INVESTIGATING CARBOHYDRATE UTILIZATION AND VIRULENCE IN *ERWINIA AMYLOVORA*

By

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ABSTRACT

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Fire blight, caused by the gram-negative bacterium *Erwinia amylovora*, is a destructive disease of apple and pear trees worldwide. A unique aspect of apple and pear physiology is the presence of sorbitol in the leaves and shoots, glucose on the flower stigma surface and sucrose in the flower nectary. *Erwinia amylovora* cells encounter all three carbohydrates at different stages of infection, and it is unknown how the carbohydrate utilization genes are regulated between these changing nutrient environments. This thesis explores carbohydrate utilization by *E. amylovora* in relation to virulence, regulatory small RNAs (sRNAs), other virulence factors and host specificity. The findings presented here indicate that sorbitol utilization (*srl*) gene mutants of *E. amylovora* are amylovoran-deficient, and they are unable to obtain the energy base needed to infect apple shoots and immature pear fruits. Additionally, the sRNA Spot 42 does not regulate sorbitol as it does in *Escherichia coli*, and we hypothesize that *E. amylovora* has evolved to evade Spot 42 regulation in order to adapt to the high-sorbitol content of apple and pear hosts.

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KEY TO ABBREVIATIONS

CPC	Cetylpyridinium chloride
DPI	Days Post Inoculation
EPS	Exopolysaccharide
FRT	Flippase recognition target
GFP	Green fluorescent protein
Hrp	Hypersensitive response and pathogenicity
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
LB	Luria-Bertani
MCS	Multiple cloning site
PAI	Pathogenicity island
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RBS	Ribosomal binding site
sRNA	Small RNA
T3SS	Type III secretion system

Chapter 1. Literature Review

I. Introduction to *Erwinia amylovora*, causal agent of fire blight

Erwinia amylovora (Burrill) Winslow et al. is a bacterial pathogen of plants in the Rosaceae family, including apple, pear, hawthorn, raspberry and blackberry. Fire blight is the highly infectious disease caused by *E. amylovora*, and is typically recognized by the development of necrosis, wilting shoots and the emergence of bacterial ooze from blighted tissues. On apple, fire blight symptoms can occur on most tissues of the tree, including flowers, shoots, large branches and rootstock crowns (Norelli *et al*., 2003). During the infection cycle, the bacterium has a brief epiphytic cycle on flower stigmas, but, following flower infection, the disease progression involves systemic migration through host tissues downward toward the roots. In severe cases, fire blight can kill an entire orchard in a single growing season, with modern high-density plantings especially vulnerable to devastating losses. For example, in the year 2000, a particularly severe fire blight epidemic occurred in southwestern Michigan, resulting in losses estimated at $42 million including complete loss of approximately 20% of the apple acreage in this region (Longstroth, 2001). Fire blight disease has had a long history in the United States. The pathogen is endemic to the US, where it was first observed in New York State in the 1780s. Since then, fire blight has spread to most apple-growing regions of the world, including parts of Europe, the middle East and New Zealand (Bonn & Van der Zwet, 2000), and has more recently been reported in Central Asia and South Korea (Myung *et al.*, 2016).

Virulence determinants of *E. amylovora*

The coordinated use of virulence factors like type III secretion, biofilm formation, exopolysaccharide production and motility facilitate rapid infection by *E. amylovora* (Malnoy *et al*., 2012). The type III secretion system (T3SS), used by many gram-negative bacteria, plays an important role in host-pathogen interactions by injecting bacterial effector proteins into host cells. In *E. amylovora*, the Hrp (hypersensitive response and pathogenicity)-T3SS is responsible for secreting and delivering effector proteins to the plant apoplast or cytoplasm (Oh & Beer, 2005). Hrp-T3SS mutants of *E. amylovora* are nonpathogenic and cannot elicit a hypersensitive response (HR).

Host xylem colonization and biofilm formation are key virulence traits and adaptations that allow *E. amylovora* to thrive in the host vascular system. Koczan *et al.* (2009) determined that *E. amylovora* forms a biofilm spanning individual xylem vessels, which protects bacteria against external stressors such as environmental fluctuations, host defense responses and antibiotics. The exopolysaccharides (EPS) amylovoran and levan were found to be major components of *E. amylovora* biofilms (Koczan *et al*., 2009).

