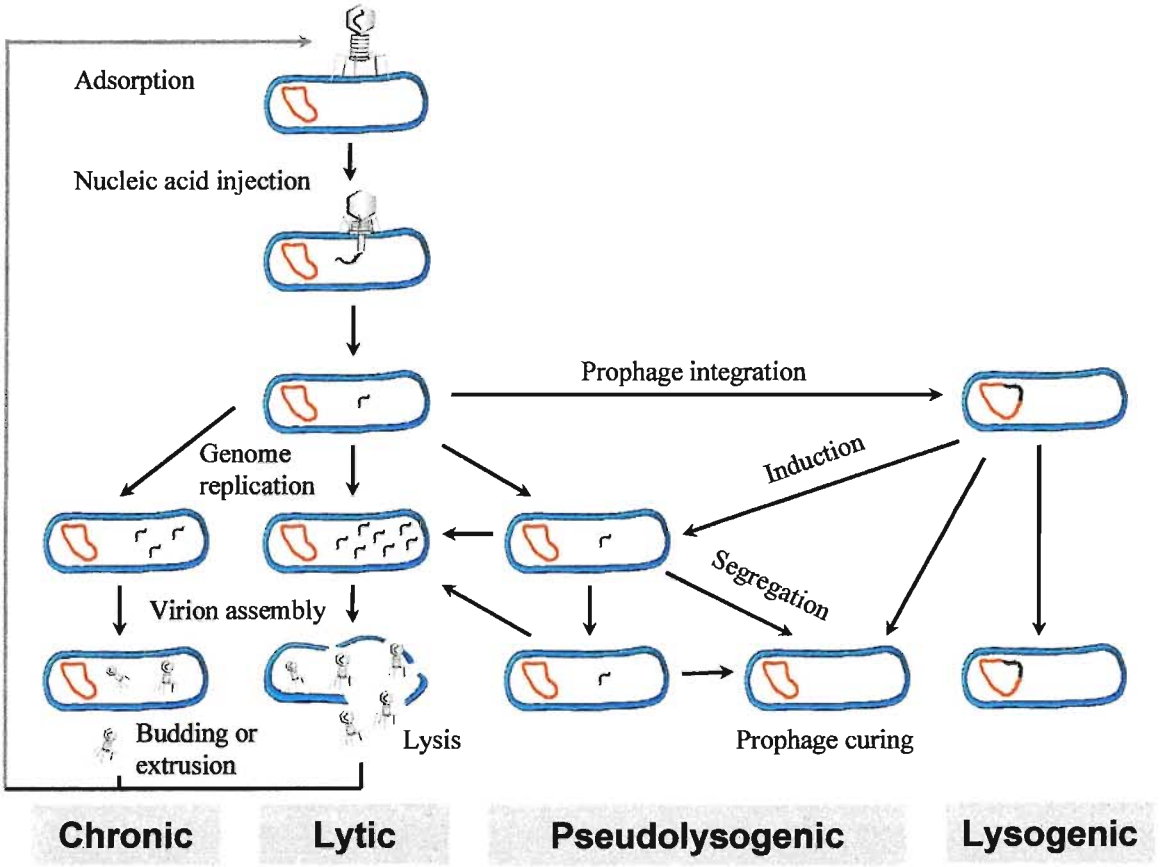

Figure 2-2. Bacteriophage life cycles. Model adapted from Weinbauer (2004).

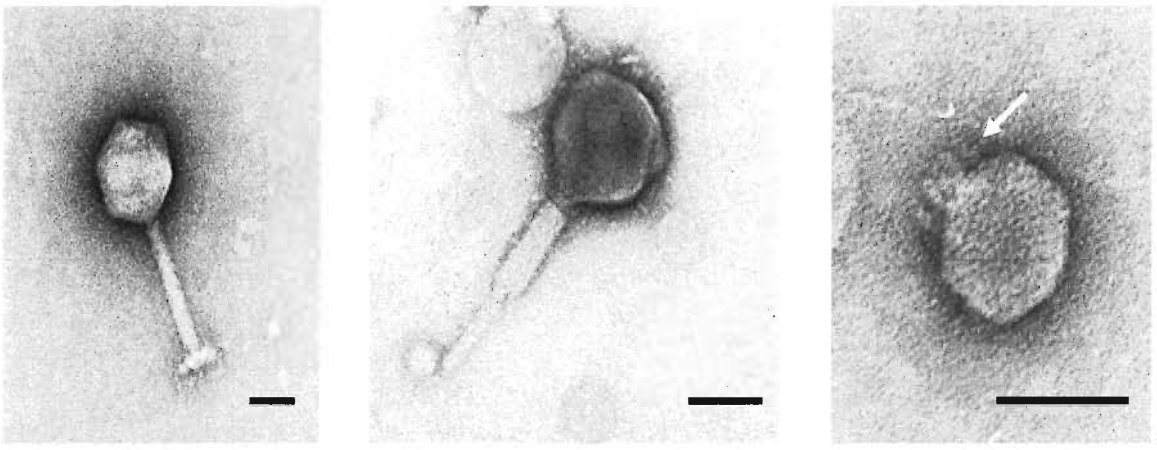

Figure 2-3. *Erwinia amylovora* bacteriophages belonging to the order *Caudovirales.* The virus particles possess a distinct icosahedral symmetric head that contains a single molecule of linear dsDNA and a tail-like structure. The tail-like structure can be non-contractile (*Siphoviridae*), contractile (*Myoviridae*), or short (arrow; *Podoviridae*). Micron marker = 50 nm. TEMs courtesy of A.M. Svircev.

2.5.1 Lysogeny

Lysogeny is a phase in the temperate phage life cycle where the phage nucleic acid integrates into the host chromosome (Fig. 2-2). The integrated nucleic acid, called a prophage, does not promote virion production and the bacterial viability is maintained. The only prophage genes that are transcribed are those that maintain a repressed state. The prophage is vertically transmitted to daughter cells at each subsequent cell division until a later environmental cue or cell stress (such as UV radiation) disrupts repressor expression, induces prophage separation from the host chromosome and promotes production of new phage progeny via the lytic cycle (Lwoff 1953; Campbell 2006; 2007; Abedon 2008).

Since the prophage replicates along with the host chromosome, it produces far fewer copies of the original prophage at a much slower rate than a lytic infection. The advantage of this cycle is greater security as the host provides a safe haven for the prophage. This arrangement protects the prophage for long periods of relative inactivity whereas free phages may be inactivated by harsh environmental stresses (Iriarte *et al.* 2007). Mittler (1996) used a chemostat model to demonstrate that in variable environments, phages with a higher frequency of lysogeny were favored. Lysogenic phages remained dormant until environmental factors became favorable for host proliferation. This increases their probability for survival. The opposite was true in constant environments where host proliferation was abundant. Lytic phage population growth outperformed their lysogenic counterparts due to a lack of dormancy periods. Therefore, when conditions become unfavourable for phage multiplication through lytic infections or host abundance is low, lysogeny may provide refuge for temperate phages (Marsh and Wellington 1994; Ashelford *et al.* 1999; Weinbauer 2004; Pasharawipas *et al.* 2008a).

Lysogeny may also be advantageous to the bacterial host. Novel genes may be introduced into the bacterial cell by transduction. Transduction is the transfer of genetic material by horizontal gene transfer (HGT) from bacterium to bacterium as mediated by phages. This ultimately results in the modification of bacterial phenotype. Specialized transduction occurs as a result of mistakes in prophage excision. Flanking bacterial genes are excised along with the prophage and transferred to a new host bacterium. This is followed by expression of the genetic traits in the progeny of the recipient (Weinbauer 2004). Generalized transduction occurs when errors in DNA packaging occur during virion assembly. After the empty phage capsules are constructed, instead of phage DNA

packaging, a DNA fragment from the host genome is packaged in error. This results in functional phage particles, which can attach and deliver bacterial DNA into other bacterial cells. Due to the absence of the phage DNA, generalized transduction does not harm the bacterium, and the injected foreign bacterial DNA can be incorporated into the host genome. Transduction can affect bacterial phenotype or fitness, as introduced genes may offer novel virulence and fitness factors, affect cell metabolism, adhesion, colonization, invasion, distribution, toxin production, and/or antibiotic resistance (Abedon 2008). Therefore, phage-mediated transduction increases bacterial genetic diversity through horizontal gene transfer.

Bacterial diversity may be affected through lysogenic conversion. Lysogenic conversion represents the expression of prophage-encoded genes that results in changes in bacterial phenotype (Miao and Miller 1999; Canchaya *et al.* 2004; Brussow *et al.* 2004). The most common form of lysogenic conversion is superinfection immunity (Abedon 2008). Superinfection is the process by which a bacterial cell that has been previously infected by a lysogenizing phage gets co-infected by a second phage at a later point in time. Superinfection immunity is where the lytic properties of the second phage are inhibited and bacterial viability is maintained. In *E. coli* phage λ, superinfection immunity is a byproduct of the dormant maintenance of the prophage within the bacterial cell. The prophage encodes CI repressor protein. CI binds to a specific site on the phage DNA (the operator) and inhibits transcription of almost all phage genes except the CI repressor gene. The result is a stable repressed phage genome which is integrated into the host chromosome (Campbell 2006; 2007; Abedon 2008). Prophage-encoded repressor proteins are only able to repress its own operators and not those from heterologous phage and therefore immunity is limited to homologous phages (Ptashne 2004). Examples of

superinfection immunity are limited in phytopathogens. Lysogenic relationships found in *Bacillus amyloliquefaciens* with phage PK rendered the bacterium resistant to superinfection by other PK type phages (Jonasson *et al.* 1969).

2.5.2 Pseudolysogeny

Pseudolysogeny has been alluded to since the beginning of phage research. This phenomenon has been clearly described in *Bacillus subtilis* by Romig and Brodetsky (1961). This phage life cycle has only recently received serious scientific consideration in the last few years (Fig. 2-2) (Ripp and Miller 1998). Although still poorly understood, pseudolysogeny is generally described as a phage–host relationship where post-infection nucleic acid of the phage (also referred to as a preprophage) neither establishes a long-term stable relationship as seen with lysogeny nor produces virions and the lytic response. The phage nucleic acid simply resides within the cell in a non-active state. Unlike true lysogeny, the nucleic acid is not replicated by the host nor segmented equally amongst daughter cells during replication (Ackermann and Dubow 1987; Ripp and Miller 1997; 1998).

Researchers have been unable to agree on a definition of this unusual infection process. According to Los et al. (2003), pseudolysogeny is characterized by a stalled but potentially active phage in non-growing infected cells where the phage nucleic acid 'waits' until environmental conditions are more conducive to propagation. Lysogeny differs by being a stably maintained prophage, capable of causing host cell lysis when triggered (Abedon 2008). Khemayan *et al.* (2006) reserves the term pseudolysogeny

specifically for bacterial clones that have undergone transient lysogeny and retain some phenotypic characteristics of the lysogenic state despite the loss of a functional prophage. Weinbauer (2004) suggests that pseudolysogeny is a common name that has been given to describe a number of different phage phenomenon, and such a classification is probably a simplification of the diversity of phage life cycles. Regardless of the lack of definitive consensus, studies are beginning to show that pseudolysogeny is widely spread among bacterial hosts (Ripp and Miller 1997; Williamson *et al.* 2002; 2008; Pleteneva *et al.* 2010).

The ecological relevance of pseudolysogeny is thought to allow for increased longevity of the phage nucleic acid by protecting it during hostile times. It is thought that this potential of enduring poor conditions in a dormancy state allows some plasticity before either a lytic or lysogenic infection strategy depending on phage type and host physiology. Pseudolysogenic conversions have been shown to confer host phenotypic changes such as pigment production, nutritional changes, toxin production, and superinfection immunity (Ripp and Miller 1997; Williamson *et al.* 2002; Khemayan *et al.* 2006).

2.6 Bacteriophage resistance mechanisms

The development of phage resistance in the bacterial host is a major concern when phages are used in agriculture (Okabe and Goto 1963; Vidaver 1976; Goodridge 2004; Jones *et al.* 2007). Bacteria have developed several mechanisms which interfere with phage multiplication at various points in the infection cycle. The mechanisms include the

prevention of phage adsorption; inhibition of DNA injection, replication and transcription; restriction of incoming phage DNA; and interference with phage proteins synthesis, particle assembly and cell lysis (Fig. 2-4) (Lenski 1988; Allison and Klaenhammer 1998; Bohannan and Lenski 2000; Coffey and Ross 2002; Labrie *et al.* 2010).

The inhibition of adsorption involves a number of host mechanisms that interfere with phage attachment to the bacterial cell surface. One of the common modes of adsorption interference for phage is through mutation or recombination of bacterial genes. This mechanism allows the bacterial cell to become invulnerable to phage infection (Fig. 2-5a) (Delbruck and Luria 1943; Lenski 1988; Hyman and Abedon 2010). Bacterial phage resistant mutants often achieve this through loss or modification of cell surface carbohydrate moieties or receptor proteins. These receptor sites serve as the initial binding sites for the phages. Bohannan and Lenski (2000) found that phage-resistant derivatives of *E. coli* O157:H7 had either lost or altered the OmpC protein that was acting as the phage receptor. The resistant bacteria do not always displace their sensitive counterparts in the phage population. In most cases mutation causes a reduction in the cell's competitiveness, presenting a trade-off between competitive ability and resistance to phage infection (Abedon 2006; 2008). A competitive cost to phage resistance has been shown for *E. coli* resistant to phage T2 (Lenski and Levin 1985), phage T4 (Lenski and Levin 1985; Lenski 1988), and phage T7 (Chao *et al.* 1977; Lenski and Levin 1985).

The production of EPS by the bacterial cell may also provide resistance to phage infection by providing a physical barrier between the phage and the bacterial cell surface receptors (Fig. 2-5b) (Valyasevi *et al.* 1990; Cerning 1990; Forde and Fitzgerald 1999a;

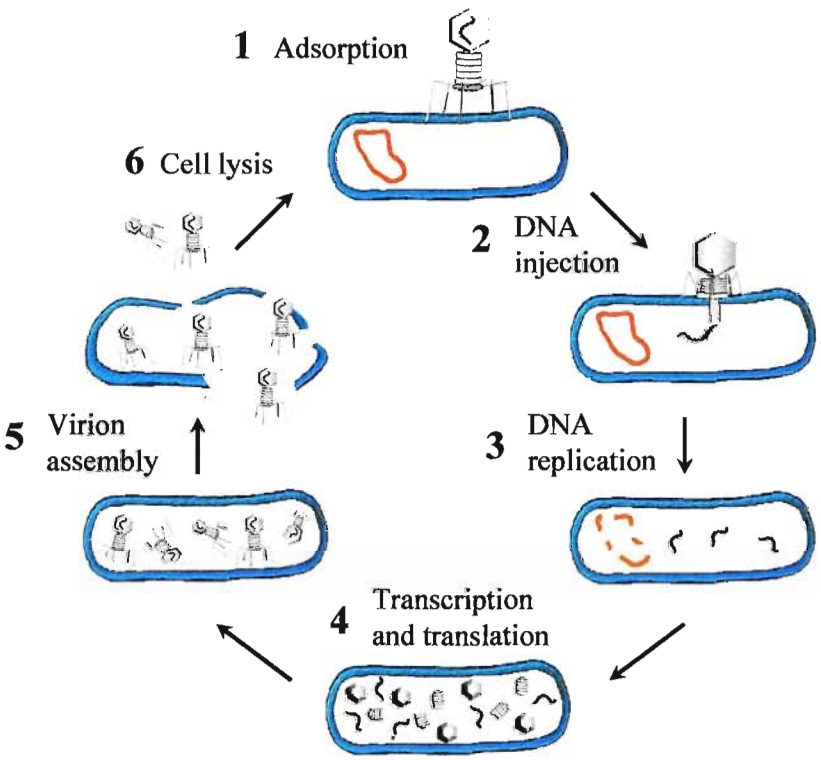

Figure 2-4. Bacteriophage lytic life cycle. Bacteria target any of the 1-6 stages with several anti-phage defense mechanisms.

Looijesteijn *et al.* 2001). Several investigations have demonstrated the role EPS plays in blocking or at least interfering with phage adsorption to lactococcal cells (Forde and Fitzgerald 1999a; Looijesteijn *et al.* 2001). *Rhizobacterium meliloti* inhibited phage adsorption by coating the cell surface receptors with EPS (Defives *et al.* 1996). Scholl *et al.* (2005) showed that *E. coli* cells which produced a K1 capsule could block phage T7 infection. Infection could be restored by the artificial removal with virion-associated endosialidases that can hydrolyze the capsule. Similar observations between capsule producing cells and phages have been reported with *Streptococcus pneumoniae* (Bernheimer and Tiraby 1976) and *Staphylococcus aureus* (Wu and Park 1971). The EPS may not always be an effective mechanism for preventing phage adsorption (Deveau *et al.* 2002). Certain phages have evolved to specifically recognize these extracellular polymers and even to degrade them (Fig. 2-5c) (Leiman *et al.* 2000; Deveau *et al.* 2002; Scholl and C. Merril 2005). *E. coli* produces a capsular EPS with a serotype-specific surface sugar, K antigen. Certain *E. coli* phage types are able to recognize and bind specifically to the K antigen. Mutants void of capsular EPS are resistant K antigen-specific phage lysis (Stirm 1968).

Following phage attachment to the bacterial cell surface, the cell wall is penetrated and the phage DNA enters into the cell. Although not well characterized, bacteria have been shown to employ different mechanisms which prevent phage DNA from reaching the cytoplasm. Superinfection exclusion systems (sie) can block phage DNA entry (Lu and Henning 1994; Forde and Fitzgerald 1999a; Labrie *et al.* 2010). In *E. coli*, T4 phage infection involves degradation of the cell wall to allow translocation of phage DNA into the cell cytoplasm. After T4 is attached to the cell, *E. coli* produces proteins which block

the further degradation of the cell wall, inhibiting translocation of phage DNA into the cytoplasm (Lu and Henning 1994).

Following successful phage adsorption and penetration infection may be inhibited by the presence of a restriction-modification (R-M) systems (Lenski and Levin 1985; Lenski 1988; Lu and Henning 1994; Allison and Klaenhammer 1998; Labrie *et al.* 2010). R-M systems are composed of two complementary enzymatic actions that work in unison to differentiate between endogenous and exogenous DNA. The first functional component uses host-encoded restriction endonuclease, which cleaves site-specific dsDNA. The second component is a modification enzyme, a methylase that covalently modifies DNA at sequence specific *loci* located throughout the genome. The majority of incoming phage DNA is digested by the endonuclease, while the cell itself remains protected by modifying its own DNA through methylation. Although methylation spares cellular DNA from autodegradation, a small number of phage genomes may escape restriction by becoming modified by the methylase. This leakiness, allows modified phage progeny to be resistant to bacterial cells with the same R-M system (Lenski and Levin 1985; Lenski 1988; Enikeeva *et al.*2010). The majority, if not all, bacterial genera possess R–M systems (Allison and Klaenhammer 1998; Labrie *et al.* 2010; Enikeeva *et al.*2010).

Abortive infection (Abi) defense systems can also act as an intracellular defense against phage infection (Smith *et al.* 1969; Allison and Klaenhammer 1998; Tran *et al.* 1999; Durmaz and Klaenhammer 2007; Haaber *et al.* 2008; Fineran *et al.* 2009). Abi systems promote cell death and limit phage replication within a bacterial population. Abi systems often are highly effective once activated. They are capable of inhibiting phage replication at a variety of stages, from viral genome transcription to viral genome replication, morphogenesis, assembly and burst (Snyder 1995; Coffey and Ross 2002).

Another unique characteristic of an Abi system is that bacterial cell lysis follows inhibition of phage replication. Abi systems typically do not provide individual cell resistance to phage infection, rather they provide population protection by promoting "altruistic suicide" of the phage infected bacterium (Forde and Fitzgerald 1999b). The majority of Abi systems are encoded on plasmids of Gram positive bacteria that include *Bacillus licheniformis* (Tran *et al.* 1999), *Lactococcus lactis* (Yang *et al.* 2006a; Durmaz and Klaenhammer 2007; Haaber *et al.* 2008). Gram negative bacterial species that have

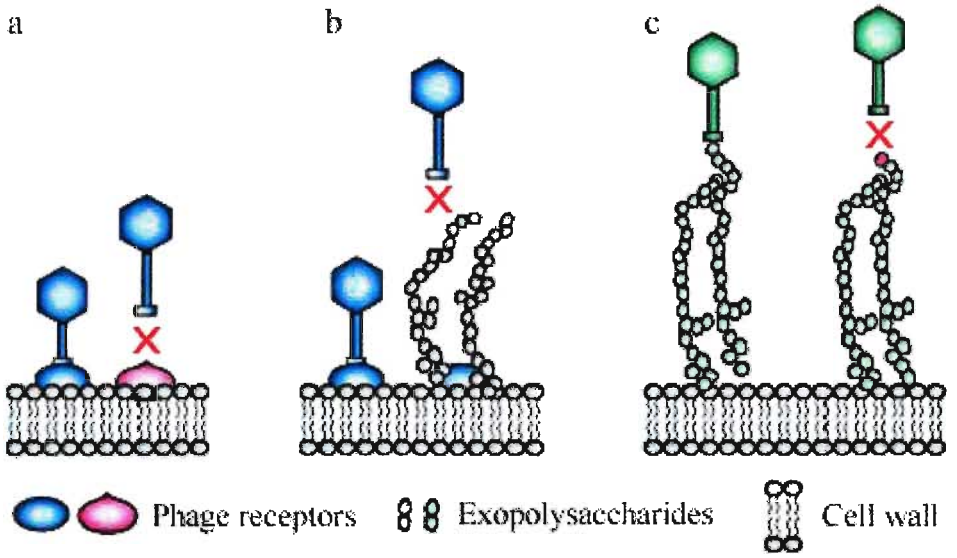

Figure 2-5. Defense mechanisms used by bacteria to inhibit phage adsorption. (a) Phage adsorption occurs through recognition of a phage receptor on bacterial cell surface. Resistance to phage adsorption can occur by host modification of the cell surface receptors. (b) Phage adsorption may be inhibited by the production of exopolysaccharides. (c) Phage adsorption may occur through recognition of specific EPS components. Modifications to the EPS may prevent virion attachment.

Abi systems include *E. coli* (Snyder 1995), *Shigella dysenteriae* (Smith *et al.* 1969), *Vibrio cholerae* (Chowdhury *et al.* 1989), and *Pectobacterium carotovora* (formerly *Erwinia carotovora*) (Fineran *et al.* 2009). Abi have been shown to inhibit of phage DNA replication (Coffey *et al.* 1991; Garvey *et al.* 1997; Boucher *et al.* 2000; Yang *et al.* 2006b; Haaber *et al.* 2008), prevent transcription (Dai *et al.* 2001; Domingues *et al.* 2008) and interfere with phage particle maturation (Garvey *et al.* 1995; 1997). The mechanisms of action for most recognized Abi systems are unknown.

2.7 *Erwinia* spp. bacteriophages

In the early 1970s, Erskine (1973a) conducted the first study on *E. amylovora* phages. Phage S1 was collected from the soil beneath fire blight diseased plants, and characterized as producing small clear plaques on *E. amylovora* isolate PRI. The optimal infection temperature for S1 was found to be 10°C on PRI and the virions became inactivated when stored at temperatures typical of summer or by exposure to ultraviolet (UV) irradiation. The study also showed that phage S1 could lysogenize a closely related yellow saprophytic orchard bacterium, presumably *P. agglomerans*. Using pathogenicity tests on pear slices, Erskine (1973a) demonstrated that phages could be used to reduce disease incidence when mixtures of *E. amylovora* PRI and phage S1 were co-inoculated.

Shortly thereafter, Ritchie and Klos (1977; Ritchie 1978; 1979) demonstrated that there was variability in *Erwinia* spp. phage plaque morphologies and host ranges. Phages produced large plaques with expanding translucent halos [e.g. ΦEa1(h)], produced pin-point plaques with expanding translucent halos [e.g. ΦEa7] and produced plaques without halos [e.g. ΦEa1(nh)] (Ritchie and Klos 1979). Host range studies found that no single

phage isolate could lyse all *E. amylovora* isolates assayed. Host ranges were not limited to *E. amylovora,* as *P. agglomerans* isolates could also be lysed (Ritchie and Klos 1979). Schnabel and Jones (2001) were able to further demonstrate the diversity of *Erwinia* spp. phages by collecting 5 distinct phage types. Phages were classified based on genome size estimates, restriction fragment length polymorphisms (RFLP) and PCR fingerprinting. Of the five phages, two were similar to those previously characterized isolated by Ritchie and Klos (1977), ΦEa1(h) and ΦEa7 and the remaining phages ΦEa100, ΦEa125 and ΦEa116C were novel. The host ranges of the five phages varied and one was able to infect all 52 *E. amylovora* isolates tested (Schnabel and Jones, 2001).