The amylovoran EPS is a pathogenicity determinant in *E. amylovora.* Amylovoran is a heteropolymer consisting of branched repeated units of glucose, galactose and pyruvate (Nimtz *et al.,* 1996). When produced in xylem vessels, EPS blocks water movement through the shoot, resulting in wilt symptoms (Sjulin & Beer, 1978). The *ams* operon encodes the amylovoran biosynthesis genes, and mutations of the *ams* genes result in a loss of pathogenicity (Bellemann & Geider, 1992).

Levan, another EPS produced by *E. amylovora*, is an important virulence factor and a homopolymer of fructose residues formed in the breakdown of sucrose by the levansucrase

enzyme. The *lsc* gene encodes levansucrase and is regulated by the transcriptional activator RlsA (Zhang & Geider, 1999). Levan is required for biofilm formation, and mutation of the *lsc* gene leads to impaired bacterial virulence.

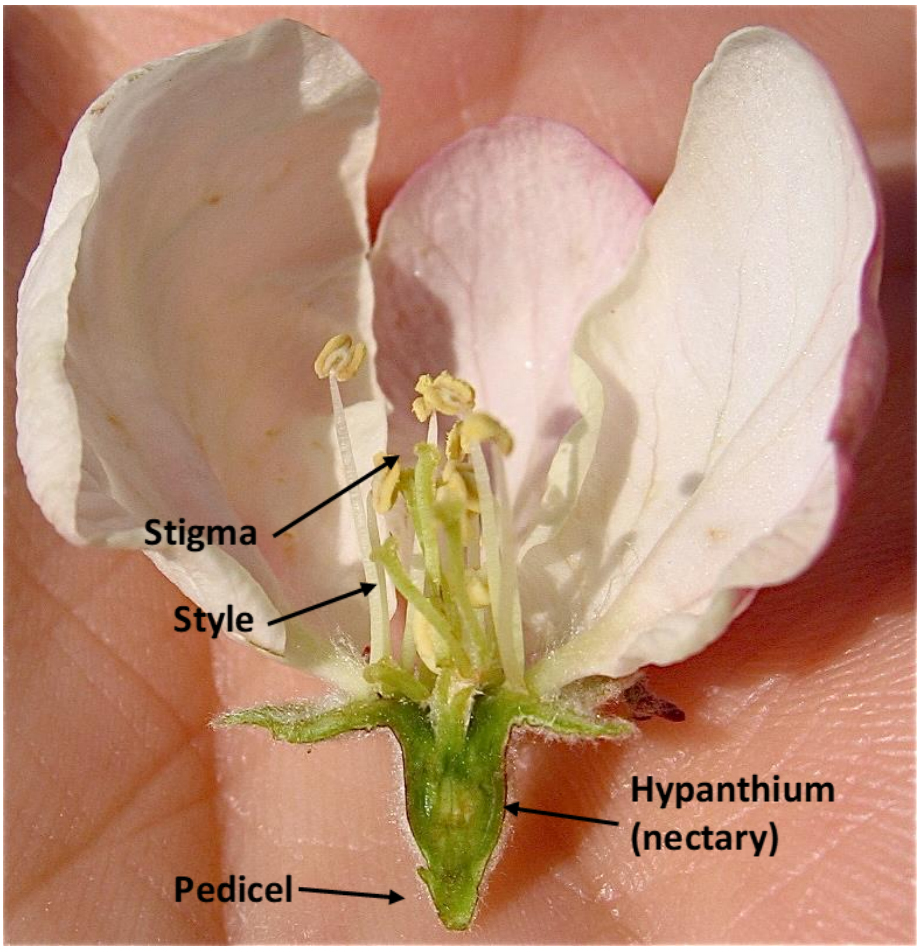
Motility, driven by peritrichous flagella (Raymundo & Ries, 1981) is another known virulence factor in *E. amylovora.* Motility is important for bacterial blossom colonization, but not disease progression within the shoots (Bayot & Ries, 1987).