Gill *et al.* (2003) conducted a study to collect and estimate the diversity of *Erwinia* spp. phages in Southern Ontario orchards. Over 42 phages were collected from orchard soils (Gill 2000; Gill *et al.* 2003) and make up the Vineland Phage Collection located at Agriculture and Agri-food Canada, Vineland ON Canada (Table 2-1). Based on restriction patterning, molecular identification, plaque morphology, and electron microscopy, the phages were identified and organized into six groups (Gill *et al.* 2003). Virion morphologies placed all phages into the virus order *Caudovirales,* with members in the *Podo-, Myo-* and *Siphoviridae* families (Fig. 2-3). Phages in group 1 had contractile tails and belonged to the *Myoviridae*. Within the *Myoviridae* two distinct morphologies were evident, a smaller contractile phage and a larger contractile phage but both produced similar plaque morphology. The phages belonging to group 2 were *Siphoviridae* with noncontractile-tails. The phages in groups 3 (including all three subgroups), 4, 5, and 6 were short-tailed phages to belong to the family *Podoviridae*. Group 3 phages produced distinctive, large plaques with expanding haloes. Group 6 phages had unique restriction pattern but shared similar phage morphological characteristics as groups 3, 4, and 5 (Gill

et al. 2003).

Genomic information for *Erwinia* spp. phages is limited to a 3.3 kb genome region of phage ΦEa1(h) (Kim and Geider 2000), and complete genomes of phages Era103 (GenBank accession no. NC_009014) and ΦEa21-4 (Lehman *et al.* 2009). The 3.3 kb fragment of genomic DNA from phage ΦEa1(h) encodes three open reading frames (ORFs). The first ORF encodes a lysozyme, the second a holin and the third an EPS-degrading enzyme the authors called depolymerase (Kim and Geider 2000). ΦEa1(h) fragment shares homology with the unpublished sequence of Era103 which is most likely due to both being the same phage type or isolate (Ritchie and Klos 1979; Vandenbergh *et al.* 1985). The only published complete genome is of *Erwinia* spp. phage is of ΦEa21-4 (Lehman 2007; Lehman *et al.* 2009). This *Myoviridae* phage has a genome size of ~85kbp, a GC content of 43.8%, contains 117 putative protein-coding genes and 26 tRNA genes. A unique feature of ΦEa21-4 is 24 copies of conserved rho-independent terminators found dispersed throughout the genome. ΦEa21-4 shares 56% homology with the *Salmonella* phage Felix 01. ΦEa21-4 genome has a total of 43 of the 117 genes which are unique to ΦEa21-4, and 32 of the Felix 01-like genes do not appear in any phage genome sequences other than Ea21-4 and Felix 01. N-terminal sequencing and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis resulted in the identification of five virion structural proteins, including the major capsid protein (Lehman *et al.* 2009).

2.8 Bacteriophages for control of plant pathogens

Several studies have been conducted that examined the possibility of using phages as biopesticides. Civerolo & Keil (1969; Civerolo 1973) demonstrated that spraying phage on peach foliage 1 hour before inoculation with *Xanthomonas campestris* pv. *pruni* (*Xcp*) reduced bacterial spot by ~35% under greenhouse conditions. Saccardi *et al.* (1993) revisited the potential of using phages to reduce *Xcp*. The authors found weekly spraying of peach trees with phage suspension reduced the percentage of diseased fruits compared with untreated controls by 55%. Out of the 53 orchard isolates of *Xcp* only 1 was not controlled by the phage treatment. Kuo *et al.* (1971) conducted a survey of phages applied 1, 3, and 7 days before *X. oryzae* pathogen inoculation. Leaf blight on rice was reduced 100%, 96%, and 86%, respectively. The phages were found to maintain viability for the seven days in the absence of host bacteria on control plants (Kuo *et al.* 1971). Munsch and Olivier (1995) found that applying phages to compost prior to mushroom production resulted in a ~55% reduction of bacterial blotch caused by *Pseudomonas tolaasii*. Fourteen phages were assessed for their ability to control *E. carotovora* subsp. *carotovora*, the causal agent of bacterial soft rot on calla lily. On plugs of tuber tissue and in greenhouse trials, phages were able to reduce the incidence of soft-rot symptoms by 50% (Ravensdale *et al.* 2007).

Studies have incorporated phage treatment in combination with integrated pest management (IPM) strategies. Tanaka *et al.* (1990) treated tobacco bacterial wilt, caused by *Ralstonia solanacearum*, by co-application of an antagonistic avirulent *R. solanacearum* strain and bacteriophages active against both the pathogen and the antagonist. Application of the antagonist alone reduced the number of wilted plants by

56%, whereas the co-application of the antagonist and phages resulted in a 77% reduction (Tanaka *et al.* 1990). Obradovic et al. (2004; 2005) compared various combinations of bacteriophage-based biopesticides with plant growth-promoting rhizobacteria (PGPR), SAR inducers (harpin and acibenzolar-S-methyl [ASM]) and antagonistic bacteria were evaluated for control of tomato bacterial spot caused by *X. campestris* pv. *vesicatoria* in greenhouse experiments. Experiments showed that phages applied in combination with ASM or harpin significantly reduced bacterial spot compared with the other treatments (Obradovic *et al.* 2004). Lang (2007) reported that treatment efficacies were improved when phages were mixed with ASM to treat leaf blight on onion caused by *X. axonopodis* pv. *allii*. Many of these studies have not progressed beyond small scale greenhouse and field trials nonetheless there is merit to using phage biopesticides in plant pathogen systems.

A major challenge for phage biopesticides in agriculture is that field conditions are highly detrimental to phage survival (Civerolo and Kiel 1969; Schnabel *et al.* 1999; Balogh *et al.* 2003; Balogh *et al.* 2005; Iriarte *et al.* 2007). Phage viability may be lost due to excessive heat, pH extremes, and desiccation, but the most significant environmental factor mediating phage inactivation is ultraviolet (UV) light (Iriarte *et al.* 2007). The phage biopesticides that have gained the highest treatment efficacy in field trials are those that have devised protection for the phages during treatments (Balogh *et al.* 2003; 2005; Lehman 2007; Balogh *et al.* 2008). Several formulations with pregelatinized corn flour, casecrete, sucrose and/or powdered skim milk were tested with phages to control *X. campestris*, the causative agent of bacterial spot of tomato. These formulations increased phages treatment efficacy by 61% in greenhouse trails and 20% in field trials, over unprotected phages application (Balogh *et al.* 2003; Jones *et al.* 2005).

These studies led to the development of the only phage biopesticide that is EPA registered for agricultural use. It is commercially available as Agriphage (OmniLytics Inc., Salt Lake City, UT, EPA Registration # 67986-1) for the suppression of bacterial spot on tomato (Jones *et al.* 2007).

To the best of my knowledge, the only other high efficacy phage biopesticide that has utilized a phage protection system is currently being developed for *E. amylovora* (Gill *et al.* 2003; Lehman 2007; Lehman *et al.* 2009; Svircev *et al.* 2010) as described below.

2.8.1 Bacteriophage biopesticide for *Erwinia amylovora*

Independently, Ritchie and Klos (1977; 1979); Schnabel and Jones (2000), and Gill (2000) conducted studies to examine the possibility of using phages as biopesticides for control of *E. amylovora.* These authors reported that phages specific for *E. amylovora* were abundant in orchards experiencing disease symptoms. Host range studies demonstrated that individual phage isolates had broad host ranges within *E. amylovora* isolates. Co-culturing the bacterium with phages resulted in reductions of the bacterial populations. This led researchers to suggest that *Erwinia* spp. phages may be useful for the control of *E. amylovora* (Schnabel and Jones 2000; Gill 2000; Gill *et al.* 2003).

Lehman (2007) used phages in the AAFC Vineland Phage Collection devised by Gill *et al.* (2003), to develop a phage biopesticide for *E. amylovora.* Control of the blossom-infection stage of fire blight is critical to the overall control of fire blight (Steiner 2000; Vanneste 2000). To achieve control of *E. amylovora,* phages have to be present on the blossoms prior to pathogen arrival (Lehman 2007; Svircev *et al.* 2010). The concern that applying unprotected phages would rapidly succumb to inactivation from harsh field

conditions (Zacchardelli *et al.* 1992; Schnabel and Jones, 2001; Balogh, 2002; 2006) and not increase phage populations in the blossom, Lehman (2007) used a phage-carrier system. The phage-carrier system consists of virulent *Erwinia* spp. phages and the carrier *Pantoea agglomerans* Pa21-5. *P. agglomerans* is a non-pathogenic orchard epiphyte that is also a suitable host to *Erwinia* spp. phages. The carrier enabled the establishment and maintenance of replicating phage populations in the blossom prior to pathogen arrival. Without the carrier the phage populations declined rapidly, presumably due to UV light or desiccation effects (Lehman 2007).

The phage-carrier system was shown to provide a significant reduction in fire blight symptom severity on isolated pear blossoms *in vitro* when it was applied several hours prior to pathogen inoculation (Lehman 2007). In follow up field trials, six of twelve treatments of the phage-carrier system significantly reduced the incidence of blossom blight when tested using a randomized complete block design. In successful trials, it was demonstrated that the phage-carrier system could significantly reduce disease incidence by 50% in apple and 33% in pear trees. These successful treatments were statistically not different from the antibiotic streptomycin (Lehman 2007).

Over the course of selected treatments, Lehman (2007) used multiplex real-time PCR to follow population sizes of the phage, carrier and pathogen simultaneously. Monitoring population dynamics correlated with the disease outcome of trees treated with the biopesticide confirmed that the both the phage and carrier became established on the blossom and their populations increased significantly prior to pathogen inoculation. In treatments exhibiting a significantly reduced incidence of fire blight, the average blossom population of *E. amylovora* had been reduced to pre-experiment epiphytic levels. To achieve this reduction, the average phage population had to be greater than 1×10^5

PFU/blossom at the time of pathogen arrival (Lehman 2007; Svircev *et al.* 2010).

Chapter 3

Multiplex Quantitative Real-time PCR Assay Design and Validation

3.1 Introduction

The development of a biopesticide requires a number of sequential steps that have to be taken before a product is registered for commercial use (Bailey *et al.* 2009; Kabaluk *et al.* 2010). The main steps are: prospecting the biologicals; determination of biological and environmental fate; biopesticide optimization; and technology scale-up (Bailey *et al.* 2009). To achieve these milestones, it is necessary to have a diagnostic system in place which would function to accurately identify and monitor the biopesticide during laboratory and field trials. Traditional diagnostic approaches in bacteriology generally involve lengthy isolation and enrichment protocols, costly media, and limited detection capabilities. Real-time PCR (rtPCR) technologies offer a flexible molecular based diagnostic and quantitative capability. Real-time PCR provides a rapid, streamlined process with high specificity and sensitivity since it monitors the progression of nucleic acid amplification in real-time (Arya *et al.* 2005; Roach and Svircev 2010). By defining a set threshold cycle (C_t), as the greatest change in amplification rate, a fluorogenic response can be used to indicate intended target amplification by target specific primers and probes. This C_t value is also used to determine the initial reaction copy number providing template titres are standardized. This latter utilization is referred to as quantitative rtPCR (qPCR) (Hegedus *et al.* 2006; Roach and Svircev 2010).

Lehman (2007) developed a biopesticide model that employed a combination of *Pantoea agglomerans* (carrier) and *Erwinia* spp. phages. *P. agglomerans* as the carrier

increases the phage population on the blossom surface. Under field conditions, the phage-carrier system reduced disease incidence by 50% in apple and 33% in pear blossoms. These reductions were comparable to that of the streptomycin control (Lehman 2007). Multiplex quantitative rtPCR (qPCR) was used to monitor population sizes of the pathogen, the carrier and *Podoviridae* phages (Lehman 2007; Svircev *et al.* 2009). After the phage-carrier system was applied to open flowers at 75-100% bloom, the phage and carrier populations increased simultaneously at 20-24°C. The pathogen was applied 3 days post-phage-carrier at full bloom. Lehman (2007) demonstrated that the biopesticide (phage-carrier) efficacy correlated with the increase in phage populations (>$10E^8$ PFU/ml) and the phage infection preference was the pathogen over the carrier.

The AAFC Vineland Phage Collection contains 50 *Erwinia* spp. phage belonging to the *Podo-, Myo-* and *Siphoviridae* (Gill *et al.* 2003). Previous rtPCR protocols only included *Podoviridae* phages (Lehman 2007; Svircev *et al.* 2009). This limited the inclusion of *Myo-* and *Siphoviridae* phages in the study. The aim of my study was to design and validate rtPCR primers and probes that could detect, quantify, and discriminate between *Erwinia* spp. *Podo-, Sipho-* and *Myoviridae* phages. The primers and probes were designed to complement existing *E. amylovora* and *P. agglomerans* primers/probes (Lehman 2007). This allowed target amplification of up to four gene targets simultaneously in a single reaction. In addition, the assay was optimized to provide high sensitivity while reducing diagnosis turnaround. Several validations were performed to ensure that multiplexing did not interfere or reduce assay sensitivity and specificity. This new qPCR assay was used to monitor the AAFC Vineland Phage collection phage master stocks for purity and titre. QPCR provided a highly sensitive, specific and versatile technique, suitable for incorporation into a number of studies when

developing a phage biopesticide.

3.2 Methods

3.2.1 Phages, bacterial isolates and media

Phage isolates used in this study are listed in Table 3-1. High titre phage stocks were stored in 0.8% nutrient broth (NB; Difco Laboratories, Sparks, MD) at 4°C. The bacterial isolates listed were stored in a medium containing 0.8% NB, 0.25% yeast extract (Difco), 0.5% sucrose, 0.25% K_2HPO_4, 0.05% KH_2PO_4, 0.025% $MgSO_4$ and 50% (v/v) glycerol and held at -80°C. *E. amylovora* and *P. agglomerans* isolates were cultured from frozen and incubated on nutrient agar (NA; Difco) at 26°C for 48 h prior to use. Serial dilutions of phage lysates or bacterial suspensions were done in 0.01 M phosphate buffer (PB) (10X PB, pH 6.8 was prepared as by combining 49 ml of 0.2M Na_2HPO_4, 51 ml of 0.2M NaH_2PO_4, and 100 ml of dH_2O).

3.2.2 Design of oligonucleotide primers and probes

Erwinia spp. phage primers and probes are listed in Table 3-2. Oligonucleotide design was based on the following sequences deposited in the NCBI nucleotide database: NC_009014; AJ278164; and EU710883. Nucleotides were aligned using Clone Manager Professional Suite (Scientific & Educational software, Cary, NC, USA) to determine possible target sequences. Candidates were designed to avoid hairpin, heterodimer or selfdimer structures with IDT analysis software (http://scitools.idtdna.com). The Basic Local Alignment Search Tool (Blast) revealed that no sequence homologies were present outside the reference sequence deposits. TaqMan chemistry probes were designed for

addition with primer sets STS3, PUN45, TER7 and ENDO2 using the same sequence information for primer design. Fluorescent reporter molecules Cy5, 6-FAM, HEX, and ROX, with excitation and emission maxima of 643/667 nM, 494/518 nM, 535/553 nM and 587/607 nM, respectively, were selected to avoid overlapping emission spectra. Corresponding manufacturer's suggested quenchers NHS Ester, Iowa Black RQ-Sp (IAbRQSp) and Black Hole Quencher (BHQ_1) were incorporated for probe labeling of the 3' end (IDT, Coralville, IA, USA).

3.2.3 End-point PCR and SYBR Green I real-time PCR

End-point PCR reactions were run in 25 µl amplification reactions which contained a final concentration of 200 nM each primer, 1X polymerase buffer, 2.5 U *Taq* (NEB, Ipswich, MA. USA), 300 µM of each dNTP (NEB), 3 mM $MgCl_2$ and 2 µl of template suspended in 0.01 M PB (pH 6.8). Reactions were run in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 95°C for 5 min; and 30 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s. Amplification products were visualized using 0.80 - 2.5% agarose gel electrophoresis and stained with ethidium bromide.

Similar reaction volume and reagent concentrations were used for SYBR Green I chemistry real-time PCR. In addition, reactions contained 1.25 µl of fresh SYBR Green I dye 1:3000 dilution in nuclease-free PCR-grade H_2O. SYBR Green I reactions were run in a Stratagene MX4000 Quantitative PCR thermocycler (Stratagene, La Jolla, CA) under the following conditions: 95°C, 5 min; then 95°C for 10 s and 60°C for 16 s repeated for

40 - 50 cycles. Dissociation profiles were generated by subjecting the PCR products to a stepwise increase in temperature while monitoring fluorogenic responses. Positive, negative and no template controls (NTC) were included with each run.

3.2.4 Quantitative real-time PCR

E. amylovora, *P. agglomerans*, and *Erwinia* spp. phage specific oligonucleotide primers and TaqMan chemistry probe sequences are listed in Table 3.2. Reactions were run in a singleplex, dualplex or multiplex qPCR format. All contained 1X ThermoPol buffer (NEB, Ipswich, MA), 0.2 mM of each primer, 0.1 mM of probe, 0.2 mM each of dNTP, 2 mM $MgCl_2$, 1.3 U *Taq* (NEB) and 2 µl of template suspension in 0.01M PB (pH 6.8). Reactions were centrifuged briefly and run in a Stratagene MX4000 Quantitative PCR thermocycler (Stratagene) under the following conditions: 95°C, 5 min; then 45 - 50 cycles of 95°C, 10 s, 60°C, 16 s. Fluorogenic responses were recorded during each elongation step with positive detection when responses crossed a calculated threshold cycle (C_t) as determined by the Mx4000 software. Positive, negative and no template controls were included with each run.

3.2.5 Quantitative real-time PCR specificity and sensitivity

Specificity of the *Erwinia spp.* phage primers and probes were initially assessed by means of Basic Local Alignment Search Tool (Blast) to exclude the presence of similar sequence homology. Primer set specificity was determined by SYBR Green I

qPCR with the addition of a melt curve analyses and 2% agarose gel electrophoresis and ethidium bromide staining. Primers/probes were added to primer sets that lacked non-specific amplification or primer-dimer artifacts. False-positives were monitored for sample cross-contamination during preparation of mastermix. Negative controls consisting of either water or buffer were included. Aliquots from serial dilutions of known CFU/ml of bacterial suspensions or PFU/ml for phage lysates were used to determine assay limits of detection (LOD) of qPCR reactions. C_t values for the dilutions were plotted against the starting target sequence copy number to generate a standard curve.

Table 3-1. Bacterial isolates and bacteriophages.

Isolate	Plant Host	Source	Source
Erwinia amylovora			
Ea6-4	*Pyrus communis*		(Jeng *et al.* 2001)
EaD-7	*Pyrus communis*		(Jeng *et al.* 2001)
Ea29-7	*Malus* X *domestica*		(Gill *et al.* 2003)
EaG-5	*Pyrus communis*		(Jeng *et al.* 2001)
Ea17-1-1	*Pyrus communis*		(Jeng *et al.* 2001)
Ea110R	*Malus* X *domestica*		(Ritchie and Klos 1977)
***Erwinia* spp. phages**			
ΦEa1(h)	*Malus* X *domestica*	symptomatic plant tissue	(Ritchie 1978)
ΦEa9-2	*Pyrus communis*	soil	A.M. Svircev[b]
ΦEa9-3	*Pyrus communis*	soil	"
ΦEa9-4	*Pyrus communis*	soil	"
ΦEa9-5	*Pyrus communis*	soil	"
ΦEa10-1	*Malus* X *domestica*	symptomatic plant tissue	"
ΦEa10-2	*Malus* X *domestica*	symptomatic plant tissue	"
ΦEa10-3	*Malus* X *domestica*	symptomatic plant tissue	"
ΦEa10-4	*Malus* X *domestica*	symptomatic plant tissue	"
ΦEa10-5	*Malus* X *domestica*	symptomatic plant tissue	"
ΦEa10-6	*Malus* X *domestica*	symptomatic plant tissue	"
ΦEa10-7	*Malus* X *domestica*	soil	"
ΦEa10-8	*Malus* X *domestica*	soil	"
ΦEa10-9	*Malus* X *domestica*	soil	"
ΦEa10-10	*Malus* X *domestica*	soil	"
ΦEa10-11	*Malus* X *domestica*	soil	"
ΦEa10-12	*Malus* X *domestica*	soil	"
ΦEa10-13	*Malus* X *domestica*	soil	"
ΦEa10-14	*Malus* X *domestica*	soil	"
ΦEa10-15	*Malus* X *domestica*	soil	"
ΦEa10-16	*Malus* X *domestica*	soil	"
ΦEa10-17	*Malus* X *domestica*	soil	"
ΦEa21-1	*Pyrus communis*	soil	"
ΦEa21-2	*Pyrus communis*	soil	"
ΦEa21-3	*Pyrus communis*	soil	"
ΦEa21-4	*Pyrus communis*	soil	"
ΦEa31-2	*Malus* X *domestica*	soil	"
ΦEa31-3	*Malus* X *domestica*	soil	"
ΦEa31-4	*Malus* X *domestica*	soil	"
ΦEa35-2	*Pyrus communis*	soil	"
ΦEa35-3	*Pyrus communis*	soil	"
ΦEa35-4	*Pyrus communis*	soil	"
ΦEa35-5	*Pyrus communis*	soil	"
ΦEa35-6	*Pyrus communis*	soil	"
ΦEa35-7	*Pyrus communis*	soil	"
ΦEa45-1A	*Pyrus communis*	symptomatic plant tissue	"
ΦEa45-1B	*Pyrus communis*	symptomatic plant tissue	"
ΦEa45-3	*Pyrus communis*	soil	"
ΦEa46-1A2	*Malus sylvestris*	symptomatic plant tissue	"
ΦEa46-2	*Malus sylvestris*	symptomatic plant tissue	"
ΦEa50-3	unknown	unknown	"
ΦEa51-1	*Magnolia* sp.	soil	"
ΦEa51-2	*Magnolia* sp.	soil	"
ΦEa51-3	*Magnolia* sp.	soil	"
ΦEa51-4	*Magnolia* sp.	soil	"
ΦEa51-6	*Magnolia* sp.	soil	"
ΦEa51-7	*Magnolia* sp.	soil	"
ΦEa51-8	*Magnolia* sp.	soil	"

[a] Original isolation host (Gill et al., 2003)

[b] Vineland Phage Collection. AAFC, Southern Crop Protection and Food Research Centre. Vineland, ON

Table 3-2. Sequences, product sizes, and target gene of the primers (F & R), and TaqMan chemistry probes (P) used for end-point and real-time PCR.

Name	Species (target gene)[a]	Sequence (5' - 3')[b]	Amplicon Size	Reference / Source
Ea-Lsc-F	*E. amylovora*	cgctaacagcagatcgca		
Ea-Lsc-R	(levansucrase)	aaatacgcgcacgaccat	105 bp	Lehman *et al.* 2008
Ea-Lsc-P		**Cy5**/ctgataatccgcaattccaggatg/**IAbRQSp**		
ENDO2-F	*Erwinia spp.* phage	cccacgttatacgcgaagat		
ENDO2-R	*(endolysin)*	aactcgctctggacttgcat	118 bp	this study
ENDO2-P		**Cy5**/caaggtatttttcaacacctgattta/**IAbRQSp**		
DPO2-F	*Erwinia spp.* phage	Intellectual property of AAFC		
DPO2-R	*(depolymerase)*		84bp	AAFC[c]
DPO2-P				
Pa-Gnd2-F	*P. agglomerans*	Intellectual property of AAFC		
Pa-Gnd2-R	(gluconate-6-		73 bp	AAFC
Pa-Gnd2-P	dehydrogenase)			
PUN45-F	*Erwinia* phage	aacgaacagcgccttgac		
PUN45-R	*(unknown)*	ccagttgcagccagtgtg	140 bp	this study
PUN45-P		**ROX**/actgagaagtacggtatcaaaccttc/**IAbRQSp**		
STS3-F	*Erwinia spp.* phage	gacaaacaagaacgcggcaactga		
STS3-R	*(small terminase*	atacccagcaaggcgtcaacctta	96 bp	this study
STS3-P	*subunit)*	**FAM**/agatgaagtaggttatcttcacagtgccct/**BHQ_1**		
TER7-F	*Erwinia spp.* phage	tgagaggtgattggaacgatgcct		
TER7-R	*(packaging*	ctttggaaagcatcactggtggca	106 bp	this study
TER7-P	*terminase subunit)*	**HEX**/ttcacaggcgatggtaaacgcaagga/**BHQ_0**		

[a] Design was based on the following sequences deposited in the NCBI nucleotide database: NC_009014 (STS3) AJ278164 (DPO,HOL), X75079 (Lsc), AF208633 (Gnd) and Lehman (2007) (ENDO2,TER7,PUN45)

[b] Designations: Cy5™, 6-FAM™, HEX™, ROX™ NHS Ester, Iowa Black RQ™-Sp (IAbRQSp) and Black Hole Quencher™ (BHQ_1) (IDT, Coralville, IA, USA)

[c] Agriculture and Agri-Food Canada, ON, CAN

3.3 Results

3.3.1 Optimization of real-time PCR reagents

Lehman (2007) demonstrated that multiplex real-time PCR may be effectively used for in situ detection of phages, carrier and pathogen. In this study, the PCR reagent "mastermix" was further optimized by varying the DNA polymerase and $MgCl_2$ concentrations (Fig. 3-1). Reagent concentrations were reduced from 2.5 to 1.3 U of Taq polymerase, and 3 mM $MgCl_2$ to 2 mM $MgCl_2$. The other reagent concentrations remained the same and the H_2O volume was changed to make up the difference in final reaction volume. Sensitivity effects were monitored by running two reactions simultaneously with identical titres of *E. amylovora* Ea6-4 or *Erwinia spp.* phage ΦEa31-3 differing only by a reduction in polymerase and $MgCl_2$. Ea6-4 produced C_t values of 18 and 19 for the initial and amended mixes, respectively, while ΦEa31-3 maintained the same C_t of 18. Reduced reagent concentration did display a reduction in total fluorescence between the initial and amended mixes for Ea6-4. This change was not observed for ΦEa31-3.

The assay time was also decreased while maintaining reliability and quantitative accuracy for the qPCR thermal profile (Fig. 3-2). The original thermal profile consisted of 1 cycle at 95 °C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 30 s (Lehman 2007). The amended thermal profile consisted of a cycle at 95°C for 5 min and 40 cycles of 95°C for 10 s and 60°C for 16 s. This allowed for a reduction of the qPCR assay time by about 20 min. Reduction in denaturing and annealing times did not alter C_t values.

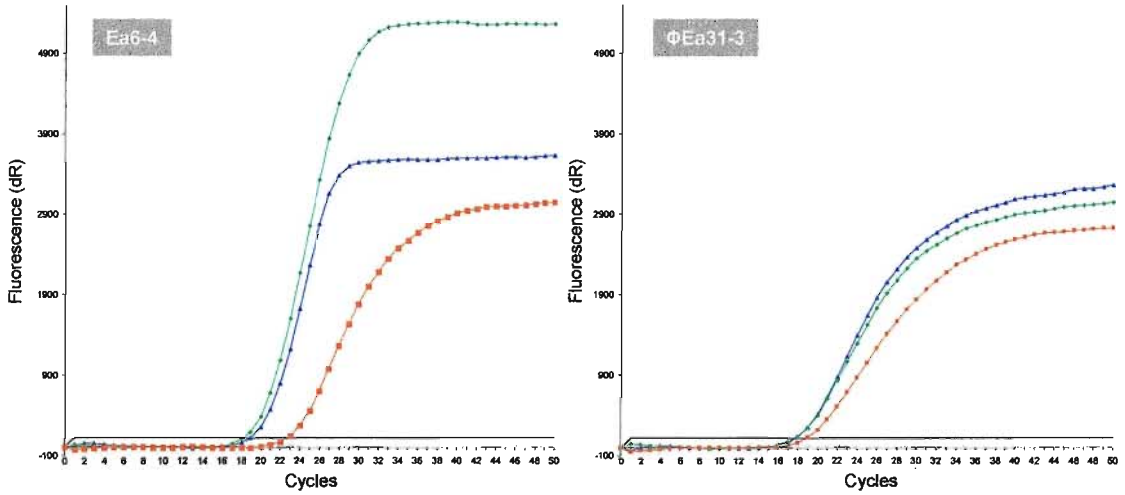

Figure 3-1. Real-time PCR sensitivity change from reductions in *Taq* polymerase and $MgCl_2$. Three reactions were monitored simultaneously with identical titres of *E. amylovora* Ea6-4 or *Erwinia* spp. phage ΦEa31-3; (i) Original reagent concentrations (green) as described by Lehman (2007), (ii) Reduced reagents (blue), (iii) Commercial Brilliant Multiplex QPCR mastermix (Stratagene) was used as positive control (red). NTC were included.

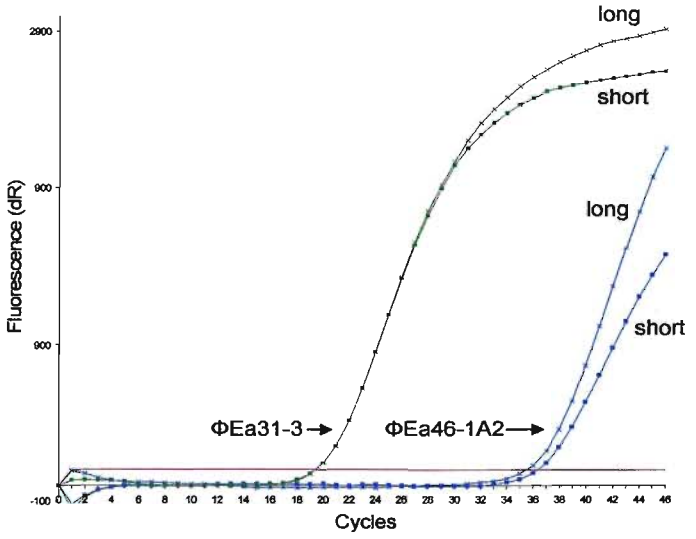

Figure 3-2. Typical threshold cycle value comparison from real-time PCR two step thermal profiles. Lehman (2007) described thermo profile (long) time was compared to the amended thermo profile by reducing denaturing and annealing times (short). This resulted in an overall assay time reduction of about 20 min (short). Here phages ΦEa31-3 (high titre) and ΦEa46-1A2 (low titre) were used as representative templates.

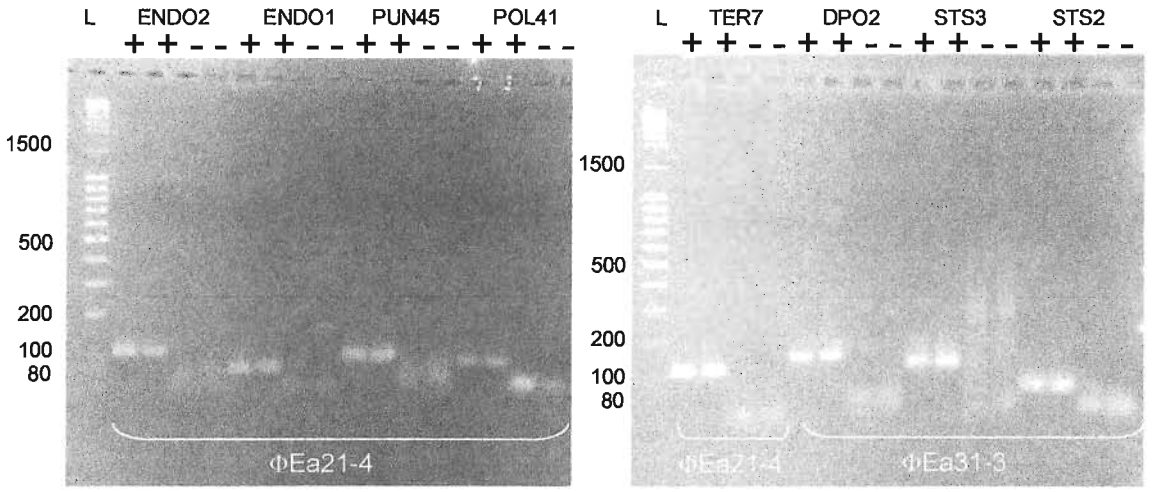

Figure 3-3. Gel electrophoresis of PCR products. Primer sets ENDO1, ENDO2, PUN45, POL41 and TER7 designed for *Myoviridae* (ΦEa21-4) PCR. DPO2, STS2 and STS3 designed for *Podoviridae* (ΦEa31-3) PCR. (+) DNA template, (-) NTC and (L) Fermentas MassRuler™ DNA Ladder (L).

3.3.2 Confirmation of qPCR specificity

Primer specificity was assessed by end-point PCR followed by gel electrophoresis (Fig. 3-3). Two *Erwinia* spp. phages ΦEa31-3 and ΦEa21-4 were used as positive controls for *Podo-* and *Myoviridae* phage families, respectively. When ΦEa31-3 was added as template, single PCR amplicons produced by primer sets STS2, STS3 had estimated sizes of 94 bp, 96 bp respectively. No amplicons were produced with these primers from ΦEa21-4 templates. When ΦEa21-4 was added as template, single amplicons produced from primers sets POL41, TER7, ENDO2, PUN45, and ENDO1 had estimated sizes of 83 bp, 106 bp, 118 bp 140 bp, and 149 bp, respectively. No amplicons were produced with these primers from ΦEa31-3 templates. Overall, amplicon generation and sizes corresponded well to predicted amplicon sizes based on known sequence

information from ΦEa31-3 and ΦEa21-4.

Template specificity for each primer set was also assessed by SYBR Green I rtPCR, with the addition of melt curve analyses (MCA) (Fig. 3-4). Phages ΦEa21-4, ΦEa31-3 ΦEa45-1B ΦEa10-6 ΦEa35-4 ΦEa46-1A2 were added as templates, and MCA determined the similarity of their amplicons produced by a given primer set. MCA also determined the singularity of amplicons produced by a specific primer set for each template. For primer set STS2, single peaks were displayed for ΦEa31-3, ΦEa10-6 and ΦEa45-1B, whereas ΦEa46-1A2 displayed a double peak. During MCA, the NTC also produced a small temperature peak at a lower temperature then previously described peaks from primer dimer dissociations. Phages ΦEa21-4, ΦEa10-1 and ΦEa35-4 produced profiles similar to the NTC. Primer set STS3 produced similar peak profiles as STS2 with the same templates with one exception; ΦEa46-1A2 produced a single peak which differed from the other templates (Fig. 3-5).

Primer set TER7 displayed a strong, single peak for ΦEa21-4, and ΦEa10-1 (Fig. 3-4). Weak, lower temperature dissociation peaks were displayed for ΦEa35-4 ΦEa46-1A2. Phages ΦEa31-3, ΦEa10-6 and ΦEa45-1B produced peaks which were similar to the NTC and considered to be from a lack of amplicon production and the presence of primer dimers. Primer set PUN45 also displayed a single peak for ΦEa21-4 and ΦEa10-1 and peaks for ΦEa31-3, ΦEa10-6, ΦEa35-4, ΦEa46-1A2 and ΦEa45-1B were similar to the NTC (Fig. 3-5). There was no discernable profile grouping from primer sets ENDO1, ENDO2 and POL41 between similar phage templates (Fig. 3-4). STS3 and PUN45 were chosen for further study since they generated PCR products with single peaks that were significantly greater than background fluorescence. POL41 and TER7 were also chosen for further study since they generated peaks for all isolates tested.

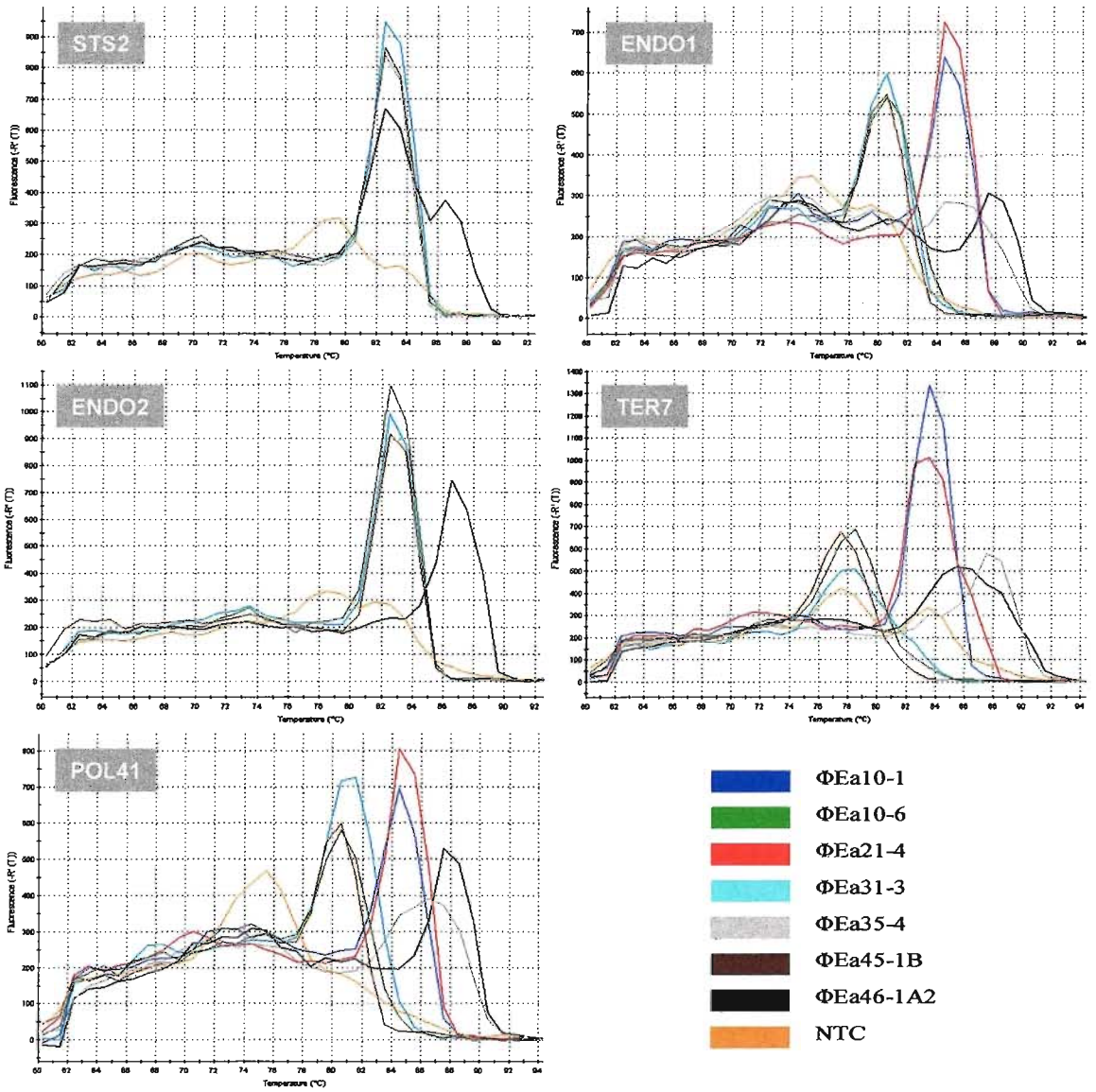

Figure 3-4. SYBR Green I dissociation curves for *Erwinia* spp. phages with real-time PCR primer sets. Multiple peaks in a single profile indicate non-specific amplification. No template controls (NTC) offer primer-dimerization profiles.

Sequence specific TaqMan probes complementary to the amplicons produced by STS3, PUN45, POL41 and TER7 primers were added to the qPCR assay and labeled with 6-FAM, ROX, Cy5 and HEX, respectively. Specificities of PUN45 and STS3 were determined against the entire Vineland *Erwinia* spp. phage collection (Table 3-3). PUN45 amplified and detected all known *Myoviridae* isolates and STS3 all known *Podoviridae*. The isolates which were not amplified and detected by either PUN45 or STS3 included all *Siphoviridae* isolates. The addition of probes increased assay specificity for primers/probe POL41 and TER7 as isolate detection positives were the same as those generated by PUN45, amplifying and detecting only *Myoviridae* phages.

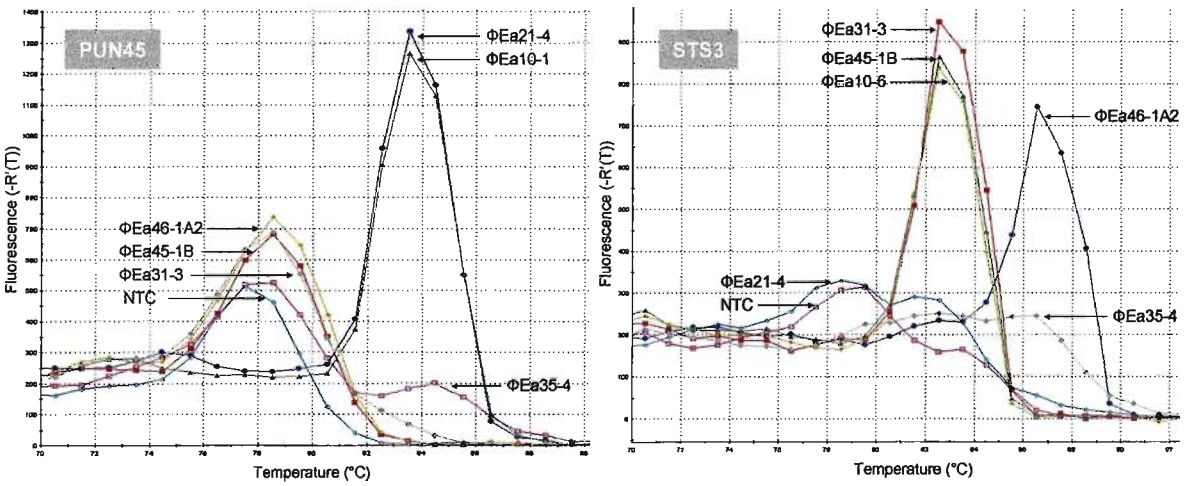

Figure 3-5. SYBR Green I dissociation curves for several *Erwinia* spp. phages with real-time PCR primers sets PUN45 and STS3. These primers were selected for further development with TaqMan probes. Overlapping profiles from differing templates indicate PCR products with a conserved nucleotide sequence.

Table 3-3. Family determination for *Erwinia* spp. bacteriophage. Identification based on real-time PCR detection (+) of *Podo-* or *Myoviridae* specific genes with primers/probes STS3 or PUN45, respectively. Undetectable phage (-) could not be identified.

		real-time PCR primers/probe		
Group[a]	Isolate	STS3	PUN45	Family[b]
1	ΦEa10-2	-	+	*Myoviridae*
	ΦEa10-3	-	+	*Myoviridae*
	ΦEa10-4	-	+	*Myoviridae*
	ΦEa21-1	-	+	*Myoviridae*
	ΦEa21-2	-	+	*Myoviridae*
	ΦEa21-3	-	-	**unknown**
	ΦEa21-4		+	*Myoviridae*
	ΦEa35-2	-	+	*Myoviridae*
2	ΦEa10-5	-	-	unknown
	ΦEa10-6	+	-	***Podoviridae***
	ΦEa31-2	-	-	unknown
	ΦEa31-5	-	+	***Myoviridae***
	ΦEa35-4	-	-	unknown
	ΦEa35-6	-	-	unknown
	ΦEa35-7	-	-	unknown
3a	**ΦEa10-7**	-	+	***Myoviridae***
	ΦEa10-8	+	-	*Podoviridae*
	ΦEa10-9	+	-	*Podoviridae*
	ΦEa10-10	+	-	*Podoviridae*
	ΦEa10-11	-	-	**unknown**
	ΦEa10-13	-	-	**unknown**
	ΦEa10-14	+	-	*Podoviridae*
	ΦEa10-15	+	-	*Podoviridae*
	ΦEa31-3	+	-	*Podoviridae*
3b	ΦEa46-2	+	-	*Podoviridae*
3c	ΦEa1	+	-	*Podoviridae*
4	**ΦEa10-16**	-	-	**unknown**
	ΦEa10-12	-	+	*Myoviridae*
5	**ΦEa9-2**	-	-	**unknown**
	ΦEa9-4	+	-	*Podoviridae*
	ΦEa9-5	-	+	***Myoviridae***
6	ΦEa51-1	+	-	*Podoviridae*
	ΦEa51-2	-	+	***Myoviridae***
	ΦEa51-3	-	-	**unknown**
	ΦEa51-4	-	-	**unknown**
	ΦEa51-6	-	-	**unknown**
	ΦEa51-7	+	-	*Podoviridae*
Ungrouped	ΦEa9-6	-	+	*Myoviridae*
	ΦEa10-1	-	+	*Myoviridae*
	ΦEa10-17	-	+	*Myoviridae*
	ΦEa45-3	-	-	unknown
	ΦEa46-3	-	-	unknown
	ΦEa50-3	-	-	unknown
	ΦEa50-4	-	+	*Myoviridae*
	ΦEa35-8	-	+	*Myoviridae*
	ΦEa35-9	+	-	*Podoviridae*
	ΦEa35-10	+	-	*Podoviridae*
	ΦEa45-1B	+	-	*Podoviridae*
	ΦEa46-1A1	+	-	*Podoviridae*
	ΦEa46-1A2	+	-	*Podoviridae*

[a] Groups designated by Gill *et al.* (2003).

[b] Classification based on real-time PCR.
Bold designations are discrepancies from reported phage family by Gill *et al.* (2003)

3.3.3 Sensitivity of qPCR

Serial dilutions of purified samples were used to determine the sensitivity of a singleplex qPCR assay for each of the isolates (Fig. 3-6). Simultaneously, the same reactions were run but with the addition of a non-target DNA sample. The standard curve and amplification efficiency of ΦEa10-1 with primers/probe PUN45 did not change with the addition of non-target ΦEa31-1 at 10^8 PFU/ml to all dilutions. Alternatively, primers/probe STS3 amplification of ΦEa31-1 was not affected by the addition of the non-target ΦEa10-1 at 10^8 PFU/ml. Mixtures of ΦEa45-1B and *E. amylovora* Ea29-7 at 10^4 and 10^9 CFU/ml generated standard curves that were identical to that of pure ΦEa45-1B. Similarly, the standard curve generated for Ea29-7 was also identical to that produced for Ea29-7 in a mixture with ΦEa45-1B at 10^8 PFU/ml.

Serial dilutions of purified samples from 6 *Erwinia spp.* phages were used to determine the limits of detection (LOD) for each primers/probe set (Fig. 3-7). The sensitivity and LOD varied for individual phage isolates even when using the same primers/probe. The LOD was 100 PFU/ml for ΦEa10-1 and 300 PFU/ml ΦEa21-4 using primers/probe PUN45. The detection limit was different for each phage species. For primers/probe DPO2, ΦEa46-1A2, ΦEa45-1B, ΦEa31-3, ΦEa10-6, had LOD of 100, 400, 400, 1000 PFU/ml, respectively. Finally for primers/probe STS3, ΦEa46-1A2, ΦEa45-1B, ΦEa31-3, ΦEa10-6 had LOD of 100, 100, 40, 100 PFU/ml, respectively. These latter sensitivities are equivalent to 1 virion per 2 µl sample. The line equations for the phages are provided in Table 3-4. For *E. amylovora* isolates, LOD was calculated to be on average 1000 CFU/ml or 20 bacterial cells per 2 µl sample.

Amplification % efficiency is calculated from the slope of the linear regression using the equation: % efficiency = $(10^{(-1/\text{slope})} - 1) \times 100\%$ (Suslov and Steindler 2005).

For primers/probe STS3 qPCR amplification efficiency for *Podoviridae* isolates ΦEa46-1A2, ΦEa45-1B, ΦEa31-3, and ΦEa10-6 was 76%, 87%, 97% and 90%, respectively. For the same isolates with primers/probe DPO2, amplification efficiency was 92%, 86%, 89% and 90% respectively. For the *Myoviridae* isolates ΦEa21-4 and ΦEa10-1 amplified by primers/probe PUN45, amplification efficiency was 99%. All linear regressions also had a coefficient of determination (R^2) value greater than 0.98.

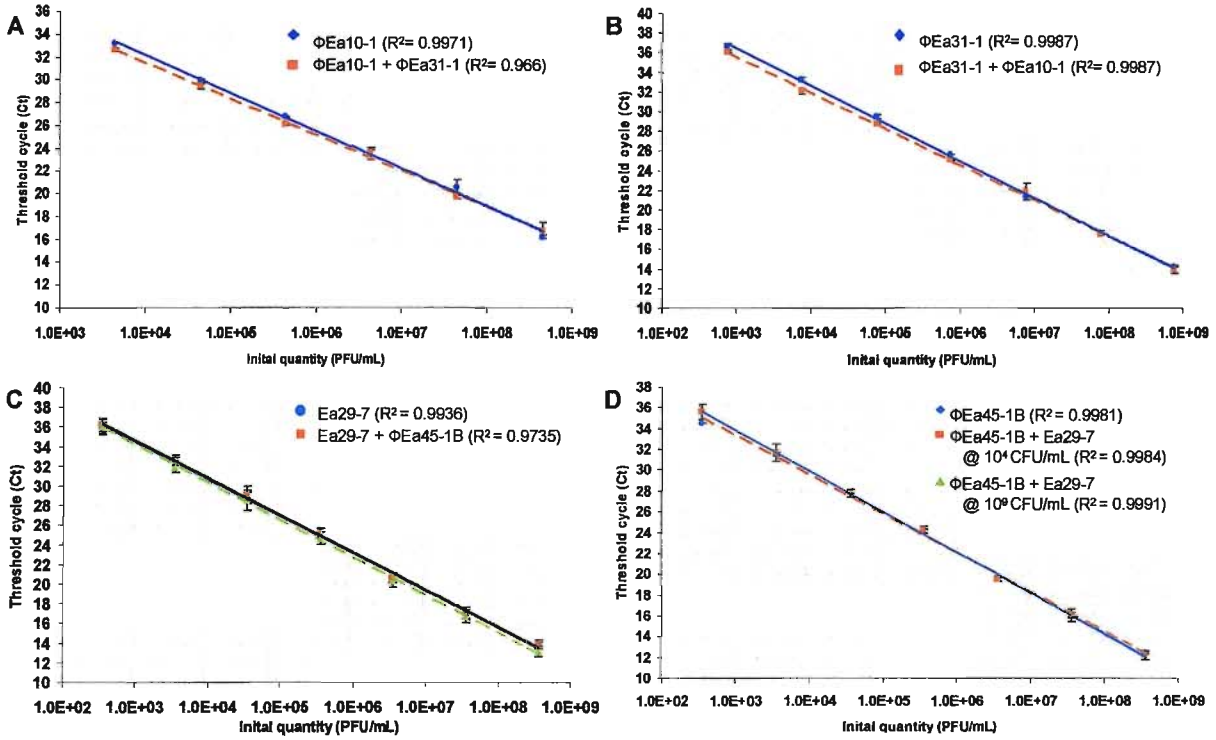

Figure 3-6. Limits of detection for quantitative real-time PCR protocols of *Erwinia amylovora* and *Erwinia* spp. phage for mixed template reactions.