Infection progression and carbohydrate consumption

Fire blight infection in apple trees typically begins in the flowers, with rapid progression from the stigma to the nectary and into the pedicel (Figure 1.1). At each of these stages in the flower infection process, *E. amylovora* cells encounter a different primary carbohydrate source: glucose in the stigma, sucrose in the nectary and sorbitol in the pedicel, leaves and shoots. It is not known how the bacteria regulate transitions between these host carbohydrate zones. In addition to sugar variations within a single host, different hosts of *E. amylovora* are known to have different sugar profiles. For example, while sorbitol is the predominant photosynthate of apple trees, raspberry plants use the sugar sucrose. It has not been determined whether host carbohydrates play a role in the host specificity of *E. amylovora.*

The following review will summarize current knowledge of carbohydrate utilization in *E. amylovora* focusing on 1) the importance of sorbitol utilization via the *srl* operon 2) *Rubus*-infecting strains of *E. amylovora* and 3) small RNAs as regulators of *E. amylovora* carbohydrate utilization.

Figure 1.1. Anatomy of apple (*Malus domestica*) flower with arrows indicating stigma, style, nectary and pedicel.



Fire blight disease cycle

Erwinia amylovora cells overwinter in cankers formed in the branches or trunk of the tree (Schroth, 1974). When spring temperatures reach between 21°C and 28°C (Pusey & Curry, 2004), the bacteria emerge from the cankers in the form of ooze droplets. Ooze, a matrix of very large populations of *E. amylovora* cells embedded in amylovoran (Eden-Green & Billing, 1974; Slack *et al*., 2017), is dispersed via wind, rain and insects to flower stigmas, and serves as primary inoculum to promote new flower and shoot infections (Thomson, 2000).

Open flowers are highly susceptible to fire blight infection and remain so until petal fall. Symptoms of flower infection include water-soaking, wilt and necrosis. The infection process typically begins with the epiphytic establishment of *E. amylovora* on the stigma surface; here, the bacteria rapidly multiply and can reach populations of $1x10^{6-7}$ cfu/µl (Koczan *et al*., 2009). After stigma colonization, the bacteria use flagellar motility to migrate down the style into the hypanthium, or nectary, of the flower, a movement facilitated by rain or heavy dew (Thomson, 2000). Natural openings at the base of the hypanthium called nectarthodes serve as the primary bacterial entry point into the tree where the pathogen uses type III secretion to initiate infections and begin moving systemically within the host. As the systemic infection progresses, the bacteria can also emerge via ooze to provide secondary inoculum for further infections.

Shoot blight is the result of secondary infection from blighted flowers or cankers (Vanneste, 2000). In addition to entry though flowers, the bacteria enter through natural openings in the shoot tips (hydathodes) or through wounds in damaged leaves. Symptoms of shoot infection are first observed as necrosis along the main leaf vein and culminate in the characteristic “shepherd’s crook” wilt. This is the direct result of biofilm formation in the xylem, which effectively blocks the movement of water (Koczan *et al.,* 2009). Systemic infection and further movement in the trees is accomplished by *E. amylovora* cells migrating via intercellular

spaces in the cortical parenchyma utilizing type III secretion to cause plant cell death and provide energy resources for the bacteria. Ultimately, infection can continue systemically into rootstocks, or spread into rootstocks through wounds, and can kill the tree in a single season.

Carbohydrate zones in the apple tree

The carbohydrate composition of pome flowers likely promotes the epiphytic survival and success of *E. amylovora*. In an analysis of stigma exudates, glucose and fructose were the predominant free sugars detected in all apple and pear varieties tested (Pusey *et al.*, 2008). Although free glucose and fructose are minor components of stigma exudates in terms of mass, Pusey *et al.* (2008) suspect that these carbon sources are important for microbial growth. Free monosaccharide quantities available to bacteria were estimated to be greater than 3 µg, which the authors suggested is enough to support bacterial growth of 10^6 or 10^7 cfu per flower. Increases of glucose and fructose on the stigma were also found to occur at the same time as bacterial growth and sugar consumption.

Analysis of free sugars in both stigma exudates and nectar from the same flower showed that sucrose is a major constituent of nectar while largely absent in stigma exudates (Pusey *et al.*, 2008). As the bacteria spread from the nectary into the vascular system of the tree, however, they encounter sorbitol, which is the predominant translocation carbohydrate of apple and pear trees (Aldridge *et al.*, 1997).