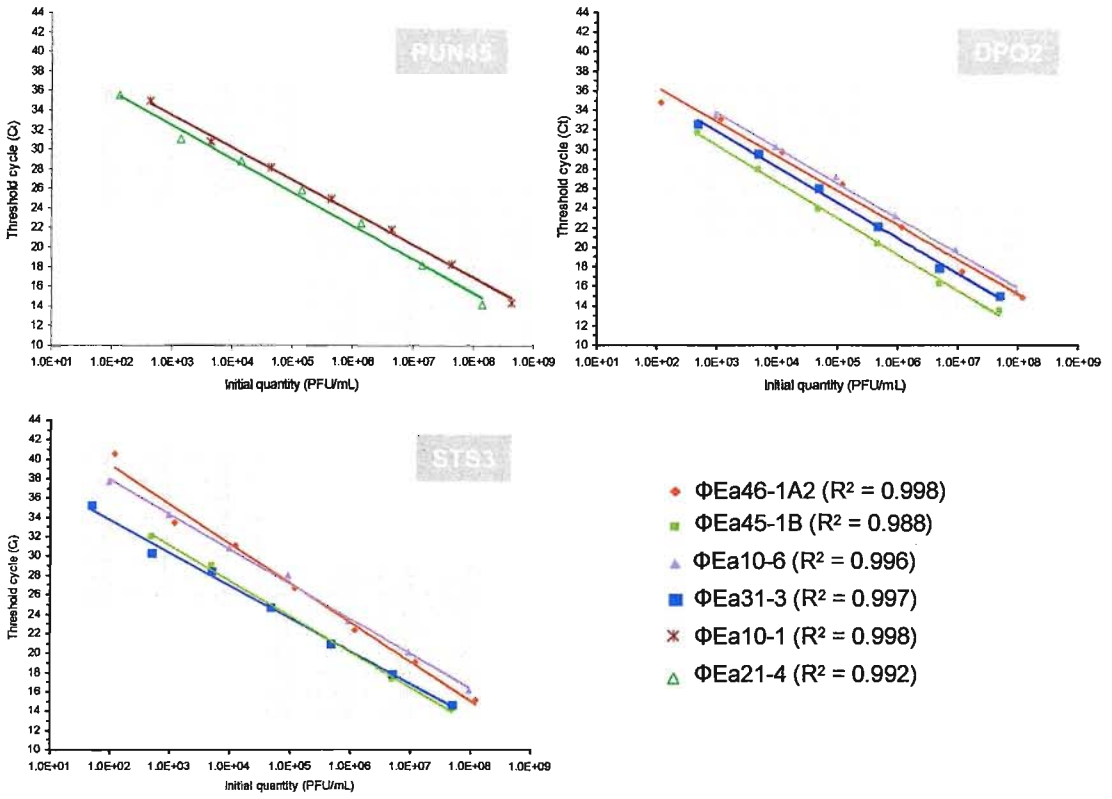

Figure 3-7. Comparison of linear regressions produced by primers/probe PUN45, DPO2 and STS3 for *Erwinia* spp. phage templates. (For line equations see Table 3-4)

Table 3-4. TaqMan chemistry primers/probe regression line equation and R^2 value for *Erwinia* spp. phage isolates.

	qPCR primers/probe		
Isolate	**DPO2**	**STS3**	**PUN45**
ΦEa46-1A2	y = -1.526Ln(x) + 43.374 R^2 = 0.9881	y = -1.764Ln(x) + 47.54 R^2 = 0.9892	N/A
ΦEa45-1B	y = -1.604Ln(x) + 41.472 R^2 = 0.9975	y = -1.592Ln(x) + 42.115 R^2 = 0.9977	N/A
ΦEa10-6	y = -1.554Ln(x) + 44.478 R^2 = 0.9964	y = -1.561Ln(x) + 45.122 R^2 = 0.9975	N/A
ΦEa31-3	y = -1.569Ln(x) + 42.663 R^2 = 0.9969	y = -1.469Ln(x) + 40.505 R^2 = 0.9946	N/A
ΦEa10-1	N/A	N/A	y = -1.441Ln(x) + 43.45 R^2 = 0.9976
ΦEa21-4	N/A	N/A	y = -1.491Ln(x) + 42.782 R^2 = 0.9927

3.4 Discussion

Rapid and accurate diagnostic methods are essential to study the interactions between phages and their targeted pathogen to devise effective biopesticide treatment strategies and to follow the environmental fate of phages. The environmental fate data is critical information required by Health Canada, Pest Management Regulatory Agency during the biopesticides registration process. In the current work, a nucleic acid based diagnostic approach using quantitative rtPCR (qPCR) technology was designed for the sensitive and specific detection of two families of *Erwinia* spp. phages, *Podo-* and *Myoviridae*. The qPCR protocol described for each phage is compatible with each other as well as the qPCR primers/probes for *E. amylovora,* and *P. agglomerans* which were previously described by Lehman (2007). This allowed for the simultaneously diagnosis and quantification of up to four target microbes in a single reaction tube, without any reduction in sensitivity or specificity.

To our knowledge, this is the first report of the use of fluorogenic probes for the detection and differentiation of two family members of *Caudoviridae*. Real-time PCR is a complex technique with substantial difficulties associated with interpreting its true sensitivity, specificity and reproducibility. Several nucleotide sequences were selected for targeting which could uniquely identify each family from one another as well as having no significant homology with non-target sequences found on the NCBI nucleotide database (http//www.ncbi.com). RFLP data have shown that sequence variability exists between *Erwinia* spp. phages isolates of the same family (Gill *et al.* 2003). This variability has the potential to affect detection and produce false-negative results since the primers and/or probe will not be homologous the target sequence. To account for this,

qPCR primers/probes were designed only from highly conserved sequences among multiple known *Podoviridae* isolate sequences. Unfortunately, this could not be done for *Myoviridae,* as only a single isolate sequence information was available (Lehman *et al.* 2009).

The PCR products generated by the primers were further scrutinized by gel electrophoresis and negative first-derivative melting curve analysis (Ririe *et al.* 1997). All the primers produced PCR products that generated single bands of predicted base pair lengths from target templates and lacked amplification from non-target templates. Negative first-derivative melting curves generated with the DNA-associating dye SYBR green I showed the double stranded dissociation transitions as temperature peaks for each primer. This dissociation profile gives an indication of uniformity between PCR products generated by different templates (Ririe *et al.* 1997), in this case isolates within a phage family. From the primers tested, STS3 and PUN45 were found to produce ideal, narrow overlapping dissociation peaks suggesting homology between sequences from isolates within a family. There was one exception, ΦEa46-1A2 had a slightly different profile than other *Podoviridae* isolates tested. The profile was consistent and considered to be the result of nucleotide variability in the target sequence rather then from non-specific amplification of a non-target sequence. The addition of TaqMan chemistry probes with the STS3 and PUN45 primers produced similar fluorogenic responses as seen with SYBR green I. This confirmed that each primers/probe was able to discriminate between *Podo-* and *Myoviridae* phages.

The qPCR assay was developed to be compatible with the use of templates from crude samples, not purified nucleic acid preparations. This approach was taken because inefficiencies in phage DNA isolations resulted in false-negatives and false-positives

from pre-sample processes prone to cross-contamination by easily aerosolized phage particles. This approach is not without drawback, as false negatives can still occur as a result of low levels of target DNA or amplification inhibition from PCR inhibitors present in the crude preparation (Wilson 1997; Radstrom *et al.* 2004; Kox *et al.* 2005). To address this possibility, assay sensitivity was determined by calculating the LOD for each primers/probe set. On average, the LOD for *Erwinia* spp. phages was about 125 PFU/ml and 1000 CFU/ml for *E. amylovora.* As far as assay reliability, PCR amplification efficiencies between the two primers/probe sets were >90 for 4 out of 6 phage templates, which is a good indicator of an optimal and reproducible assay (MacKay 2007; Sloan 2007). The remaining phage isolates showed a slight reduction in PCR efficiencies which was to be expected. Multiplex design employs a universal reagent concentration and thermal profile, which may not lend itself to be the best conditions for all PCR amplifications. However, all R^2 values showed minimal variability between samples offering regression reproducibility and template serial dilutions that produced C_t values that were evenly distributed. In a multiplex reaction the LOD and amplification efficiencies did not change by addition of multiple primers/probe sets or templates. This suggested that there was no competition between PCR reactions resulting in failed or diminished fluorogenic responses, or non-specific binding producing a false-positive response (Henegariu *et al.* 1997; Polz and Cavanaugh 1998).

In an attempt to construct a universal *Erwinia* spp. phage qPCR diagnostic method, several primers were designed from the known *Myoviridae* sequence. Negative first-derivative melting curves generated with SYBR green I produced an array of dissociation peaks for multiple *Podo-*, *Myo-* and *Siphoviridae* isolates. This suggested multiple PCR products were generated but each isolate had a different amplicon

sequence. Inclusion of TaqMan probes increased specificity to the extent that only *Myoviridae* phages produced fluorogenic responses. The addition of TaqMan chemistry probes to qPCR inherently increased specificity which was the result of adding a third hybridization event (MacKay 2007; Roach and Svircev 2010). This allowed the assay the ability to exclusively identify only *Myoviridae* phage from false-positives generated from the *Podo-* and *Siphoviridae,* and a universal *Erwinia spp.* phage primers/probe was not attained. So far *Erwinia* spp. phage sequence information has shown little homology of members between families, therefore it is unlikely that a universal primers/probe can be developed. The benefit of the lack of homology is increased confidence in the ability to prevent false-positives due to non-specific amplification between the phage families, which can be problematic with rtPCR.

To reduce assay time and cost, several factors were considered when optimizing qPCR protocol (Kolmodin and Birch 2002; Exner and Lewinski 2002; Gunson *et al.* 2006). These included the concentrations of PCR reagents and template, thermal cycling parameters, and the compatibility of each set of primers and probes. Comparison of two reactions differing in only reductions in PCR reagents or thermal profile duration resulted in no significant difference in C_t values for either the phage or bacterium. Compared with the commercially available mastermix for multiplex qPCR, the optimized protocol provided a 10% increase in sensitivity for bacterial quantification. There was nominal difference between the mastermixes for phage quantification. Overall, a higher reagent concentration or longer thermal profile duration did not improve the assay sensitivity and was deemed unnecessary. This translates to a lower cost per sample by reducing reagent use and assay time by about twenty minutes.

Traditionally, culture-based double agar layer techniques are used to confirm

plaque morphology leading to isolate identity confirmation (Adams 1959), an approach used in the past to monitor the AAFC Vineland Phage Collection. Based on TEM studies of the original collection, phages in Group 1, 2, 3-6 were classified as *Myo-*, *Sipho-* and *Podoviridae,* respectfully (Gill *et al.* 2003). Comparison of rtPCR screening for phage family (Table 3-3) with the Vineland Collection published by Gill *et al.* (2003) shows that phage isolates 21-2,10-6, 31-5, 10-16, 9-2, 9-5, 51-2, -3, -4, -6,and -7 (highlighted in bold) differ from the original characterized phage family and/or are uncharacterized. This led to the discovery that reliance on plaque morphologies to maintain stock purities of phage library maintenance was inadequate. This was most likely due to the inability to visually discriminate between similar plaque types and/or to detect low concentrations of contaminating phages which led to the subsequent subculturing of the incorrect phage isolate. Targeting specific phage family genes with qPCR was able to distinguish an isolates family identity with ease. The developed qPCR assay is also capable of detecting low level contaminants when the contaminant is of a different phage family to the intended original isolate, another limitation of the double agar layer technique. Nonetheless, evidence that the master stock has been compromised has sent investigators back into old stock collections to retrieve the original phage isolates.

Screening the entire library of *Erwinia* spp. phages did present some limitations of the developed qPCR assay. None of the designed primers/probes could distinguish between isolates within each phage family; therefore it does not negate the requirement to conduct RFLPs to ensure phage isolates belong within their respective group. QPCR was unable to identify any of the known *Siphoviridae* phages. This may be remedied as new *Siphoviridae* sequence information becomes available to allow additional primers and probe development. The LOD for the phages had C_t values in the range of 35-41 cycles.

All C_t values produced in this range were capable of true-positive diagnosis, but the absence of C_t values could not discriminate no template from low template. For accurate true-negative diagnosis C_t values had to occur at <35 PCR cycles.

Microbial diagnostics and quantification have undergone remarkable improvement since the advent of rtPCR technologies. Real-time PCR has introduced additional specificity and sensitivity when compared to conventional PCR methods, as well as streamlined sample handling and result interpretation (Holland *et al.* 1991; Makay 2007; Roach and Svircev 2010). This study outlines a second-generation validated qPCR assay to discriminate and quantify *Erwinia* spp. *Podo-* and *Myoviridae* phages, the pathogen *E. amylovora* and the carrier, *P. agglomerans* simultaneously in a multiplex format. The qPCR assay was highly sensitive and specific, and provided a valuable tool to facilitate the development of the phage-based biopesticide to control the fire blight pathogen, *E. amylovora*. QPCR may be used further to address current diagnostic issues by providing a more versatile and accurate platform to diagnose and quantify biological titers in sample preparations over traditional culturing methods. Future applications of qPCR will also be instrumental in biopesticide registration. For example, qPCR may be used to determine the environmental fate of the biopesticide in the orchard environment.

Chapter 4

Incidence of Lysogeny in *Erwinia amylovora* and the Epiphyte *Pantoea agglomerans*

4.1 Introduction

To date, studies have not addressed the impact of artificially applied phages to the environment during biopesticide treatments. Phages have the capacity to alter bacterial adhesion, colonization, invasion, spread, resistance to host immune defenses and bacterial exotoxin production (Bossi *et al.* 2003; Brussow *et al.* 2004; 2008). Commonly, this enhanced bacterial virulence occurs due to horizontal gene transfer (HGT) through lysogeny, via transduction or lysogenic conversion (Miao and Miller 1999; Wagner and Waldor 2002; Brussow *et al.* 2004). Specialized transduction occurs when host genes are erroneously excised along with the prophage, packaged into a phage virion and carried to another bacterial host. Lysogenic conversion occurs when genes on the phage genome are expressed and alter the host phenotype, the most common form being immunity against lytic phage infection (Brussow *et al.* 2004; 2008). To date, studies have not reported the recovery of temperate phages nor the occurrence of lysogeny in *E. amylovora* (Erskine 1973b; Ritchie 1978; Schnabel and Jones 2001; Gill *et al.* 2003; Lehman 2007; Svircev *et al.* 2010). However, *E. amylovora* isolates have been reported to be insensitive to phage infection as indicated by a variable phage host range (Schnabel and Jones 2001; Gill *et al.* 2003). One explanation for the variable host range within the *E. amylovora* isolates may be that cells harbour temperate phages that render them resistant to other lytic phages via lysogenic conversions.

Typically, lysogens are identified by inducing the prophage lytic cycle which initiates the production of phage virions which are visualized as plaques on indicator bacterial hosts (Erskine 1973b; Ritchie 1978). This approach requires an appropriate prophage induction techniques and indicator bacterial hosts (Williamson *et al.* 2007). My work provides an alternative approach in detecting lysogens. The use of molecular based screening for prophage genes in the quiescent state, avoids the need for lytic induction or plaque formation on indicator bacterial hosts (O'Sullivan *et al.* 2000; Lunde *et al.* 2003). This chapter describes a large-scale screening approach for prophages, which utilizes real-time PCR (rtPCR) technologies to assess the incidence of lysogeny within *E. amylovora.* Included in this assessment was *P. agglomerans,* an orchard epiphyte with a similar niche as *E. amylovora* and involvement in the phage-based biopesticide. This assessment of the incidence of lysogeny provides critical information on the potential impact and long term efficacy of the phage biopesticide.

This study is the first to demonstrate that lysogeny exists in *E. amylovora.* The host cells that harboured prophages were found to be immune to further phage infection. However, wild type bacterial populations showed no evidence of native lysogeny suggesting that lysogeny may be disadvantageous under natural conditions.

4.2 Materials and methods

4.2.1 Bacteria and bacteriophages

The bacterial and bacteriophage isolates are listed in Table 4-1. Bacteria were stored at -80°C in a medium containing 0.8% nutrient broth (NB) (Difco Laboratories, Sparks, MD), 0.25% yeast extract (Difco), 0.5% sucrose, 0.25% K_2HPO_4, 0.05% KH_2PO_4, 0.025% $MgSO_4$ and 50% (v/v) glycerol. Phages were stored at 4°C in either 0.8% NB or 0.01 M phosphate buffer (PB, pH 6.8).

4.2.2 Media and culture conditions

Modified Miller-Schroth media (MMS) (0.8% NB), 5% sucrose, 9 ml of 0.5 % bromothymol blue, 2.5 ml of 0.5% neutral red, 2% Bacto-agar, and 0.5% cycloheximide solution; pH 7.4) was used as a semi-selective medium for initial isolation of *E. amylovora* and *P. agglomerans* isolates (Brulez and Zeller 1981). Inoculated MMS plates were incubated at 27°C for 24 h. Single colonies from MMS were subcultured on nutrient agar (NA, Difco) for 72 h and bacterial lawn was aseptically scraped into storage medium. Frozen bacterial solutions were grown on 2.3% NA, streaked to isolate single colonies and incubated at 27°C for 48 h prior to use. Single colony forming units (CFU) were subcultured on 2.3% NA or in flasks containing 50 ml of 0.8% NB, 0.25% yeast extract and 0.5% sucrose and incubated at 27°C for 18-20 h prior to use. Phages were enriched using the double agar overlay technique (Adams 1959). Phage lysate was mixed with host suspensions adjusted to 1 x 10^9 CFU/ml (OD_{600} = 0.6) in 0.01 M PB

Table 4-1 Bacterial isolates and bacteriophage used in study.

Isolate	Isolation plant or details	Source or Reference
Erwinia amylovora		
Ea6-4	*Pyrus communis*	(Jeng *et al.* 2001)
EaD-7	*Pyrus communis*	(Jeng *et al.* 2001)
Ea29-7	*Malus* X *domestica*	(Gill *et al.* 2003)
Ea29-7RΦ1	BID[a] of Ea29-7	this study
Ea29-7RΦ2	BID of Ea29-7	this study
Ea29-7RΦ3	BID of Ea29-7	this study
Ea29-7RΦ4	BID of Ea29-7	this study
Ea29-7RΦ5	BID of Ea29-7	this study
Ea29-7RΦ6	BID of Ea29-7	this study
EaG-5	*Pyrus communis*	(Jeng *et al.* 2001)
EaG-5Φ1	BID of EaG-5	this study
EaG-5Φ2	BID of EaG-5	this study
Ea17-1-1	*Pyrus communis*	(Jeng *et al.* 2001)
Ea17-1-1RΦ1	BID of Ea17-1-I	this study
Ea17-I-1RΦ2	BID of Ea17-1-1	this study
Ea17-1-1RΦ3	BID of Ea17-1-1	this study
Ea110R	*Malus* X *domestica*	(Ritchie and Klos, 1977)
Ea110RΦL	Ea110R lysogen harbouring ΦEaT100	this study
92 Global isolates		see Table 4-5.
Pantoea agglomerans		
Eh 1	*Malus* X *domestica*	A.M. Svircev[b]
Eh 1-28a	*Malus* X *domestica*	"
Eh 1-28b	*Malus* X *domestica*	"
Eh 17-17	*Malus* X *domestica*	"
Eh21-5	*Pyrus communis*	"
Eh 7-5	*Malus* X *domestica*	"
71 isolates[c]	*Pyrus communis* or *Malus* X *domestica* blossom washings	"
***Erwinia* spp. bacteriophage**		
ΦEa9-2	*Myoviridae*	A.M. Svircev
ΦEa9-4	*Myoviridae*	"
ΦEa10-1	*Myoviridae*	"
ΦEa10-4	*Myoviridae*	"
ΦEa10-6	*Myoviridae*	"
ΦEa10-16	*Siphoviridae*	"
ΦEa21-2	*Podoviridae*	"
ΦEa21-4	*Myoviridae*	"
ΦEa31-3	*Podoviridae*	"
ΦEa35-4	*Siphoviridae*	"
ΦEa45-1B	*Podoviridae*	"
ΦEa46-1A2	*Podoviridae*	"
ΦEa50-3	*Siphoviridae*	"
ΦEaT100	*Podoviridae*, temperate	this study

[a] BID - bacteriophage insensitive derivative
[b] Agriculture and Agri-Food Canada. Vineland, ON, CAN
[c] See Table 4-6 for details

(pH 6.8). After 10 min, 3 ml of top agar consisting of 0.8% NA, 0.25% yeast extract (Difco), 0.5% sucrose heated to 55°C was added and poured over solidified 2.3% NA and incubated for 18-20 h at 27°C. Plates were flooded with 0.8% NB and the soft agar was scraped into a chloroform resistant centrifuge tube containing 20ml of NB. After agitation for 20 min, 2% (v/v) of chloroform was added and the sample was agitated for an additional 20 min. Centrifugation at 8000 x *g* for 25 min removed bacterial debris and agar. The supernatant was filtered using 0.2 µm filters (Nalgene, Rochester, NY). Phages were enriched by inoculating liquid bacterial cultures prepared in 0.8% NB and incubated at 27°C on an orbital shaker (100 rpm) for 18-20 h. Chloroform was added then the sample centrifuged and the supernatants filtered using 0.2 µm filters. To achieve higher titres, phage enrichments were concentrated by centrifugation at 16000 x *g* for 45 min and pelleted. The phage pellet was re-suspended in 100 µl.

4.2.3 Bacteria and bacteriophage titering

Phage stocks were titred by counting plaques produced on a uniform host lawn, prepared using the double layer agar technique (Adams 1959). Countable plaques were produced from 10 fold serial dilutions of the stock, removal of 100 µl from each dilution and mixing it with 100 µl of bacterial host at 10^9 CFU/ml. Three millilitres of top agar was added and contents poured over 30ml of solidified 2.3% NA in a 90 mm Petri plate. After an incubation period of 18-20 h at 27°C, plaques were counted and titres were calculated in PFU/ml. Routine lysate titres were confirmed by spot lysis technique where 10 µl of serial dilutions were aliquoted onto pre-poured bacterial lawns seeded with 100

µl of isolation host at 10^9 CFU/ml. Bacterial suspensions in 0.01 M PB (pH 6.8) were measured by mixing serial dilutions with 3ml of top agar. Mixtures were poured over 30 ml of 2.3% NA. After incubation for 18-20 h at 27°C, titres were calculated in CFU/ml. A standard curve was generated by relating each dilution measured CFU/ml with absorption at OD_{600}.

4.2.4 Blossom isolation of *Pantoea agglomerans*

Ten blossoms with removed petals were sonicated in 0.01 M PB and cultured on semi-selective MMS medium to isolate *Erwinia* sp. and *Pantoea* sp.[1] *E. amylovora* and *P. agglomerans*, colonies growing on MMS which had the characteristic orange appearance and produce large amounts of EPS were subcultured on NA to isolate single colonies. Colonies which produced a yellow pigmentation on NA were tentatively identified as *P. agglomerans*.

4.2.5 Real-time PCR

Multiplex real-time PCR was used to detect and diagnose *E. amylovora*, *P. agglomerans*, and *Erwinia* spp. phage targets. Target oligonucleotide primer and probe sequences are listed in Table 4-2. Multiplex rtPCR were run in 25 µl volumes using 10X ThermoPol buffer (New England Biolabs, Ipswich, MA), 0.2 mM of each primer, 0.1 mM

[1] Orchard bacterial isolates collected by A.M. Svircev (Agriculture and Agri-Food Canada, Vineland ON)

of probe, 0.2 mM each of dNTP, 2 mM $MgCl_2$, 1.3 U *Taq* polymerase (New England Biolabs). Two µl of a single CFU was suspended in PB and added as template to reactions to be analyzed in a Stratagene Mx4000 Quantitative PCR thermocycler (LaJolla, CA) under the following conditions; 95°C, 5 min; then 45 cycles of 95°C, 10s, 60°C, 16 s, with fluorogenic responses recorded during each elongation step. Positive and no template controls were included during each run. Positive diagnosis occurred when fluorogenic responses generated during reactions crossed a threshold cycle (C_t) as determined by the Mx4000 software.

Table 4-2. Primers (F/R) and TaqMan™ probes (P) used for multiplex real-time PCR.

Name	Diagnosis	Sequence (5' - 3')[a]	Reference / Source
Ea-Lsc-F	*Erwinia amylovora*	cgctaacagcagatcgca	(Lehman *et al.* 2007)
Ea-Lsc-R		aaatacgcgcacgaccat	
Ea-Lsc-P		**CY5**/ctgataatccgcaattccaggatg**IAbRQSp**	
Pa-Gnd2-F	*Pantoea agglomerans*	Intellectual property of AAFC	AAFC[b]
Pa-Gnd2-R			
Pa-Gnd2-P			
PUN45-F	*Erwinia* spp. phage	aacgaacagcgccttgac	this study
PUN45-R	(Myoviridae)	ccagttgcagccagtgtg	
PUN45-P		**ROX**/actgagaagtacggtatcaaaccttc/**IAbRQSp**	
STS3-F	*Erwinia* spp. phage	gacaaacaagaacgcggcaactga	this study
STS3-R	*(Podoviridae)*	atacccagcaaggcgtcaacctta	
STS3-P		**FAM**/agatgaagtaggttatcttcacagtgccct/**BHQ_1**	

[a] Designations: Cy5™, 6-FAM™, HEX™, ROX™ NHS Ester, Iowa Black RQ™-Sp (IAbRQSp) and Black Hole Quencher™ (BHQ_1) (IDT, Coralville, IA, USA)

[b] Agriculture and Agri-Food Canada. Vineland ON, CAN

4.2.6 Spontaneous liberation and induction of prophage

Two methods were used for the recovery of a prophage from phage-resistant bacterial isolates, spontaneous liberation and induction. An overnight nutrient broth culture of isolate was centrifuged at 12,000 x *g* for 4 min. The supernatant was mixed with a sensitive host in nutrient soft agar and then overlaid on NA. After incubation at 27°C for 24 h phage particles released spontaneously had produced plaques. Mitomycin C (MMC) treatment was used to induce prophage activity. Five µl of overnight culture was diluted into 45 ml of fresh NB in separate flasks, one for a control and one for a treatment. When the culture's OD_{600} reached 0.4, MMC (0.5 µg/ml or 1 µg/ml) (Jiang and Paul 1998) was added and all tubes were incubated at 27°C. After 18 h, absorbance measurements were obtained prior to centrifugation at 12,000 x *g* for 4 min. Supernatant was mixed with a sensitive host in soft NA and then overlaid on 2.3% NA. After incubation at 27°C for 24 h, phage particles released by induction were visible as plaques. From both methods, single phage types were isolated by removing an agar plug with a sterile glass pipette, transferred to PB. This passage was repeated three times and phages stored at 4°C in NB.

4.2.7 Isolation of bacteriophage insensitive derivatives

To isolate phage insensitive bacterial cells two challenge methods were used, the agar plate method and the secondary culture method (Guglielmotti *et al.* 2006). Aliquots of overnight bacterial cultures grown in nutrient broth were infected with phages at different multiplicity of infections (MOI) of 10, 1 and 0.1. Infected cells were mixed with

top agar, poured over 2.3% NA and incubated at 27°C for 24-48 h. Single colonies on an otherwise confluently lysed lawn were tested for phage resistance. The secondary method was performed in a similar manner, except after 24 h streaks were taken from cultures exhibiting no colony growth and sub-cultured on fresh 2.3% NA medium at 27°C for an additional 24 h. Colony formation indicated bacteriophage insensitive derivatives (BID) of their parental strain. BIDs were subcultured 4 times on 2.3% NA in the absence of phages and stored at −80°C.

4.2.8 Host range analysis

Sensitivity of bacterial isolates to phage isolates was determined by spotting 10 µl of 10^8 PFU/ml phage lysate on bacterial lawns. Lawns were prepared by seeding 3 ml of top agar with 100 µl from a 10^9 CFU/ml bacterial culture suspended in 0.01 PB and poured over solidified 2.3% NA. After an incubation period of 18-20 h at 27°C, isolates were considered sensitive to a phage isolate if plaques had formed. If an inoculation area appeared the same as the surrounding untreated lawn, the bacterial isolate was considered to be phage-resistant.

4.2.9 DNA manipulation

DNA from high-titre purified phage lysates containing at least 10^{10} PFU/ml were extracted using the Phage DNA Isolation kit (Norgen Biotek, St. Catharines, ON). DNA was eluted in sterile dH_2O and stored at -20°C. DNA (1 µg) was digested using with 1 U

of restriction enzyme. Digestions were conducted with *Eco*RI (Invitrogen Canada, Burlington, ON), *Bam*HI (New England Biolabs) or *Bgl*II (MBI Fermentas, Hanover, MD) according to the supplier's instructions. After digestion, restriction patterns were visualized on a 1% agarose gel run in Tris-acetate-EDTA buffer and stained with 0.5 μg/ml ethidium bromide.

4.3 Results

4.3.1 Screening for prophages

Multiplex rtPCR was used for simultaneous isolate identification and prophage detection in several *E. amylovora* isolates originating from 9 geographic locales across the globe (Table 4-3). All 102 *E. amylovora* isolates were confirmed by rtPCR with the Ea-lsc primers/probe combination. Simultaneously, these isolates were shown to be negative for detectable *Podo-* and *Myoviridae* prophages as no C_t values were generated. Samples were collected from *Pyrus* sp. blossom washings, isolated and screened on semi-selected MMS growth medium. Isolates expressing orange colony morphology on MMS were identified as being either *E. amylovora* or *P. agglomerans*. Subsequent culturing on NA showed that of 82 isolates, 74 were yellow pigmented and the remaining 8 were non-pigmented or white. Multiplex rtPCR with primers/probe Pa-gnd identified all the yellow and white pigmented colonies as being *P. agglomerans*. Simultaneously, all isolates were shown to be negative for detectable *Podo-* or *Myoviridae* prophages as no C_t values were generated (Table 4-4).

The natural spontaneous induction of prophage's lytic cycle was not observed in any of the *E. amylovora* isolates or BIDs during monitoring broth cultures for visual clearing. Supernatants from these cultures also did not produce plaques when cultured on lawns of bacterial indicator isolates (data not shown). Supernatants from cultures of 82 *P. agglomerans* isolates that were originally purified from orchard blossom washings and treated with 0.5 μg/ml MMC did not produce plaques on *E. amylovora* indicator isolates Ea6-4, Ea17-1-1, Ea110 and Ea29-7 (Table 4-4). Treatment with 1 μg/ml MMC on a subgroup of 22 *P. agglomerans* did not yield visible plaques on the same indicator

isolates.

Table 4-3. Genomic screening for lysogeny in *Erwinia amylovora*. C_t values achieved with real-time PCR were used to determine *E. amylovora* identity (Ea-Lsc) and identify presence of a *Myoviridae* (PUN45) or a *Podoviridae* (STS3) prophage target gene.

Isolate	*E. amylovora*	Prophage	Origin	Source	Isolate	*E. amylovora*	Prophage	Origin	Source
Ea6-4	+	-	ON, Canada	A.M. Svircev[a]	661	+	-	Poland	P. Sobiczewski
Ea29.7	+	-	ON, Canada	"	367	+	-	Poland	"
Ea17-1-1	+	-	ON, Canada	"	1526-1	+	-	Spain	M. Lopez
EaG-5	+	-	ON, Canada	"	2349-2	+	-	Spain	"
EaD-7	+	-	ON, Canada	"	1777-1	+	-	Spain	"
F1	+	-	France	J.-P.Paulin[b]	1892-1	+	-	Spain	"
F2	+	-	France	"	2397-5.4	+	-	Spain	"
F3	+	-	France	"	1739-1	+	-	Spain	"
F4	+	-	France	"	1924-4	+	-	Spain	"
F5	+	-	France	"	2303-3	+	-	Spain	"
F6	+	-	France	"	2311-6	+	-	Spain	"
F7	+	-	France	"	Ea If	+	-	CA, USA	S. Lindow[f]
F8	+	-	France	"	Ea Apl 1	+	-	CA, USA	"
F9	+	-	France	"	Ea12	+	-	CA, USA	"
F10	+	-	France	"	Ea91	+	-	CA, USA	"
250/07	+	-	Germany	E. Moltmann[c]	Ea120	+	-	CA, USA	"
280	+	-	Germany	"	Ea168	+	-	CA, USA	"
294	+	-	Germany	"	Ea176	+	-	CA, USA	"
245	+	-	Germany	"	Ea184	+	-	CA, USA	"
234/07	+	-	Germany	"	Ea205	+	-	CA, USA	"
227/07	+	-	Germany	"	Ea21	+	-	CA, USA	"
224/07	+	-	Germany	"	Ea43	+	-	CA, USA	"
221/07	+	-	Germany	"	Ea60	+	-	CA, USA	"
213/07	+	-	Germany	"	Ea7a	+	-	CA, USA	"
243/07	+	-	Germany	"	Ea8	+	-	CA, USA	"
M1	+	-	Morocco	J.-P.Paulin	Ea110	+	-	MI, USA	G. Sundin[g]
M2	+	-	Morocco	"	EL0	+	-	MI, USA	"
M3	+	-	Morocco	"	RB02	+	-	MI, USA	"
M4	+	-	Morocco	"	RB07	+	-	MI, USA	"
M5	+	-	Morocco	"	RN8	+	-	MI, USA	"
M6	+	-	Morocco	"	MK1	+	-	MI, USA	"
M7	+	-	Morocco	"	A7	+	-	MI, USA	"
M8	+	-	Morocco	"	A-11	+	-	MI, USA	"
M9	+	-	Morocco	"	A-22	+	-	MI, USA	"
M10	+	-	Morocco	"	A-24	+	-	MI, USA	"
Ea 501	+	-	New Zealand	J. Vanneste[d]	B-3	+	-	MI, USA	"
Ea 315-1	+	-	New Zealand	"	B-51	+	-	MI, USA	"
Ea 412-1	+	-	New Zealand	"	B-58	+	-	MI, USA	"
FB 29	+	-	New Zealand	"	B-65	+	-	MI, USA	"
FB 97b	+	-	New Zealand	"	B-95	+	-	MI, USA	"
FB 78a	+	-	New Zealand	"	B-110	+	-	MI, USA	"
462	+	-	Poland	P. Sobiczewski[e]	MI 6-2	+	-	MI, USA	"
464	+	-	Poland	"	MI 14-2	+	-	MI, USA	"
E10	+	-	Poland	"	P-C-1	+	-	MI, USA	"
660	+	-	Poland	"	SL	+	-	MI, USA	"
650	+	-	Poland	"	T-1	+	-	MI, USA	"
694	+	-	Poland	"	D-1	+	-	MI, USA	"
614a	+	-	Poland	"	BB-1	+	-	MI, USA	"
616a	+	-	Poland	"	Root C-2	+	-	MI, USA	"
624a	+	-	Poland	"	GM-1	+	-	MI, USA	"
633a	+	-	Poland	"	BJN	+	-	MI, USA	"

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Table 4-4. Screening for lysogeny in *Pantoea agglomerans* isolated from orchard collected blossoms. Isolate identity confirmed with morphology and real-time PCR. Presence or absence of prophage was determined by phage gene detection and lytic induction.

	Colony morphology[a]		Real-time PCR diagnosis[b]		Induction[c]		Colony morphology		Real-time PCR diagnosis		Induction
Isolate	MMS	NA	*P. agglomerans*	Prophage	Prophage	Isolate	MMS	NA	*P. agglomerans*	Prophage	Prophage
Eh 1	NT	Y	+	-	-	Pa31-3	O	Y	+	-	-
Eh 1-28a	NT	W	+	-	-	Pa31-5	O	Y	+	-	-
Eh 1-28b	NT	Y	+	-	-	Pa31-6	O	Y	+	-	-
Eh 17-17	NT	W	+	-	-	Pa31-7	O	Y	+	-	-
Eh21-5	NT	Y	+	-	-	Pa31-8	O	Y	+	-	-
Eh 7-5	NT	W	+	-	-	Pa39-1	O	Y	+	-	-
Pa2-2	O	Y	+	-	-	Pa39-2	O	Y	+	-	-
Pa9-1	O	Y	+	-	-	Pa39-3	O	Y	+	-	-
Pa9-2	O	W	+	-	-	Pa39-4	O	Y	+	-	-
Pa9-4	O	W	+	-	-	Pa39-5	O	Y	+	-	-
Pa13-1	O	Y	+	-	-	Pa39-6	O	Y	+	-	-
Pa13-2	O	Y	+	-	-	Pa39-7	O	Y	+	-	-
Pa13-3	O	Y	+	-	-	Pa39-8	O	Y	+	-	-
Pa13-4	O	Y	+	-	-	Pa39-9	O	Y	+	-	-
Pa13-5	O	Y	+	-	-	Pa39-10	O	Y	+	-	-
Pa17-1	O	Y	+	-	-	Pa39-11	O	Y	+	-	-
Pa17-2	O	Y	+	-	-	Pa39-28	O	Y	+	-	-
Pa17-3	O	Y	+	-	-	Pa39-12	O	Y	+	-	-
Pa17-4	O	Y	+	-	-	Pa39-13	O	Y	+	-	-
Pa17-5	O	Y	+	-	-	Pa39-29	O	Y	+	-	-
Pa21-2	O	Y	+	-	-	Pa39-14	O	Y	+	-	-
Pa21-3	O	Y	+	-	-	Pa39-15	O	Y	+	-	-
Pa21-4	O	Y	+	-	-	Pa39-16	O	Y	+	-	-
Pa21-5	O	W	+	-	-	Pa39-17	O	Y	+	-	-
Pa21-6	O	Y	+	-	-	Pa39-18	O	Y	+	-	-
Pa21-7	O	W	+	-	-	Pa39-19	O	Y	+	-	-
Pa21-9	O	Y	+	-	-	Pa39-20	O	Y	+	-	-
Pa21-10	O	Y	+	-	-	Pa39-21	O	Y	+	-	-
Pa21-11	O	W	+	-	-	Pa39-22	O	Y	+	-	-
Pa21-12	O	Y	+	-	-	Pa39-23	O	Y	+	-	-
Pa21-13	O	Y	+	-	-	Pa39-26	O	Y	+	-	-
Pa21-14	O	Y	+	-	-	Pa39-24	O	Y	+	-	-
Pa21-15	O	Y	+	-	-	Pa39-25	O	Y	+	-	-
Pa21-16	O	Y	+	-	-	Pa39-27	O	Y	+	-	-
Pa21-17	O	Y	+	-	-	Pa39-30	O	Y	+	-	-
Pa21-18	O	Y	+	-	-	Pa45-1	O	Y	+	-	-
Pa21-19	O	Y	+	-	-	Pa45-2	O	Y	+	-	-
Pa21-20	O	Y	+	-	-	Pa45-3	O	Y	+	-	-
Pa21-21	O	Y	+	-	-	Pa46-2	O	Y	+	-	-
Pa31-1	O	Y	+	-	-	Pa49-1	O	Y	+	-	-
Pa31-2	O	Y	+	-	-	Pa-HJ	O	Y	+	-	-

[a] MMS (modified Miller-Schroth agar), NA (nutrient agar), O (orange), Y (yellow), W (white), NT (not tested)
[b] Ct value acheived from either Ea-Lsc (*E. amylovara*) or STS3/PUN45 (prophage) primers/probe
[c] Induction of prophage's lytic cycle with mitomycin C, determined by plaque formation on 6 indicator isolates

4.3.2 Bacteriophage insensitive derivative

After numerous trials using the phage challenge methods and various combinations of phages and hosts (data not shown), the secondary culture method was found to be more efficient at isolating BIDs over the agar overlay method. In total 53 BIDs were isolated from one of the sensitive *E. amylovora* Ea29-7, Ea17-1-1, G-5 and Ea110 isolates. All BIDs were identified by rtPCR as *E. amylovora*. *Podoviridae* and *Myoviridae* phage genes were not detected as C_t values were not generated by either primers/probe STS3 or PUN45 with the 6, BIDs from Ea29-7, 3 BIDs from Ea17-1-1 and 2 BIDs from G-5. Of the 42 BIDs isolated from Ea110R, 27 survived the subculturing process. These survivors were cultured from frozen three times and individual colonies assayed with multiplex rtPCR (Table 4-5). The target *Podoviridae* gene was amplified from within all 27 purified BIDs during the first culture trial. All BIDs were negative for the target *Myoviridae* gene. Of the 27 BIDs, 12 remained stable and produced C_t values in the next two trials that were similar in titre ratio between host and prophage as the first trial. The remaining BIDs showed a decrease in or no prophage detection over the course of the two subsequent trials.

Table 4-5. Dualplex real-time PCR of isolated *Erwinia amylovora* Ea110R bacteriophage insensitive derivatives. Threshold cycle (C_t) values were derived from purified Ea110R BID colonies by amplifying *Podoviridae* prophage DNA with primers/probe STS3. The recovered putative lysogens were assayed from three cultures cultivated from frozen stocks.

	Culture 1		Culture 2		Culture 3			
BID	CY5	FAM	CY5	FAM	CY5	FAM	CY5	FAM
8	17.21	18.17	17.08	No Ct	17.30	19.28	17.39	19.01
10	15.38	19.62	16.40	11.59	18.73	14.18	18.31	13.60
18	16.50	16.63	17.85	12.39	17.01	13.16	16.83	13.11
19	17.64	18.06	19.59	17.27	18.96	16.89	19.06	16.63
24	16.15	17.00	20.47	25.91	18.63	24.06	18.38	23.67
26	15.52	15.30	16.28	14.89	19.66	21.77	19.60	21.74
27	16.89	14.59	15.90	13.07	16.24	16.99	16.79	16.09
34	15.49	26.09	18.88	14.75	20.15	16.40	20.19	16.66
35	16.48	30.64	19.80	13.71	19.11	13.63	19.28	13.63
39	16.66	16.25	17.48	9.43	18.16	15.35	18.29	15.26
40	16.12	16.80	18.58	12.99	16.73	13.18	16.91	13.26
41	16.84	20.94	17.13	16.65	20.02	21.23	20.45	21.82
1	18.27	32.76	17.15	*No Ct*	20.63	38.85	20.30	*No Ct*
6	15.93	27.46	20.87	37.82	18.95	44.63	19.11	*No Ct*
7	17.03	36.02	19.53	*No Ct*	17.44	38.63	17.47	*No Ct*
16	15.95	35.32	18.17	39.24	17.01	No Ct	17.17	39.90
21	15.75	22.15	16.99	*No Ct*	17.89	No Ct	18.27	*No Ct*
22	15.26	33.30	18.96	*No Ct*	18.43	38.31	16.11	36.96
23	17.18	30.31	17.20	*No Ct*	17.33	39.21	17.63	*No Ct*
25	16.28	19.71	16.38	39.84	19.24	No Ct	19.33	*No Ct*
29	16.16	31.54	18.63	*No Ct*	16.67	No Ct	16.79	31.31
37	17.04	32.60	18.12	*No Ct*	20.84	37.42	20.85	36.96
38	17.20	29.63	19.63	37.04	18.59	38.15	18.50	*No Ct*
42	17.14	29.40	17.55	37.55	17.92	37.33	14.44	*No Ct*

4.3.3 BID host range change

Plaque formation was used to assess the host range and plaque morphology of several *Erwinia* spp. phages on both wild-type (*wt*) and BID *E. amylovora* isolates (Table 4-6). All BIDs exhibited a loss of sensitivity to multiple phages, including the phage that they were first challenged with, compared to the parental isolate. The BIDs were resistant to the challenge phage as well as two *Podoviridae* phage isolates when compared to parental cells. An example of these results is given in Table 4-6. BID Ea110RΦL showed complete resistance to three phages that are capable of lysing the parental strain Ea110R. A decrease in phage sensitivity was determined by a reduction in ability to confluently lyse host cells within plaques as seen by the production of turbid plaques. In general no discernable patterns in host range alterations could be determined for any of the BIDs.

Table 4-6. Host range and plaque morphology for lysogenized and non-lysogenized *Erwinia amylovora* Ea110R.

	Erwinia spp. bacteriophage (family)*						
Isolate[a]	ΦEa10-1 *(M)*	ΦEa21-4 *(M)*	ΦEa35-4 *(S)*	ΦEa10-6 *(P)*	ΦEa31-3 *(P)*	ΦEa45-1B *(P)*	ΦEa46- *(P)*
Ea110R	clear	clear	clear	clear	clear	clear	clear
Ea110RΦL	turbid	turbid	-	clear	-	clear	-

* *(M) Myoviridae, (S) Siphoviridae, (P) Podoviridae,* (-) resistant to plaque formation

4.3.4 *Erwinia* spp. temperate phage

MMC treatment of the BID Ea110RΦL #10 resulted in the induction of the prophage and the recovery of a viable temperate phage ΦEaT100. Phage ΦEaT100 produced plaque morphologies that varied depending on *E. amylovora* isolate host. ΦEaT100 produced plaques on Ea110 that were 4-6 mm in diameter with a visible halo which expanded over time (Fig. 4-1[right/top]). On Ea6-4, plaques were 2-3 mm with increased plaque clarity, pin-point in size, located in the centre of the plaque. There was also no visible halo produced (Fig. 4-1[right/bottom]). These plaque morphologies are similar to those produced by known *Podoviridae* phages (Gill *et al.* 2003).

Real-time PCR primers/probe STS3 designed to be specific for the *Podoviridae* showed typical fluorescent response patterns similar to those generated from known *Podoviridae* phages. Restriction fragment length polymorphism (RFLP) analysis of DNA from *Erwinia spp.* temperate phage ΦEaT100 showed differences between restriction site locations producing distinguishable DNA segments (FIG. 4-1 left). Restriction endonuclease *Bam*HI did not produce any segments, whereas *Eco*RI and *Bgl*II produced 3 and 9 fragments respectfully. Digestion of *Eco*RI restriction sites produced fragments approximately 10,000, 5000 and 1500 bp in length. Digestion of *Bgl*II restriction sites produced fragments approximately 12,000, 10,000, 8000, 6000, 4000, 3500, 3000, 2200, and 1500 bp. Summing the resolvable segments of *Bgl*II gives an estimated genome size of 46,000 kbp.

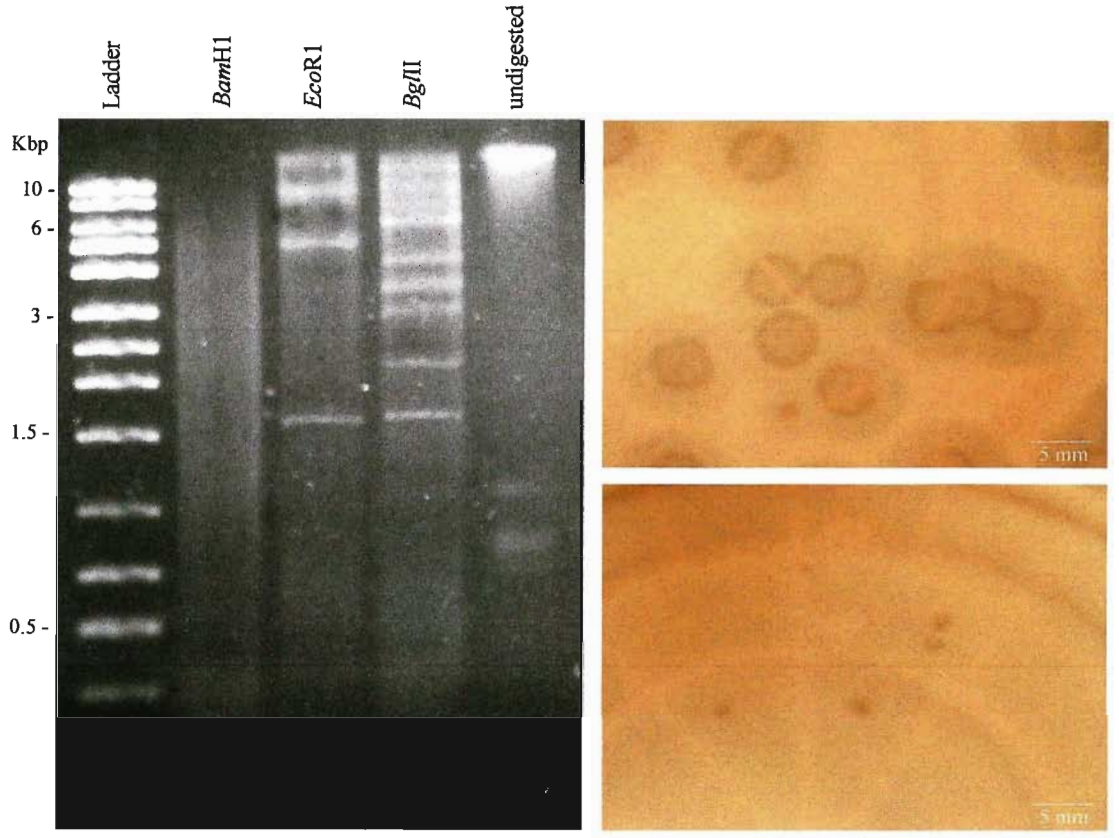

Figure 4-1. Restriction analysis of DNA from *Erwinia* spp. temperate phage ΦEaT100 (left). ΦEaT100 plaque morphologies on *E. amylovora* (right). Plaques on Ea110 (top) were 4-6 mm in diameter with a visible halo which expanded over time. On Ea6-4 (bottom) plaques were 2-3 mm with a central pin-point increased clearing and no visible halo. Ladder was HighRanger 1-kb DNA ladder (Norgen Biotek) and hosts were grown on nutrient agar (Difco).