The findings above suggest that as *E. amylovora* cells first arrive on the stigma surface, they primarily consume glucose and fructose. As monosaccharides, these sugars likely serve as efficient sources of energy important for the initial bacterial population increase. As the bacteria move into the nectar-containing hypanthium of the flower, the sugar sucrose is readily available for consumption. Finally, the bacteria navigate through the nectarthodes and into the vascular

system of the tree, where they encounter sorbitol as the predominant carbohydrate. It is largely unknown how *E. amylovora* regulates the transitions between these three carbohydrate zones.

Importance of sorbitol to fire blight infection

Although sucrose is the predominant photosynthate of many plant species, rosaceous plants such as apple use the sugar sorbitol as the major transport carbohydrate. Less commonly known as glucitol, sorbitol is a sugar alcohol obtained by the reduction of glucose. In the *Enterobacteriaceae,* hexitols such as sorbitol are transported and phosphorylated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). In *E. coli,* the sorbitol PTS is encoded by the glucitol (*gut*) operon (Yamada & Saier, 1987).

Through sequence comparison, a sorbitol-utilization operon was uncovered in *E. amylovora* that shares high similarity with the *gut* operon in *E. coli*; both are identical in length (Aldridge *et al.*, 1997). The roles of individual genes of the sorbitol utilization operon in *E. amylovora* were elucidated, and the genes were named *srlA*, *srlE*, *srlB*, *srlD*, *srlM* and *srlR* in analogy with the *gut* operon of *E. coli.*

In *E. coli*, *gutA* encodes the EIICB domain, which is responsible for sorbitol uptake (Aldridge *et al.*, 1997). In *E. amylovora,* SrlA and SrlE are highly similar to GutA, indicating that the same domains are active in the EII complex. SrlB of *E. amylovora* is only 48% identical to GutB, which is the EIIA domain that phosphorylates the incoming carbohydrate. These changes in SrlB may increase its ability to accept a phosphate group from the histidine protein (HPr), which may be beneficial in the high-sorbitol environment of the apple tree. SrlM (68% similarity to GutM) and SrlR (75% similarity to GutR) are regulators of transcription in the *srl* operon of *E. amylovora*. The low homology of *srlM* to *gutM* may also be an adaptation to the high sorbitol concentrations encountered by *E. amylovora.*

Sorbitol mutants of *E. amylovora* displayed reduced symptom formation on apple shoots, but had no delay in infection of immature pears (Aldridge *et al.*, 1997). The authors concluded that shoot infection could not take place due to the inability of the mutants to utilize sorbitol contained within the shoot tissues. The *gut* operon of *E. coli* was successfully able to complement the *E. amylovora* sorbitol mutants, restoring growth on sorbitol.

Rubus-infecting strains of *E. amylovora*

E. amylovora can be divided into two host-specific groupings: Strains that infect the sub-family Spiraeoideae including apple, pear, hawthorn and quince, and strains that infect plants in the genus *Rubus*, including raspberry and blackberry (Goesmann *et al.*, 2013). The Spiraeoideae-infecting strains of *E. amylovora* are both genetically and phenotypically homogenous, whereas the *Rubus*-infecting strains exhibit greater genetic diversity (Momol & Aldwinckle, 2000). Genome comparison of a *Rubus*-infecting strain (ATCC BAA-2158) to an apple-infecting strain (CFBP 1430) revealed that 90% of the coding sequences are conserved between the two strains (Goesmann *et al.*, 2013). In addition, no major differences exist in the amylovoran biosynthesis cluster or Rcs phosphorelay system of the two strains (Wang *et al.*, 2009).

Interestingly, the apple-infecting strains have the capability to infect both apple trees and plants in the *Rubus* genus, while the *Rubus*-infecting strains infect only *Rubus* plants and are not pathogenic to apple. In previous studies, *Rubus*-infecting strains were unable to cause disease in apple shoots, while some could cause infection in immature apple and pear fruit (Braun & Hildebrand, 2005; Ries & Otterbacher, 2005; Triplett *et al.*, 2006).

In order to find the host specificity factor of the *Rubus*-infecting strains of *E. amylovora*, the *hrp* pathogenicity islands of apple-infecting and *Rubus*-infecting isolates were compared (Asselin *et al.*, 2011). The gene *eop1* was determined to be particularly divergent between the

isolates, with only 73% identity. The cause of this divergence was a 21-base pair deletion in *eop1* of the *Rubus*-infecting isolate. In apple-infecting isolates, Eop1 is both secreted and translocated by the type III secretion system into plant cells but has no demonstrated virulence role.