4.4 Discussion

Host range studies have shown that some *E. amylovora* isolates demonstrate a varied phage infection profiles (Schnabel and Jones 2001; Gill *et al.* 2003; Lehman 2007). One explanation is that these cells were lysogenic, providing them with resistance to further lytic phage infection. Previous studies on *Erwinia* spp. phage have not been able to show that lysogeny occurs in *E. amylovora* (Ritchie 1978; Schnabel and Jones 2001; Gill *et al.* 2003; Lehman 2007). Lehman et al. (2009) also sequenced and annotated the complete genome for an *Erwinia* spp. myoviridal phage (ΦEa21-4) and found no potential known lysogeny-related genes. The only published complete sequence of an *E. amylovora* genome (CFBP 1430, NCBI NC_013961.1), was found not to encode any known bacterial attachment sites (*attB*), which may be needed for prophage integration into the bacterial chromosome (Groth and Calos 2004). In the present work, screening several isolates originating from 9 geographic locales across the globe with multiplex rtPCR and with prophage induction techniques also did not present any indication of naturally occurring lysogenic relationships between *Podoviridae* or *Myoviridae* phages and *E. amylovora*. This result suggests that lysogeny is less common for *E. amylovora* than seen for other Gram negative bacteria (Ackermann and Dubow 1987; Jiang and Paul 1998; Ghosh *et al.* 2008; Williamson *et al.* 2008).

Previous studies have reported *Erwinia* spp. phages are capable of lysogenizing the orchard epiphyte *P. agglomerans (*formerly *E. herbicola*) (Erskine 1973b; Harrison and Gibbins 1975). To investigate the incidence of lysogeny within *P. agglomerans,* 82 isolates recovered from apple and pear blossoms were screened with multiplex rtPCR. Cell cultures were also monitored for free phages released spontaneously or after MMC

induction. Similar to the *E. amylovora* findings, no evidence for lysogeny was obtained for any of the isolates.

Despite the lack of isolating native lysogens in orchard samples, by challenging *E. amylovora* isolates with high concentrations of phages *in vitro*, this study was able to select for lysogeny. The challenge methods selected for cells that were immune to phage lytic infection. Molecular screening of these BIDs for prophage genes revealed that derivatives from *E. amylovora* Ea110 gave rtPCR C_t values that confirmed lysogeny. Treatment of these lysogenized Ea110R cells (Ea110RΦL) with MMC cured cells of their resident prophage as these cured cells lost their immunity to lytic infection. RtPCR also confirmed prophage loss by the lack of C_t values. DNA restriction analysis of the lysogenic phage (ΦEaT100) showed RFLP patterning similar to that of Group 3 phages as characterized by Gill *et al.* (2003). These results suggested that ΦEaT100 is potentially a temperate member of the *Podoviridae*.

While little is known about the factors leading to the establishment of lysogeny, it is generally believed that they provide a refuge for temperate phages when conditions are unfavourable for robust replication or when host abundance is low (Marsh and Wellington 1994; Weinbauer 2004; Pasharawipas *et al.* 2008b). The results of the prophage rtPCR screening induction trials presented here are biased toward bacterial cells isolated from the phyllosphere, *E. amylovora* from plant tissues with disease symptoms and *P. agglomerans* from blossoms. As a bacterial habitat, diseased tissues and blossom stigmatic surfaces are exceptions to the generalization that aerial surfaces of plants are typically inhospitable to bacterial growth. Necrotic tissues and stigmas provide a protected, nutrient-rich, hydrated environment for the pathogen and epiphyte to multiply vigorously. These habitats do not include harsh environmental conditions or low host

abundance necessary to promote a lysogenic decision by the phage. Therefore, when *E. amylovora* and *P. agglomerans* cells were initially isolated, they were most likely in a state of vigorous growth due to ideal nutrient and environmental conditions provided by the plant. Because of this, it is no surprise that a lytic lifestyle would be favored leading to an absence of native lysogeny as seen here. To achieve a broader understanding into the incidence of lysogeny within orchard inhabiting *E. amylovora* and *P. agglomerans*, sampling of more variable environments such as those found in soils beneath plants, may reveal higher frequencies of lysogeny (Williamson *et al.* 2007; 2008). Therefore it remains unclear whether sampling the phyllosphere is representative of the genetic and phenotypic diversity of the larger orchard pathogen and epiphyte communities.

The phage challenge method resulted in a number of apparently lysogenized Ea110R cells as indicated by PCR. The host-to-prophage copy number ratios were found to be similar, almost 1:1, a trend conserved over three rtPCR trials. Several BIDs were far less stable with initial copy number ratios that indicated a high titre of bacterial cells and a low but detectable titre of prophages. However, after multiple trials, prophage copy numbers were reduced to undetectable levels. There are a few phenomena that might account for this variation in stability. These unstable lysogens could have undergone conversion to non-lysogens by mechanisms which are unclear but involves the loss of a prophage without loss of bacterial cell viability (Abedon 2008; Khemayan *et al.* 2006; Weinbauer 2004). A more plausible explanation is that these cells were initially in a pseudolysogenic state. Pseudolysogeny is an unstable coexistence of a phage genome in a host bacterium, where that genome fails to replicate either as a productive infection or with cell division as seen with lysogenic infections (Ripp and Miller 1998; 2008). Khemayan *et al.* (2006) reported a similar event where *Vibrio harveyi* lysogenized cell

stability varied widely and constantly generated large numbers of cured cells that had lost their lysogenic status. With traditional culture-dependent approaches, prophage curing or pseudolysogeny would be lost in the selection process due to their superinfection immunity instability. Since rtPCR assays are extremely sensitive, able to detect as low as 1 virion copy number per reaction (See Chapter 3), we were able to track the loss of the phages harboured by a few *E. amylovora* cells in a mixed population dominated by phage-free cells.

Currently, a phage-based biopesticide for the control of the fire blight pathogen *E. amylovora* is under development (Gill *et al.* 2003; Lehman 2007). Before such a product can be commercialized, it is important to assess the risk associated with lysogeny. Lysogenic populations, through specialized transduction and lysogenic conversion may promote new host phenotypic characteristics which can confer increased fitness, enhanced virulence and immunity to lytic phage infection (Weinbauer 2004; Brussow 2005; Abedon 2008). A consequence of lysogeny is prophage-encoded immunity against phages of the same immunity type as the resident prophage. The Ea110R cells harbouring the ΦEaT100 prophage (Ea110RΦL) were found to be superinfection immune to other but not all *Podoviridae* phages. Immunity was also gained for one of the *Siphoviridae* phages but not for any of the *Myoviridae* phages tested. From studying the incidence of lysogeny in orchard isolates, lysogeny seems to occur very infrequently within phyllosphere indicating that the risks associated with transduction and lysogenic conversion may be low. To help prevent the potential for superinfection immunity, a mixture or 'cocktail' of phages that contain at least one heterogeneous phage to the *Podoviridae* ΦEaT100 (i.e. *Myoviridae*) should be sufficient to infect and kill lysogenic cells.

Understanding the interrelationships among the lytic, lysogeny, and

pseudolysogeny lifestyles can also ensure proper selection of phage as biological control agents for the purpose of increasing the likelihood of phage survival while providing reliable and effective disease control. An added benefit of understanding the risks and benefits associated with lysogenic relationships is that it allows an alternative to using strictly virulent phages as biocontrol agents. Temperate phages undergo more than one reproductive episode per lifetime (iteroparous) whereas virulent phages only undergo a single reproductive infection episode (semelparous). A semelparous life strategy is only effective in controlling host bacteria when the rate of phage replication is high enough to overcome the rate of phage loss, putting dependence on high host numbers for prolonged phage exposure (Gill and Abedon 2003). An iteroparous life strategy may allow lysogenic phages to become established at a much lower initial host density putting less dependence on initial host numbers. To achieve biological control, environmental cues like UV radiation could be used to promote the prophage's lytic replication, thus killing the pathogen population. This approach would increase the probability of specialized transduction but one could argue that transduction would likely have a low practical consequence as isolates of *E. amylovora* are relatively homogenous having very little pathogenic variation (Momol and Aldwinckle 2000; Smits *et al.* 2010).

To the best of our knowledge, this study represents the first description of the incidence of lysogeny in pathogenic bacteria that reside in plant tissue. This study was facilitated by the innovative use of a simple and rapid molecular based rtPCR assay which detected prophages in their quiescent state, omitting the need for induction-dependent techniques. Real-time PCR provided a highly sensitive and specific assay that avoided the challenges associated with traditional prophage induction-dependent approaches for assessing lysogeny (Erskine 1973b; Williamson *et al.* 2007). Multiplexing with rtPCR

which simultaneously discriminated signals from *E. amylovora, P. agglomerans, Podo-* and *Myoviridae* phages within a single sample, allowed for rapid screening of a large number of samples for lysogeny. It was found that lysogeny does exist for *E. amylovora in vitro*, but is rare or absent when isolates are collected from plant tissues. The large scale prophage screening with rtPCR as well as lytic induction also found lysogeny to be rare to absent in epiphytic *P. agglomerans* cells collected from *Pyrus* and *Malus* blossoms. These findings present a unique opportunity to commercialize a phage-based biopesticide for *E. amylovora* since there is an extremely low risk associated with lysogenic conversion and transduction for either the carrier *P. agglomerans* or the pathogen *E. amylovora*. In addition, the newly discovered lysogenic form of an *E. amylovora* isolate can be examined for comparative virulence and examined in detail pathogenic outcomes from lysogeny.

Chapter 5

Effects of Exopolysaccharides on Bacteriophage Pathogenesis

5.1 Introduction

Like many pathogenic bacteria, *E. amylovora* produces long-chain exopolysaccharides (EPS) that are secreted by the cell into its surroundings during growth, and are in part responsible for the bacterium's virulence (Bellemann *et al.* 1994; Bereswill and Geider 1997). *E. amylovora* EPS is not permanently attached to the cell surface but rather forms a well-delimited loosely attached capsule (Gross *et al.* 1992; Geider *et al.* 1993; Bellemann *et al.* 1994). In *E. amylovora* two distinct EPSs are produced, the homopolysaccharide levan (Gross *et al.* 1992; Geier and Geider 1993) and heteropolysaccharide amylovoran (Bellemann *et al.* 1994; Bereswill and Geider 1997).

The production of a highly hydrated EPS is advantageous for growth and survival of the bacterium because it may provide protection against toxic compounds, desiccation, osmotic stress, as well as allowing *E. amylovora* to avoid recognition by plant defense systems (Leigh and Coplin 1992). EPSs have also been reported to delay or prevent phage adsorption presumably by providing a physical barrier between the phage and cell surface receptors (Cerning 1990; Forde and Fitzgerald 1999a; Looijesteijn *et al.* 2001). EPS production is not always effective as an antiphage defense mechanism. Certain phages have evolved to specifically recognize and bind to bacterial extracellular polymers (Leiman *et al.* 2000; Deveau *et al.* 2002; Scholl *et al.* 2005; Garcia *et al.* 2008). Typically, EPS-specific phages have enzymatic activities associated with the virion tail structure which degrade polymers, thereby permitting the phage access to the cell surface. Once at the outer cell wall, the virion most likely recognizes and binds to a cell surface

receptor before injecting its DNA into the host cell (Scholl *et al.* 2005). Some *Erwinia* spp. phages carry depolymerases that can degrade amylovoran (Vandenbergh *et al.* 1985; Kim and Geider 2000). However, phage adsorption to *E. amylovora* remains poorly understood.

Lytic phages are currently being evaluated for use as biopesticides for *E. amylovora.* A concern with using phages as biopesticides is the development of phage resistance imposed by the high selective pressures on the pathogen populations (Vidaver 1976; Jones *et al.* 2007). Bacteria have or can acquire a number of different antiphage defense mechanisms to inhibit or interfere with phage infection (for review see: Labrie *et al.* 2010; Hyman and Abedon 2010). *E. amylovora* ability to produce an EPS layer may be directly involved in phage-host interactions. It is unclear whether EPS plays a role as an antiphage defense mechanism or promotes susceptibility to phage infection by providing specific recognition sites for phage adsorption. The present study describes the roles of *E. amylovora* EPS in the pathogenesis of several phages belonging to the *Podo-*, *Myo-* and *Siphoviridae* (Gill *et al.* 2003). Furthermore, the correlation between quantities of EPS production of the *E. amylovora* isolates with phage sensitivity of these isolates was evaluated.

5.2 Materials and methods

5.2.1 Bacterial isolates, bacteriophages, and plasmids

Bacterial isolates, phages and plasmids used in this study are listed in Table 5-1. *E. amylovora* wild type (wt) and mutant isolates were grown in Luria-Bertani (LB; Difco Laboratories, Sparks, MD) medium at 27°C. For induction of copious amounts of EPS, 0.5% sucrose and 1% sorbitol were added to the culture medium. For transformation selection, antibiotics were added to the culture medium at the indicated concentrations: kanamycin (Km), 20 μg/ml; chloramphenicol (Cm), 20 μg/ml; and ampicillin (Ap), 100 μg/ml. Phage isolates were stored in 0.8% nutrient broth (NB; Difco) at 4°C. Properties of the plasmids are described in detail elsewhere (Datsenko and Wanner 2000).

5.2.2 Bacteriophage manipulations

The host ranges of *Erwinia* spp. phages were determined on the basis of their ability to form plaques on the *E. amylovora* isolates. The degree of plaque clearing was measured visually by assessing plaque turbidity in comparison to the surrounding bacterial lawn. Clear plaques indicated high host sensitivity to lysis, turbid plaques indicated partial lysis and no plaques indicated a non-host. Efficiency of plating (EOP) or ratio of PFU/ml obtained with an assay host divided by PFU/ml obtained with the corresponding isolation host (Asselin *et al.* 2006). This is calculated using the double layer plaque titration method of Adams (1959). Assay host refers to the bacterial host in question and the isolation host refers to the *E. amylovora* isolate used to originally isolate

the phage (Gill *et al.* 2003). Phage population growth was monitored by inoculating liquid medium with phage and bacterium at a multiplicity of infection (MOI) of 100. The cultures were incubated for 24 h at 27°C before lysates were prepared and titres determined using the double agar overlay method (Adams 1959). The same phage master stock was used for all trials.

Table 5-1. Bacterial isolates, bacteriophages and plasmids.

Isolates or plasmids	Characteristics[a]	Reference or source
Erwinia amylovora		
Ea110R	Wild type	(Ritchie and Klos, 1977)
ΔEa110/Am$^-$	*Km*^{R},*rcsB*$^-$	this study
ΔEa110/Le$^-$	*Cm*^{R}, *lsc*$^-$	this study
Ea29-7	Wild type	(Gill *et al.* 2003)
ΔEa29-7/Am$^-$	*Km*^{R},*rcsB*$^-$	this study
ΔEa29-7/Le$^-$	*Cm*^{R}, *lsc*$^-$	this study
EaG-5	Wild type	(Jeng *et al.* 2001)
ΔEaG-5/Le$^-$	*Cm*^{R}, *lsc*$^-$	this study
Ea17-1-1	Wild type	(Jeng *et al.* 2001)
ΔEa17-1-1/Am$^-$	*Km*^{R},*rcsB*$^-$	this study
Ea6-4	Wild type	(Jeng *et al.* 2001)
ΔEa6-4/Am$^-$	*Km*^{R}, *rcsB*$^-$	this study
ΔEa6-4/Le$^-$	*Cm*^{R}, *lsc*$^-$	this study
ΔEa6-4/Am$^-$Le$^-$	*Km*^{R}, *Cm*^{R}, *rcsB*$^-$, *lsc*$^-$	this study
EaD-7	Wild type	(Jeng *et al.* 2001)
***Erwinia* spp. bacteriophage**		
ΦEa21-4	*Myoviridae*	Lehman *et al.* 2009
ΦEa1	*Podoviridae*	Gill *et al.* 2003
ΦEa35-6	*Siphoviridae*	Gill *et al.* 2003
48 isolates collected from Ontario	See Table 5-4.	Gill *et al.* 2003
Plasmids		
pKD46	Ap^R, P_{BAD} gam bet exo pSC101 oriTS	Datsenko and Wanner 2000
pKD13	Km^R, FRT cat FRT PS1 PS2 oriR6K rgbN	Datsenko and Wanner 2000
pKD32	Cm^R, FRT cat FRT PS1 PS2 oriR6K rgbN	Datsenko and Wanner 2000

[a] Km^R: kanamycin resistant; CmR: chloramphenicol resistant; ApR: ampicillin resistant; rcsB$^-$: amylovoran deficient; lsc$^-$: levan deficient

5.2.3 Generation of EPS deficient mutants

Using a modified one-step PCR deletion method (Datsenko and Wanner 2000; Zhao et al. 2009), mutants of *E. amylovora* isolates with deletions of the amylovoran regulator of capsular synthesis gene *rscB*, the levansucrase gene *lsc* or double deletions of both genes were constructed. Plasmids and primers used for PCR to generate recombination constructs are listed in Table 5-1 and Table 5-2, respectively. Each PCR contained final concentrations of 200 nM of each primer, 1X polymerase buffer, 2.5 U *Taq* polymerase (NEB, Ipswich, MA. USA), 300 µM of each dNTP (NEB), 3 mM $MgCl_2$ and 2 µl of DNA template. Reactions were run in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) 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. PCR products were gel purified using a gel-purification kit (Norgen Biotek, St. Catharines, ON, Canada).

For mutant construction, PCR generated linear recombination constructs consisting of either a kanamycin (Km^R) or chloramphenicol (Cm^R) resistance gene with its own promoter using plasmids containing the Km^R cassette (pKD13) or Cm^R cassette (pKD32) (Datsenko and Wanner 2000) and PCR primers *rcsB*_Km and *lsc*_Cm, respectively. Constructed fragments were flanked by 50-nucleotide (nt) homology arms targeting either the levansucrase gene (*lsc*) or regulation of capsule synthesis B gene (*rcsB*). Prior to introduction of the recombination construct, *E. amylovora* cells were made electrocompetent by washing them 3 times in 35 ml ice cold sterile dH_2O and then transformed with plasmid pKD46 by electroporation. The transformants harbouring pKD46 were grown overnight at 27°C, reinoculated in LB 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 1ml of SOC medium (Sambrook and Russel 2001) for 1–4 h at 27°C before being plated on LB with appropriate selective antibiotic. Electroporation were performed under at 2.5 kV, 25 uF with pulse controller set at 200 Ω. In the resulting mutants, a section of the coding region of each target gene was replaced by the Cm^R or Km^R insert marker and mutants are listed in Table 1. To evict the temperature sensitive pKD46 from mutants, cells were briefly heated to 37°C, plated nonselectively and grown overnight at 27°C. Replica plating on both selective and non-selective medium was used to differentiate the colonies that lost pKD46 encoded ampicillin resistance. The primers specific to the sequences upstream and downstream of the deletion site and coding region of the antibiotic resistance expression cassette insert (Km^R or Cm^R) were used to confirm recombination events (Table 5-2). A schematic of the recombination event and primer orientation is given in Fig. 5-1.

5.2.4 EPS measurements

E. amylovora levan and amylovoran production were measured within the supernatants of one ml of their liquid cultures grown in NB at 27°C. Monitoring levan production required isolating levansucrase in culture supernatants and measuring levan production after the addition of the same volume of assay solution consisting of 50 mM sodium phosphate, 2 M sucrose, and 0.05% sodium azide and incubated for 24 h at 27°C (Bereswill and Geider 1997). For amylovoran, supernatants were mixed with 50 µl of cetylpyridinium chloride (CPC) (50 mg/ml) in H_2O which precipitated the amylovoran after 10 min (Bellemann *et al.* 1994). Concentrations were determined by measuring

turbidity at OD_{600}.

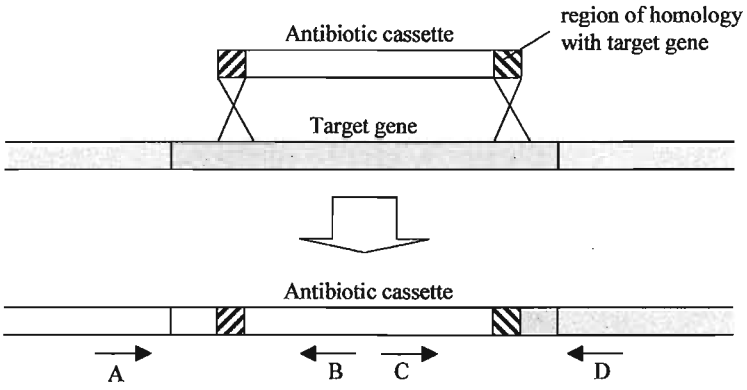

Figure 5-1. Schematic of protocol used to generate gene deletions by homologous recombination with an antibiotic resistance gene. The figure shows placement of PCR primers used to confirm insert and location.

Table 5-2. PCR primers used to generate exopolysaccharide deficient mutants by homologous recombination.

Primers	Sequence (5'—3')[a]	Function
*lsc*_CmF	TTATGTCAGATTATAATTATAAACCAACGCTGTGGA	recombination fragment for *lsc* knockout
*lsc*_CmR	CTCGTGCCGATGCA**GTGTAGGCTGGAGCTGCTTC** ACTCACTGCCTGACAGGTCTTTGGCCACAGCCAGAC CAACACAGCCTGCC**ATTCCGGGGATCCGTCGA**CC	
*rcsB*_KmF	TGAATAATCTGAATGTCATTATTGCCGACGACCATC	recombination fragment for *rcsB* knockout
*rcsB*_KmR	CTATTGTTCTGTTC**GCGATTGTGTAGGCTGGAGCT** ATCTACCGGCGTCATGCTTACTGATGACAGGTAGTT AAGCAAAGCAATAT**ATTCCGGGGATCCGTCGACC**	
*lsc*_ChF	CTGCAGCGATCATGGTTATT	flank *lsc*
*lsc*_ChR	ACCGCCAATGCGATAG	
*rcsB*_ChF	CGGCAAGCAGTTATGTG	flank *rcsB*
*rscB*_ChR	TAAGAAAGAGCCGGGAAGCGCTAA	
Cm_F	TTATACGCAAGGCGACAAGG	internal Cm^R
Cm_R	GATCTTCCGTCACAGGTAGG	
Km_F	CAGTCATAGCCGAATAGCCT	internal Km^R
Km_R	CGGTGCCCTGAATGAACTGC	

[a] Bold-antibiotic resistance cassette primer

5.3 Results

5.3.1 Analysis of EPS production

To measure the differences in EPS production between isolates of *E. amylovora*, amylovoran and levan were measured individually. For amylovoran accumulation, Ea110, Ea29-7, and EaD-7 were found to produce high levels when compared to Ea6-4, Ea17-1-1 and EaG-5 (Fig. 5-2A) Amending the growth medium with addition of 0.5% sucrose and 1% sorbitol increased amylovoran amount for all isolates except Ea6-4. For levan accumulation, levansucrase activity was measured (Fig. 5-2B). Similar to amylovoran, levansucrase activities were found to be higher for Ea110, Ea29-7, and EaD-7 and lower for Ea6-4, Ea17-1-1 and EaG-5. Except for EaG-5 which had a modest increase, amending the growth medium did change the isolates' levansucrase activity. Ea110, Ea29-7, and EaD-7 were grouped as being high EPS producers (HEP) and Ea6-4, Ea17-1-1 and EaG-5, low EPS producers (LEP).

5.3.2 Effect of EPS on phage sensitivity

The ability to cause plaque formation indicated the phage host ranges and the degree of plaque clearing was used to indicate host sensitivity to phage infection (Table 5-3). For *Myoviridae* family members, the LEP hosts Ea6-4, Ea17-1-1, and EaG-5 were highly sensitive with the formation of clear plaques, whereas the HEP hosts EaD-7, Ea29-7 and Ea110R produced turbid plaques (Fig. 5-3). The opposite was found for *Podoviridae* members, plaques produced on HEP hosts were found to be clear whereas plaques produced on the LEP hosts were turbid. The LEP host Ea6-4 showed complete

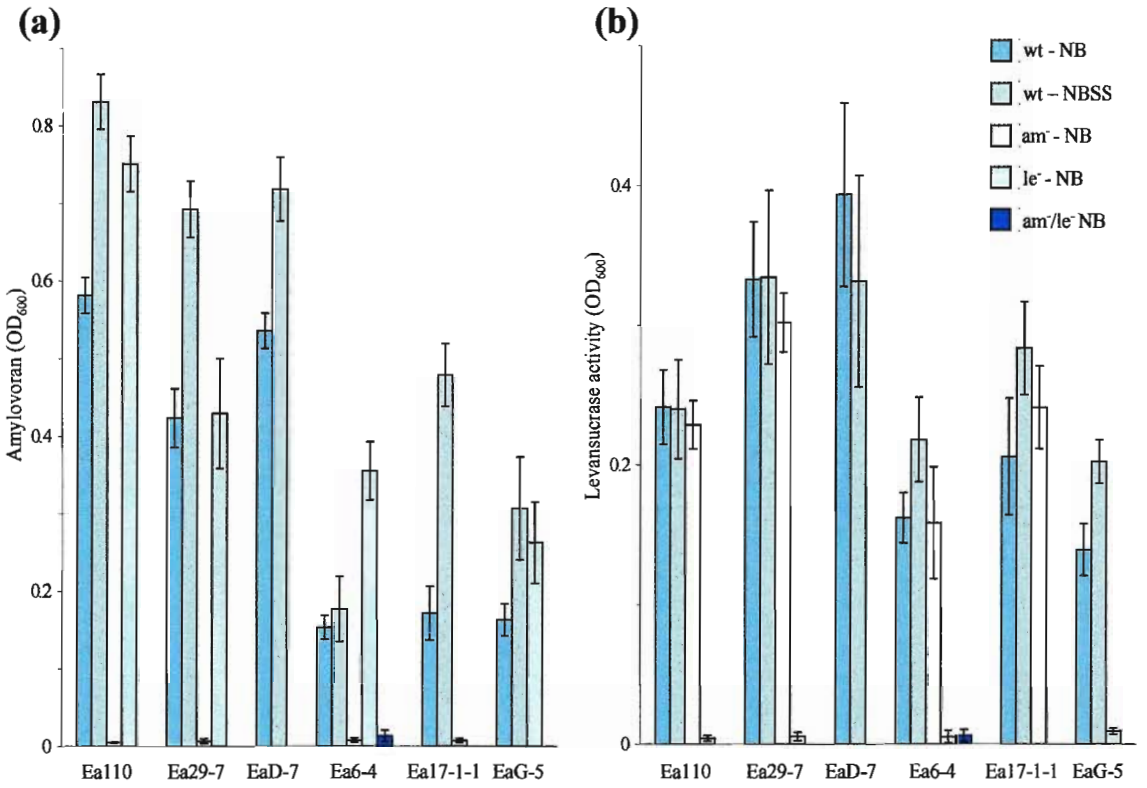

Figure 5-2. Exopolysaccharide biosynthesis for *Erwinia amylovora* wild type and deletion mutants. (a) Amylovoran amounts and (b) levansucrase activity were determined for wt isolates grown in nutrient broth (NB) and NB with added sucrose and sorbitol (NBSS) to facilitate increased EPS production, for derivatives with *rcsB* (am$^-$) or *lsc* gene (le$^-$) deletions and a derivative with both *rcsB* and *lsc* gene deletions (am$^-$/le$^-$). All mutants were grown in NB.

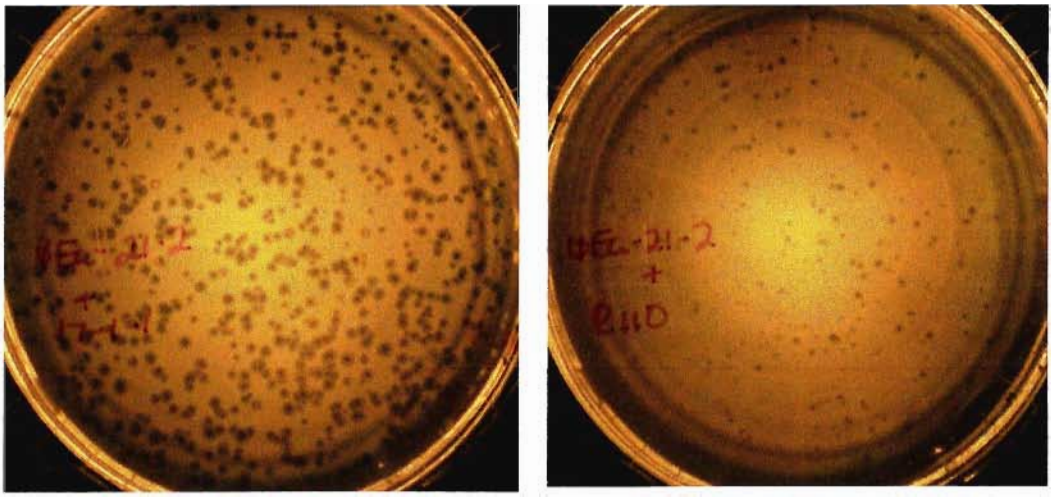

Figure 5-3. Plaque morphologies of phage ΦEa21-2 on high and low exopolysaccharide producing *E. amylovora*. Plaques were confluently lysed (clear) and small to medium in size on low EPS producing host Ea17-1-1 (left) Whereas, ΦEa21-2 plaques were turbid, smaller and less frequent on the high EPS producing host Ea110R (right).

resistance to *Podoviridae* phages ΦEa31-3 and ΦEa45-1B, as seen with the lack of plaque formation. For *Siphoviridae* members, as a family group, hosts did not show the same consistency with plaque morphologies on LEP or HEP hosts as did the other families. Instead, the phage's ability to produce clear plaques on either the LEP or HEP hosts was phage specific. For individual phages, if plaques were clear on hosts of an EPS group, they were turbid on the other.

The efficiency of plating (EOP) of several phages was also calculated (Table 5-4). In general, the EOP of *Podoviridae* phages were close to 1.0 on HEP hosts and the plaques were found to be clear, indicating a high degree of phage sensitivity. *Podoviridae* phage had poor EOP on LEP hosts and plaques were turbid, indicating a reduction in phage-sensitivity. For *Myoviridae* phages, the opposite was found, EOP of around 1.0 occurred on LEP host and producing clear plaques, whereas poor EOP was found on HEP

hosts and plaques were turbid. For *Siphoviridae* phages, no apparent relationship between EPS production and phage sensitivity was found. Isolates expressed either a high degree of pathogenicity on HEP or LEP hosts.

Table 5-3. *Erwinia* spp. phage plaque morphologies on high and low exopolysaccharide producing hosts. Clear (C), turbid (T) or no (--) visible plaques.

			Erwinia amylovora						Clear
Isolate	Host[a]	Group[a]	Ea6-4	Ea17-1-1	EaG-5	EaD-7	Ea29-7	Ea110R	plaques[b]
Myoviridae									
ΦEa10-2	Ea6-4	1	C	C	C	T	--	T	LEP
ΦEa10-4	EaG-5	1	C	C	C	T	T	T	LEP
ΦEa21-2	EaG-5	1	C	C	C	T	T	T	LEP
ΦEa21-4	Ea6-4	1	C	C	C	T	T	T	LEP
ΦEa10-12	EaG-5	4	C	C	C	T	T	T	LEP
ΦEa9-5	Ea6-4	5	C	C	C	T	T	T	LEP
ΦEa9-6	Ea17-1-1	U	C	C	C	T	--	T	LEP
ΦEa10-1	Ea17-1-1	U	C	C	C	T	T	T	LEP
ΦEa10-17	EA17-1-1	U	C	C	C	T	T	T	LEP
ΦEa35-2	Ea17-1-1	U	C	C	C	--	--	T	LEP
Podoviridae									
ΦEa1	Ea110	3	C	T	C	C	C	C	HEP
ΦEa10-8	Ea29-7	3	T	T	T	C	C	C	HEP
ΦEa31-3	Ea29-7	3	--	T	--	C	C	C	HEP
ΦEa9-4	EaG-5	5	C	C	C	T	--	T	LEP
ΦEa45-1B	Ea29-7	U	--	T	T	C	C	C	HEP
ΦEa46-1A2	EaD-7	U	T	T	T	C	C	C	HEP
ΦEa46-2	EaD-7	U	T	T	T	C	C	C	HEP
Siphoviridae									
ΦEa31-4	Ea29-7	2	T	T	T	C	C	C	HEP
ΦEa31-2	EaD-7	2	T	T	T	C	C	C	HEP
ΦEa35-7	Ea29-7	2	--	T	T	C	C	C	HEP
ΦEa10-5	Ea110	U	C	C	C	C	C	C	LEP
ΦEa10-11	Ea17-1-1	3	C	C	C	C	C	C	LEP
ΦEa10-16	Ea17-1-1	4	C	C	C	T	T	T	LEP
ΦEa51-3	Ea6-4	U	C	C	C	T	T	T	LEP
ΦEa51-4	Ea29-7	6	T	T	T	C	C	C	HEP
ΦEa51-6	Ea6-4	6	C	C	T	C	C	C	LEP

[a] As published by Gill *et al*. (2003)

[b] Hosts that phage produces clear plaque; HEP: high EPS producer, LEP: low EPS producer.

Table 5-4. Efficiency of plating for phages infecting *Erwinia amylovora*.

Isolate	Host[a]	Group[a]	*Erwinia amylovora*						Clear plaques[b]
			Ea6-4	Ea17-1-1	EaG-5	EaD-7	Ea29-7	Ea110R	
Myoviridae									
ΦEa10-2	Ea6-4	1	1.0	1.9	1.6	0.4	F	0.5	LEP
ΦEa10-4	EaG-5	1	0.6	1.8	1.0	0.3	0.6	0.4	LEP
ΦEa21-2	EaG-5	1	1.3	1.5	1.0	0.8	0.3	1.0	LEP
ΦEa21-4	Ea6-4	1	1.0	1.2	F	0.7	1.3	1.2	LEP
ΦEa10-12	EaG-5	4	0.8	1.1	1.0	0.7	F	0.5	LEP
ΦEa9-5	Ea6-4	5	1.0	1.4	1.0	0.2	0.1	1.0	LEP
ΦEa9-6	Ea17-1-1	U	1.0	1.0	1.4	0.1	N	0.5	LEP
ΦEa10-1	Ea17-1-1	U	0.8	1.0	0.5	0.1	F	0.3	LEP
ΦEa10-17	EA17-1-1	U	F	1.0	F	0.8	0.6	0.9	LEP
ΦEa35-2	Ea17-1-1	U	0.6	1.0	0.9	N	N	0.3	LEP
Podoviridae									
ΦEa1	Ea110	3	2.6	F	3.2	1.2	2.3	1.0	HEP
ΦEa10-8	Ea29-7	3	F	F	F	1.0	1.0	1.5	HEP
ΦEa31-3	Ea29-7	3	N	0.9	N	0.7	0.3	1.0	HEP
ΦEa9-4	EaG-5	5	0.3	0.9	1.0	0.2	N	0.5	LEP
ΦEa45-1B	Ea29-7	U	N	F	F	1.0	1.0	0.8	HEP
ΦEa46-1A2	EaD-7	U	F	2.5	F	1.0	0.7	3.0	HEP
ΦEa46-2	EaD-7	U	F	F	F	1.0	1.1	1.3	HEP
Siphoviridae									
ΦEa31-4	Ea29-7	2	F	F	F	1.4	1.0	0.9	HEP
ΦEa31-2	EaD-7	2	F	F	F	1.0	0.9	1.5	HEP
ΦEa35-7	Ea29-7	2	N	F	F	0.9	1.0	0.8	HEP
ΦEa10-5	Ea110	U	1.6	1.9	2.5	0.6	0.5	1.0	LEP
ΦEa10-11	Ea17-1-1	3	0.5	1.0	0.6	0.3	0.2	0.5	LEP
ΦEa10-16	Ea17-1-1	4	0.8	1.0	0.7	0.3	F	0.5	LEP
ΦEa51-3	Ea6-4	U	1.0	1.1	1.0	F	0.3	0.9	LEP
ΦEa51-4	Ea29-7	6	F	F	F	0.9	1.0	1.5	HEP
ΦEa51-6	Ea6-4	6	1.0	0.4	F	0.6	0.2	0.9	LEP

[a] As published by Gill et al. (2003); Groups 1-6, U: undetermined group

[b] Hosts that phage produces clear plaque; HEP: high EPS producer, LEP: low EPS producer.

Note - (F) faint visible plaques unable to count accurately; (N') no visible plaques: both calculated as

5.3.3 Effect of EPS on phage population growth

Representatives from the *Sipho-* and *Myo- and Podoviridae* were monitored for population propagation on five *E. amylovora* hosts. When grown on NA medium, the *Siphoviridae* phage ΦEa35-7, was found to propagate equally well on both HEP and LEP hosts when grown on amended NA medium (Fig. 5-4). With non-amended medium, the HEP Ea110R and Ea29-7 produced similar titres that were both about 10 fold greater than on the LEP. An exception to this occurred with LEP Ea17-1-1, as no changes in titres occurred due to growth medium differences. Overall propagation was slightly less than with the HEPs but greater then with other LEPs. For the *Myoviridae*, phage ΦEa21-4 exhibited different characteristics than the *Podo-* and *Siphoviridae* phages when propagated on these hosts (Fig. 5-5). Although ΦEa21-4 multiplied equally well on Ea29-7, Ea17-1-1, Ea6-4 and EaG-5, a 10 fold decrease in titre occurred with Ea110R. Amended growth medium increased titres slightly by 2-5 fold with LEPs, no titre increases were experienced by the HEPs. The *Podoviridae* phage ΦEa1 population propagation was found to be 10-100 fold greater on wt HEP Ea110R then all other wt hosts (Fig. 5-6). The addition of sucrose and sorbitol to the growth medium increased phage titres another 10 fold then growth on NA medium alone, with the exception of LEPs Ea17-1-1 and Ea6-4 which saw no titre change.

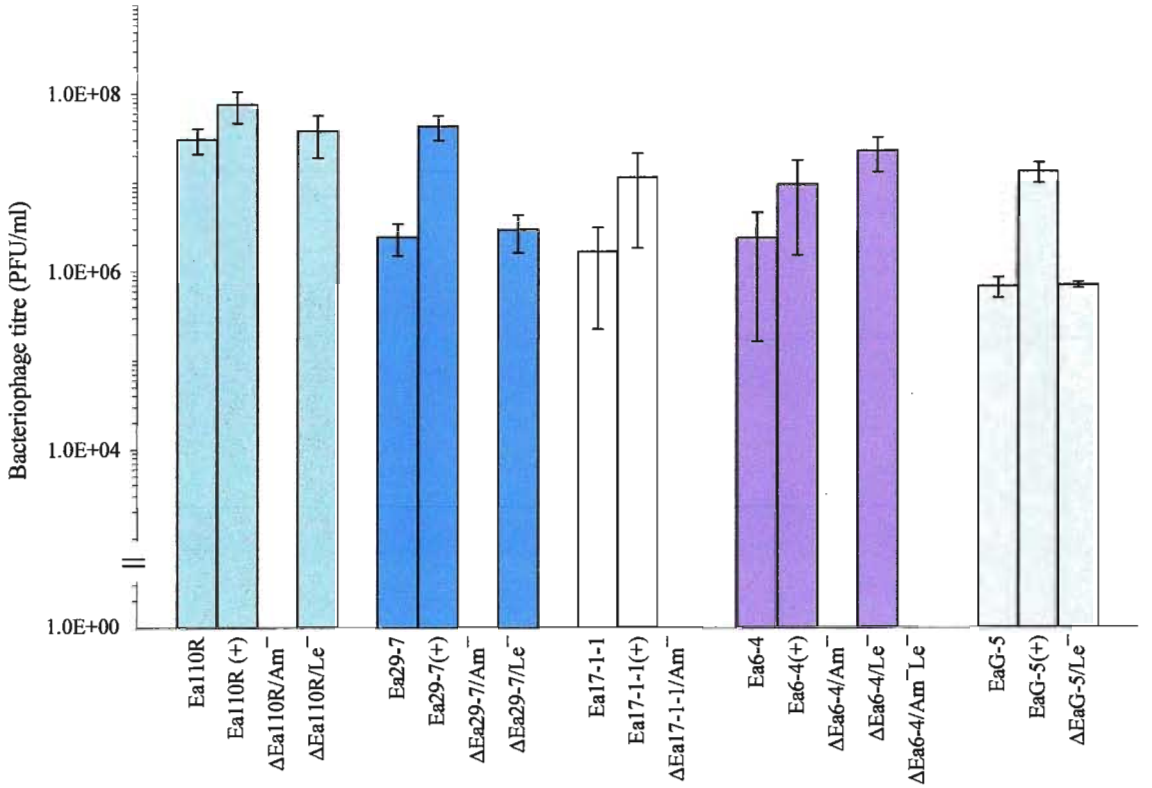

Figure 5-4. Growth of *Siphoviridae* phage ΦEa35-7 on *E. amylovora* and exopolysaccharide mutant derivatives. Titres were calculated from PFU counts on wt isolates, for wt isolates growth on amended medium to facilitate increased EPS production (wt EPS$^+$), for derivatives with *rcsB* (am$^-$) or *lsc* gene deletions (le$^-$) and a derivative with both a *rcsB* and *lsc* gene deletions (am$^-$/le$^-$). The results are from three independent trials.

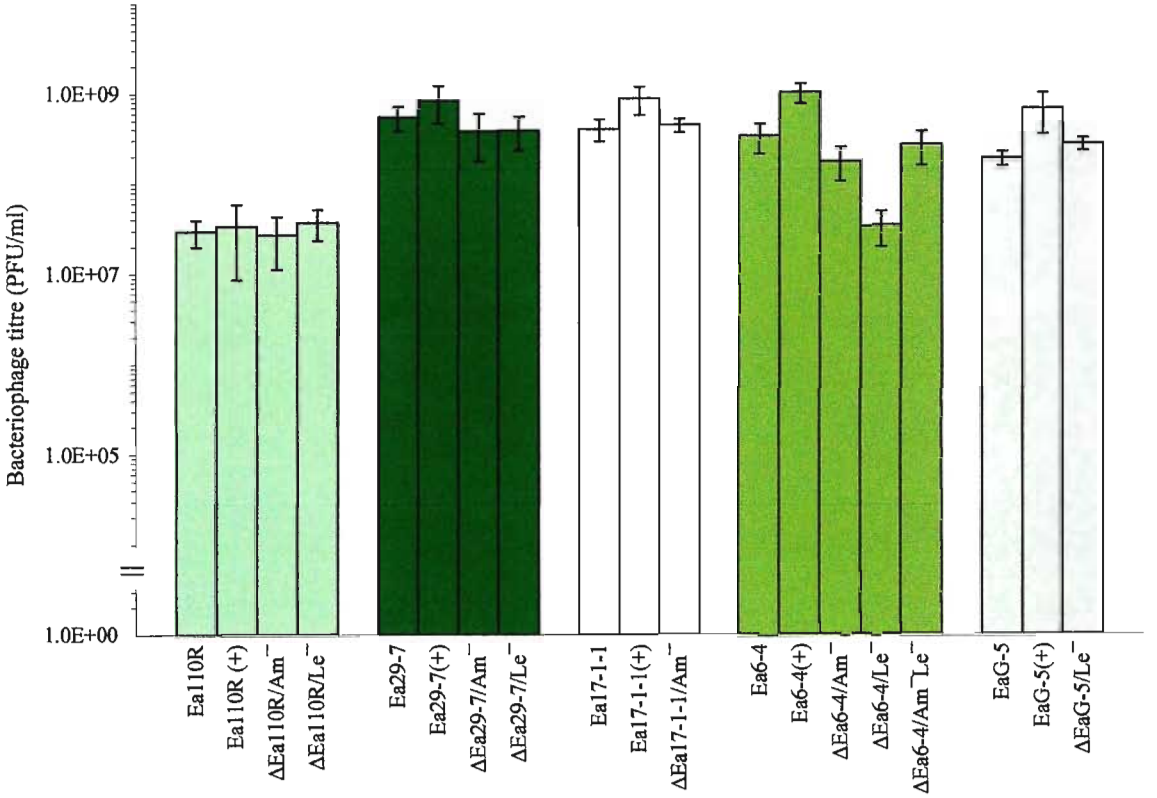

Figure 5-5. Growth of *Myoviridae* phage ΦEa21-4 on *E. amylovora* and exopolysaccharide mutant derivatives. Titres were calculated from PFU counts on wt isolates, for wt isolates growth on amended medium to facilitate increased EPS production (wt EPS^+), for derivatives with *rcsB* (am^-) or *lsc* gene deletions (le^-) and a derivative with both a *rcsB* and *lsc* gene deletions (am^-/le^-). The results are from three independent trials.

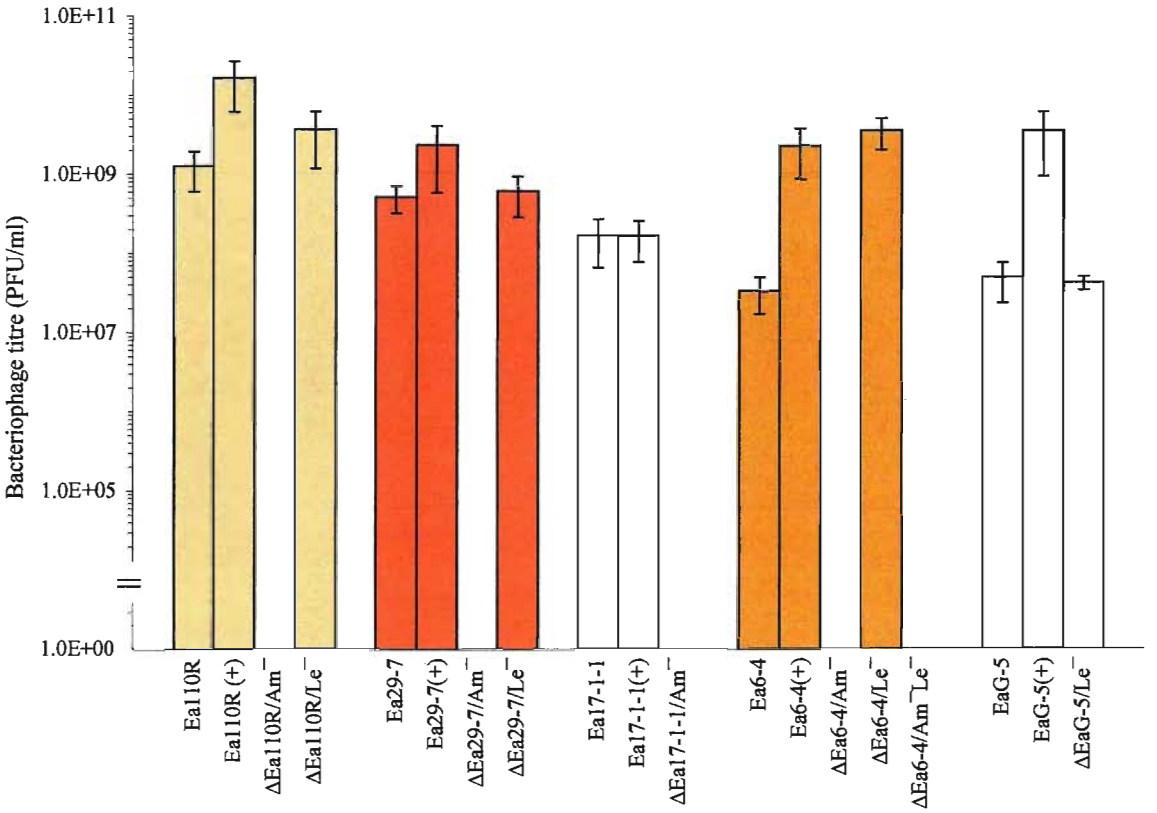

Figure 5-6. Growth of *Podviridae* phage ΦEa1 on *E. amylovora* and exopolysaccharide mutant derivatives. Titres were calculated from PFU counts on wt isolates, for wt isolates growth on amended medium to facilitate increased EPS production (wt EPS^{+}), for derivatives with *rcsB* (am^{-}) or *lsc* gene deletions (le^{-}) and a derivative with both a *rcsB* and *lsc* gene deletions (am^{-}/le^{-}). The results are from three independent trials.

5.3.4 Recovery and confirmation of EPS deficient mutants

The modified one-step PCR deletion method generated EPS deficient mutants of *E. amylovora* isolates with deletions of the amylovoran gene (*rcsB*) for Ea110R, Ea29-7, Ea17-1-1 and Ea6-4. Mutant deletions of the levansucrase gene (*lsc*) were generated for Ea110R, Ea29-7, Ea6-4 and EaG-5. After several attempts with several *E. amylovora* isolates, the only stable *rcsB* and *lsc* double deletion constructed was achieved with Ea6-4. To ensure these mutants had altered EPS biosynthesis properties, turbidity was measured. Amylovoran production was almost undetectable in all mutants with *rcsB* gene deletions (Fig. 5-2A). For amylovoran production, *lsc* mutants ΔEa110R/le$^-$, ΔEa6-4/le$^-$ and ΔEaG-5/le$^-$ showed increases by as much as 100%, compared to their wild-type parent. The remaining *lsc* mutants had unchanged amylovoran production. Levansucrase activity was almost undetectable for all mutants with *lsc* gene deletions (Fig. 5-2B). Amylovoran mutations did not alter levansucrase activity when compared to their wild type parent. EPS production was found to be almost completely abolished for ΔEa6-4/Am$^-$Le$^-$ double mutant with both *rcsB* and *lsc* gene deletions. The primers specific to the sequences upstream and downstream of the gene target site and coding region of the antibiotic resistance expression cassette insert were used to confirm gene deletion (Fig. 5-7).

5.3.5 Effect of EPS deficiency on phage population growth

EPS production was related to phage infection by monitoring population growth on the EPS deficient mutants. Hosts that had an amylovoran deficiency (Am$^-$) were found

to be resistant to phage infection by the *Siphoviridae* phage ΦEa35-7 (Fig. 5-6) and *Podoviridae* phage ΦEa1 (Fig. 5-6), as phage populations failed to grow. For the *Myoviridae* phage amylovoran deficiency did not affect ΦEa21-4 multiplication as population growth was similar to the parental cells (Fig. 5-5). For all the hosts, levan deficiency (Le^-) only affected Ea6-4 as titres were slightly higher (10 fold) over parental Ea6-4 cells when both were grown on NA medium. Levan deficiency had no effect on the virulence of any phage.

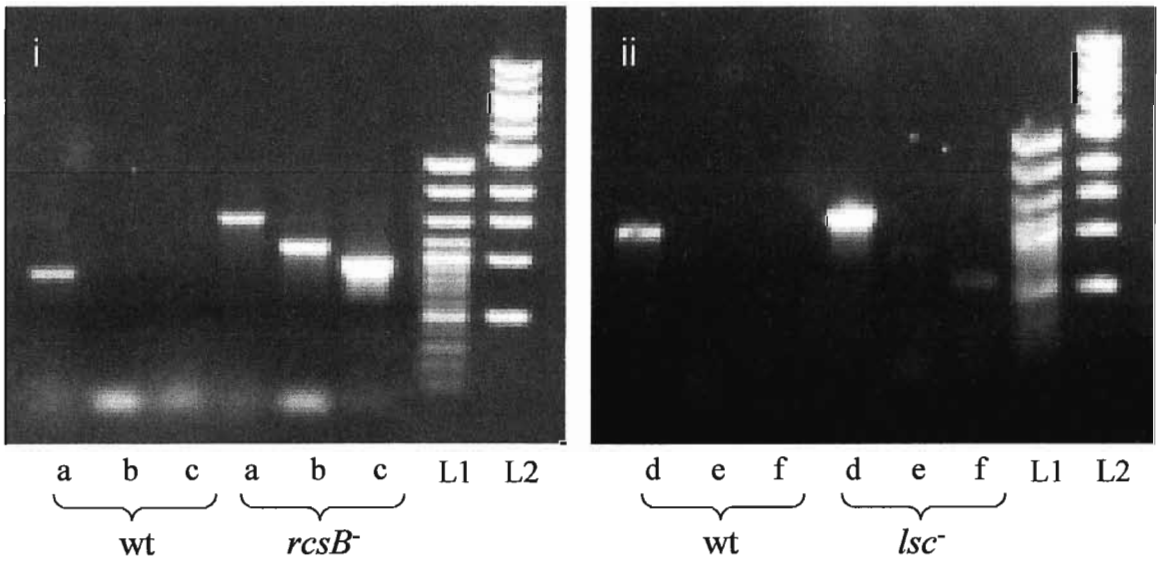

Figure 5-7. Example of PCR confirmation of homologous recombination to generate exopolysaccharide deficient mutants. Primers specific to the flanking sequences of the deletion sites for Ea110R and coding region of the antibiotic resistance insert were used to confirm successful gene deletions. (i) Amylovoran deficient mutants had primer combinations (a) rcsB_ChF/rcsB_ChR, (b) rcsB_ChF/KmR, and (c) KmF/rcsB_ChR. (ii) Levan deficient mutants had primers combinations (d) lsc_ChF/lsc_ChR, (e) lsc_ChF/CmR, and (c) CmF/lsc_ChR. Ladders MiniSizer (L1) and PCRSizer (L2) (Norgen Biotek)

5.4 Discussion

E. amylovora EPS biosynthesis has been well characterized because of its critical role in host pathogenicity (Ayers *et al.* 1979; Wang *et al.* 2009; Koczan *et al.* 2009). However, the EPS physiological role is not essential for growth nor is it used as an energy reserve and deficient mutants appear spontaneously (Bellemann *et al.* 1994; Bereswill *et al.* 1997). *E. amylovora* produces two types of EPS, amylovoran and levan. Amylovoran makes up the majority of the EPS and consists mostly of galactose and glucuronic acid residues (Bellemann *et al.* 1994; Bellemann *et al.* 1994). Levan is a polyfructan (β-2, 6-D-fructofuranan) synthesized by the secreted levansucrase which cleaves sucrose and polymerizes fructose to levan (Geier and Geider 1993; Zhang and Geider 1997; Du and Geider 2002). This work demonstrated that individual *E. amylovora* isolates produced amylovoran and levan in varying amounts when grown under the same conditions. The overall amount of EPS production was used to crudely classify 6 bacterial isolates as being either high EPS producers (HEP) or low EPS producers (LEP). By surveying a diversity of *Erwinia* spp. phages, the function of EPS with respect to phage pathogenesis was correlated between the amount of EPS produced and the infecting phage type.

EPS production could be artificially influenced by nutrient availability (Bellemann *et al.* 1994). Growth media were amended with carbohydrates to produce higher yields of EPS. This resulted in a significant increase in amylovoran production in some iolsates, for Ea17-1-1 amylovoran production doubled. An exception occurred for Ea6-4 which had no increase in amylovoran production. Increased EPS production also resulted in a slight increase in levansucrase activity for three of the isolates (Ea6-4 Ea 17-1-1 and EaG-5) but had no effect on the remaining isolates (Ea110R, Ea29-7, and EaD-7).

Bereswill *et al.* (1997) reported similar results in that changes in growth medium did not influence levansucrase activity. Based on overall EPS production, the isolates were grouped as being either high EPS producers [(HEP) Ea110R, Ea29-7, and EaD-7] or low EPS producers [(LEP) Ea6-4 Ea 17-1-1 and EaG-5].

Host range and EOP analysis of HEP and LEP hosts found that *Podoviridae* phages produced clear plaques on HEP hosts and turbid plaques on LEP hosts. In contrast, *Myoviridae* phages produced clear plaques on LEP hosts and turbid plaques on the HEP hosts. For both phage families, these clear plaques correlated with having a greater EOP than those phages which produced turbid plaques. Perhaps reduction in phage-sensitivity is due to a portion of the population which have undergone lysogeny (i.e. resistance to lysis by homologous phages) (Abedon 2008). However, none of the hosts were found to contain detectable prophage DNA by rtPCR screening or produced virions (see Chapter 3).

Another factor which could also attribute to a reduction in phage-sensitivity, is differences in bacterial cell surface receptors used by the phages (Moineau *et al.* 1996; Broadbent *et al.* 2003). Here, a PCR-based one step deletion of chromosomal genes derived from the λ phage RED system was used to generate amylovoran (*rcsB*$^-$) and levansucrase (*lsc*$^-$) deficient mutants to remove potential surface receptors on *E. amylovora* cells. Principles of the RED system (also referred as recombineering) are summarized here (Datsenko and Wanner 2000; Zhao *et al.* 2009; Sharan *et al.* 2009). The advantage of recombineering is it can be extremely efficient, has no noticeable increase of spontaneous mutation rates when RED proteins are transiently expressed and short homology sequence targeting vectors for specific DNA modifications can be easily obtained from PCR. Recombineering was able to remove amylovoran and levansucrase

production with *rcsB* and *lsc* gene deletions, respectively. This was consistent with previous mutagenesis studies (Bereswill *et al.* 1997; Wang *et al.* 2009). Since the *rcsB* genes have been found to regulate only amylovoran biosynthesis (Wang *et al.* 2009), the *rcsB*⁻ deletions did not change levansucrase activity of the mutant. In contrast, *lsc* gene deletions did result in an increase in amylovoran production for 3 out of 4 mutant isolates. Relations between lsc gene expression and the Rcs system have been reported as *rcsA* and *rcsB* genes overexpression reduces levansucrase synthesis (Bereswill and Geider 1997). In *Pantoea stewartii* subsp. *stewartii*, the synthesis of the EPS stewartan was reported to also be dependent on the Rcs system which has been found to have mediated activation of EPS synthesis by quorum sensing (Minogue *et al.* 2005). Perhaps other systems could mediate amylovoran production. Furthermore, regulated synthesis of EPS may be advantageous for the bacteria to avoid unnecessary energy expenditures by overproducing EPS.

It seems that for *Podo-* and *Siphoviridae* phages infection is dependent on their hosts producing amylovoran as all deficient mutants failed to propagate phage populations. Another indication that *Podo-* and *Siphoviridae* phages are binding to the EPS is that when EPS production was increased, phage population growth also increased, presumably by providing more phages binding sites and increasing phage infection productivity. Phages have evolved to recognize specific polysaccharide capsules (Scholl *et al.* 2005) although the involvement of EPS in phage infection is limited to a few reports (Broadbent *et al.* 2003). For *E. coli,* phage types have been found to recognize and bind only when the production of a capsular EPS with a serotype-specific surface K antigen. Removing the capsular EPS from cells resulted in a lack of K antigen-specific phage lysis (Stirm 1968). In *Vibrio cholerae*, phages have also been found to specifically lyse EPS

producing strains (Albert *et al.* 1996) and the presence of capsular polysaccharide surrounding the cells of *Streptococcus thermophilus* strains could also play a role in the adsorption of specific phages to the cells (Garcia *et al.* 2008). Typically, EPS-specific phages carry depolymerases that are associated with the tail structure of the virion which can recognize and degrade specific polymers (Lindberg 1973). The *Podoviridae* and some S*iphoviridae* phages have been reported to carry depolymerases that degrade amylovoran (Vandenbergh *et al.* 1985; Kim and Geider 2000) as seen with the formation of expanding halos around plaques. Depolymerases are thought to allow the virion to gain access to the cell surface where it most likely binds to an outer membrane receptor before injecting its nucleic acid into the host cell. This does not seem to be the case for *E. amylovora* phages as there may be no secondary binding site on the cell surface as EPS deficient mutants were resistant to phage infection. Removal of the EPS barrier should improve phage growth if there is a cell surface receptor but that was not found here. Therefore secondary binding may not occur for these phages. There is the possibility that *rcs*B deletion also alters some other cell surface component that acts as the cell surface receptor. For *E. amylovora* mutations in the Rcs system have been found to increase resistance to the antimicrobial peptide (AMP) polymyxin B which requires cell membrane binding to be effective against *E. amylovora* (Wang *et al.* 2009)

EPSs were not found to participate in infection for *Myoviridae* and certain *Siphoviridae* phages. Since phage may adsorb to more than one receptor on the surface of the host (Lindberg 1973), they maybe capable of binding at another non-EPS related cell surface receptor. However these phages are not known to possess depolymerases (Gill *et al.* 2003; Lehman *et al.* 2009), so it is unknown how these non-halo producing phages bypass the EPS barrier and infect their hosts.

The *Myoviridae* and certain *Siphoviridae* phages were also found to produce turbid plaques and had EOP decreases as much as 80% with HEP hosts compared to growth on LEP hosts. This reduction in phage-sensitivity may be due to the HEP hosts being able to surround themselves with a denser polysaccharide layer which interfered with phage adsorption compared to the LEP hosts. Most of the functions ascribed to EPS are of a protective nature including protection against phage attack (Looijesteijn *et al.* 2001). EPS is presumed to provide a physical barrier between infecting phages and the cell surface receptors (Forde and Fitzgerald 1999a; Labrie *et al.* 2010). EPSs' protective function has been reported for *Rhizobium meliloti*, as EPS non-specifically prevented phage adsorption by coating the cells (Defives *et al.* 1996). The physical removal of the K1 EPS capsule restored phage susceptibility of *Escherichia coli* (Scholl *et al.* 2005). Similarly EPS was also found to be responsible for the inhibition of phage adsorption to *Lactococcus lactis* (Forde and Fitzgerald 1999a). This suggests that the production of EPS may be a universally employed antiphage defense mechanism by bacteria by simply providing a physical barrier blocking the cell surface receptors. One explanation for only a slight reduction in phage-sensitivity is the production of EPS. It also possible that the EPS could protect the strain by coating the cell, but this protection would be limited because the *E. amylovora* EPS is loosely bound unlike the capsular EPS found with the above examples.

To utilize lytic phages as biological control agents for *E. amylovora* the phage-host interactions have to be properly assessed for the potential of phage resistance. In order for bacteria to gain resistance against phage adsorption, bacteria must dispose of or alter its phage binding sites (Scott *et al.* 2007). However, this strategy has been found to be costly to bacteria, which often become less virulent or avirulent (Jensen *et al.* 2006;

Zahid *et al.* 2008). This trade-off explains why phage-resistant and phage-sensitive bacteria coexist (Bohannan and Lenski 2000). If in fact the phage receptor for *Erwinia* spp. phages is amylovoran, the only way for the cell to become resistant is to change the polymer's composition or abolish its production. However, amylovoran produced by *E. amylovora* is correlated with virulence as deficient mutants are avirulent (Geider *et al.* 1993; Bellemann *et al.* 1994; Bugert and Geider 1995). If amylovoran composition was altered it may reduce virulence. In regards to a phage-based biopesticide, no ill effect would occur from phage adsorption resistance due to the abolishment or alteration of amylovoran production, as these cells would be nonpathogentic and disease would not occur.

Considering the significance of using phages as biological control agents underlines the importance of understanding phage-pathogen interactions to provide rational screening for high performance phages. This study showed that the adsorption of *Erwinia* spp. *Podoviridae* and certain *Siphoviridae* phages was mediated by cell receptors within amylovoran. These phages are highly specialized to attack hosts that produce high amounts of the EPS, but without amylovoran cells become resistant to phage infection. EPS were found not to play a role in phage adsorption for *Myoviridae* and other *Siphoviridae* phages. A detailed understanding of the first step in the infection process will provide strategies to construct a cocktail of phages, each utilizing different cell surface receptors. This will potentially prevent the emergence of phage-resistant cells while increasing biopesticide effectiveness by selecting highly specialized high performing phages.

Chapter 6

General Discussion

Phages have the potential to become effective biopesticides. Prolonged use of phages with bacterial populations may result in bacterial resistance to phage infection (Goodridge 2004; Jones *et al.* 2007). Despite the current understanding of the abundance of mechanisms available to bacteria to achieve phage-resistance (Labrie *et al.* 2010; Hyman and Abedon 2010) this issue has been poorly studied in the context of phage therapies. The aim of these studies was to provide an improved understanding of the interactions between phages and their *E. amylovora* hosts. Efforts were focused on determining the natural or intrinsic phage resistance of *E. amylovora* by the production of EPS as well as acquired phage resistance through lysogenic relationships. To achieve this, the objectives of this research were: (i) to design a diagnostic technique utilizing real-time PCR technology; (ii) to screen natural populations of *E. amylovora* and *P. agglomerans* to determine the incidence of lysogeny; (iii) to characterize lysogeny by isolating superinfection immune cells; and (iv) to construct EPS deficient *E. amylovora* mutants and determine whether the presence and type of EPS has a role in phage adsorption.

To facilitate the study of phage-resistance in *E. amylovora*, a genomic based phage diagnostic technique utilizing rtPCR technology was designed and validated (Chapter 3). A reliable and rapid quantitative rtPCR protocol was developed, which could easily discriminate between and quantify *Erwinia* spp. *Podoviridae* and *Myoviridae* phages, the pathogen *E. amylovora* and the carrier *P. agglomerans,* simultaneously in a multiplex format. The assay was highly sensitive with detection limits on the order of 10^2

PFU/ml for virions and 10^3 CFU/ml for bacterial cells. In multiplex reactions the LOD and amplification efficiencies did not change by addition of multiple primers/probe sets or templates. This suggested that there was no competition between PCR reactions resulting in failed or diminished fluorogenic responses, or non-specific binding producing false-positive responses. The PCR amplification efficiencies were between 76%, and 99% with R^2 values greater than 0.98. All R^2 values showed minimal variability between samples offering regression reproducibility and template serial dilutions produced C_t values that were evenly distributed.

The rtPCR assay developed here was integrated into a number of important protocols involved in the development of a biopesticide. With the ability to collect data in the exponential growth phase, the power of PCR has been expanded into applications such as microbe quantitation, pathogen and biocontrol agent detection and genotyping, quantitation of gene expression, and determining biopesticide efficacy as seen with work done by Lehman (2007). This rtPCR assay does present some limitations. The primers/probes constructed were unable to distinguish between isolates within each phage family, nor identify any of the known *Siphoviridae* phages. In the future this shortfall could be remedied as new genomic information becomes available since the rtPCR protocol allows for further development of additional primers and probes specific for added DNA targets. To date the rtPCR technology resolves six DNA targets. With the current assay of four targets, cross amplification interference was not found to occur, suggesting the inclusion of additional targets will presumably have no negative effect on assay sensitivity or specificity.

Real-time PCR was used innovatively to provide a cultivation-independent approach to assess the incidence of lysogeny within naturally occurring populations of *E.*

amylovora and *P. agglomerans* (Chapter 4). Understanding the lysogenic relationship is important when developing a phage-based biopesticide because lysogeny entails an inherent risk associated with phage applications in the environment. These risks arise when the prophage excises itself from the bacterial genome, as a portion of the host DNA may be excised and incorporated into the phage genome and transduced to another host cell. This event may provide the recipient host novel virulence factors, potentially converting non-pathogenic bacteria into pathogens or enhancing the pathogenicity of the current pathogen (Brussow *et al.* 2004). Although rtPCR provided a highly sensitive and specific assay that avoided the challenges associated with induction-dependent approaches (Erskine 1973b; Williamson *et al.* 2007), the screening for prophage genes while in their quiescent state demonstrate that lysogeny within natural populations of *E. amylovora* is rare to absent. Furthermore, large scale screening of epiphytic *P. agglomerans* also failed to demonstrate lysogeny in cells colonizing apple and pear blossoms. Since lysogeny may facilitate phenotypic or metabolic changes in the characteristics of the lysogenized cell, the most common being immunity to phage attack by superinfecting phages (Abedon 2006; Deho and Ghisotti 2006), lysis immunity was used as a selection method for recovering lysogenized cells. These recovered lysogenized cells had acquired phage resistance which was determined by an alteration in their host range compared to the nonlysogenized cells. Resistance was thought to be the result of superinfection immunity. The lytic properties of a superinfection phage were inhibited and bacterial viability was maintained. Although it was demonstrated that lysogeny does occur in *E. amylovora*, it could only be demonstrated in one of the several phage-host combinations (Ea110R and ΦEaT100) that were attempted *in vitro*. This inability to select

for temperate phages on a large scale also suggests lysogeny is a rare event in *E. amylovora*.

These findings present a unique opportunity to commercialize a phage-based biopesticide for *E. amylovora* since there is a limited risk associated with lysogeny. The probability of specialized transduction or lysogenic conversion seems to be low for *E. amylovora*, and the side-effects of employing a phage-based biopesticide when applied in orchard conditions may be negligible. For added insurance the information provided here will allow for proper genomic screening of phage candidates and monitoring pathogen populations for lysogenic relationships in an effort to avoid selecting temperate phages as biological control agents. In addition, the newly discovered lysogenic form of an *E. amylovora* isolate can be examined for comparative virulence and examined in detail pathogenic outcomes from lysogeny.

To further understand the intrinsic resistance *E. amylovora* may have against phage attack, stable *E. amylovora* EPS mutants were generated by recombineering (Datsenko and Wanner 2000; Zhao *et al.* 2009). Mutant constructs had sections of their EPS coding region replaced by a selectable antibiotic resistance cassette to disrupted amylovoran and levan biosynthesis in several *E. amylovora* isolates (Chapter 5). Consistent with previous mutagenesis studies (Bereswill and Geider 1997; Wang *et al.* 2009), disruption of the *rcsB* and *lsc* genes led to the elimination of amylovoran and levan accumulation, respectively. The *rcsB*⁻ deletions did affect levansucrase activity, whereas the *lsc* gene deletions were found to increase amylovoran production for the majority of the mutants tested. It seems that there may be some interference between levansucrase activity and amylovoran production, potentially by a feedback mechanism between lsc gene expression and the Rcs system. Relations between the two systems have

been seen before as *rcsA* and *rcsB* genes overexpression reduces levansucrase synthesis (Bereswill and Geider 1997). In *Pantoea stewartii* subsp. *stewartii*, the synthesis of the EPS stewartan was reported to also be dependent on the Rcs system which has been found to have mediated activation of EPS synthesis by quorum sensing (Minogue *et al.* 2005). Therefore, it is plausible that other systems could mediate amylovoran production. Furthermore, regulated synthesis of EPS could be advantageous for the bacteria to avoid unnecessary energy expenditures to overproduce EPS.

EPS production was found to aid in the adsorption of all *Podoviridae* and certain *Siphoviridae* phages tested. *E. amylovora* cells that were deficient in amylovoran production became resistant to phage attack. This suggests that amylovoran may be a cell surface receptor that is necessary for phage adsorption for these phages. Phages that have evolved to specifically recognize these extracellular polymers are not unique to *E. amylovora*. *E. coli* produces a capsular EPS with a serotype-specific surface sugar K antigen that also acts as a cell surface receptor (Stirm 1968). However, not all *Erwinia* spp. phages have evolved to utilize amylovoran as a binding site as all *Myoviridae* and some *Siphoviridae* phages maintain population growth characteristics similar to the non-deficient parental hosts despite their amylovoran deficiency.

Phage adsorption inhibition involves a number of host mechanisms that prevent phage attachment to the cell surface. The most common is the loss or modification of the phage receptor needed for adsorption through mutation or recombination (Delbruck and Luria 1943; Lenski 1988; Hyman and Abedon 2010). If *E. amylovora* were to lose amylovoran production it would become resistant to some of its phages. However, amylovoran production is necessary for the bacterium to cause disease. This resistance mechanism would result in decreased fitness for the cell and it would be unlikely that

these cells would prevail. Another aspect of understanding phage-host interactions is the ability to combine appropriate phages in a mixture, each utilizing different cell surface receptor. From a general prospective, it would be beneficial to employ at least a *Podo-* and *Myoviridae* phage together in a treatment as one is reliant on EPS production while the other is not. This will potentially prevent the natural selection of phage-resistant cells while increasing biopesticide effectiveness by selecting specialized high performing phages.

For phage-based biocontrol, the long-term practical and treatment implications of bacterial phage resistance are currently unknown. Studies have shown there are a number of mechanisms by which a bacterium can become resistant to phage infection (Labrie *et al.* 2010; Hyman and Abedon 2010). Typically, these can be effective at a variety of points in the phage life cycle, including adsorption inhibition, phage DNA blocking, restriction-modification and abortive infection (Hyman and Abedon 2010). This study has only provided a partial understanding into the phage-host interactions that leads to phage resistance in *E. amylovora*. It is important to investigate all aspects of resistance since the selection of resistant mutants in a bacterial population previously susceptible and responsible for the infection treated with phages will undoubtedly lead to a frequent cause of biocontrol failure.

6.1 Future Recommendations

There is a number of future directions I think would be aid in the development of the phage biopesticide for *E. amylovora*. This thesis does not provide a complete rtPCR diagnostic assay that detects all phage in the AAFC Vineland Phage Collection. It would important to sequence an *Erwinia* spp. *Siphoviridae* phage. This would not only provide important genomic information about this phage type but also give a number of potential DNA target sequences that would allow for the development of rtPCR primers and probes that could be added to the multiplex rtPCR assay developed here. This new rtPCR assay could be used to include *Siphoviridae* phages in the screening for lysogens. This along with screening bacteria from other niches within the orchard will most likely give a broader understanding into the incidence of lysogeny.

There is a need to follow up on the possibility that amylovoran is a phage receptor for *Erwinia* spp. phages. Perhaps this can be done by isolating free amylovoran and exposing it to phages. If there is a decrease in free phages in the suspension after the removal of the amylovoran, this may suggest that in fact amylovoran is the receptor.

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