To assess the role of Eop1 in host specificity, *eop1* mutants were created in both the *Rubus* and apple-infecting strains (Asselin *et al.,* 2011). The authors determined that the *eop1* deletion mutant of the *Rubus* isolate gained virulence in immature pear fruit and no longer exhibited delay in symptom development as compared with the wild-type *Rubus* strain. A transposon insertion mutant of the *eop1* gene in the apple-infecting strain, however, showed no altered symptoms to the wild-type apple strain. Due to the gain-of-virulence phenotype produced by the *eop1* deletion in the Rubus-infecting strains, the authors concluded that Eop1 in *Rubus* isolates is a host range-limiting factor.

Expression of the *eop1* gene from a *Rubus* strain in an apple strain reduced its virulence, providing further evidence that this gene serves as a host range-limiting factor (Asselin *et al.,* 2011). The authors reported that at 7 days post inoculation, shoots inoculated with this altered apple strain had no measurable lesions. Additionally, *eop1* was not found necessary for virulence of either *E. amylovora* strain in raspberry plants. Over-expression of the apple *eop1* did not change the virulence of either strain.

While a gain-of-virulence phenotype was seen in immature pear fruit, the deletion of *eop1* was never successful in making the *Rubus* mutant an aggressive pathogen of apple shoots (Asselin *et al.,* 2011). The authors concluded, "…*Rubus* strains must harbor other avirulence genes or lack factors necessary to effectively infect apple shoots". Sucrose is the predominant sugar within raspberry canes, while sorbitol is the most abundant translocation carbohydrate in

apple (Bieleski, 1977). The authors did not explore this difference as a potential reason for *Rubus* isolate's failure to infect apple.

Authors Braun and Hildebrand (2005) sought to determine whether sorbitol and sucrose play a role in cross-infection for apple and raspberry isolates. Because apple isolates are equally virulent on apple, which contains predominantly sorbitol, and raspberry, which contains mostly sucrose, the authors concluded that sucrose does not play a role in host specificity. The authors also concluded that sorbitol does not influence host specificity because apple isolates can infect apple flowers. While no relationship was found between the pathogenicity of apple and raspberry isolates and host sugar content, the study predates the Eop1 study completed by Asselin *et al.* Braun and Hildebrand therefore lacked a key piece of information on Eop1 as a host-limiting factor in *Rubus* isolates.

Small RNAs (sRNAs) as bacterial regulators

Bacteria frequently experience abrupt environmental changes like fluctuations in available nutrients, and rapid physiological response to these changes requires swift coordination of regulatory networks. Such regulation is often mediated by small RNAs (sRNAs), which can be divided into four main classes: cis-encoding RNAs, trans-encoded RNAs, CRISPRs and RNAs that modulate protein activity (Storz *et* al., 2011). The largest known class of sRNA regulators are trans-encoded sRNAs, which control mRNA translation and stability by base pairing with the target mRNA. Trans-encoded sRNAs function by binding to the 5' UTR of the target mRNA, which blocks the ribosome-binding site (RBS) and inhibits translation (Sharma *et al.*, 2007). The sRNA-mRNA duplex is subsequently degraded by RNase E, which reinforces translational repression and makes it irreversible (Massé *et al.*, 2003). While most regulation by trans-encoded sRNAs in negative (Aiba, 2007; Gottesman, 2005), sRNAs can also activate the

expression of an mRNA by preventing formation of an RBS-inhibitory secondary structure (Hammer & Bassler, 2007).

Unlike cis-encoded sRNAs, which have extended regions of complementarity with their target mRNAs, trans-encoded sRNAs undergo discontinuous base pairing with targets. Only a fraction of nucleotides in the sRNA-mRNA interaction are necessary for regulation, and the region of base pairing typically involves only about 10 – 25 nucleotides (Kawamoto *et al*., 2006). In addition, the chromosomal locations of trans-encoded sRNAs and their targets are not correlated, and each sRNA can base pair with multiple mRNAs (Gottesman, 2005). Most trans-encoded sRNAs are highly expressed under specific growth conditions, such as in the presence or absence of certain nutrients, sugar phosphate accumulation and oxidative stress (De Lay *et al.*, 2013).

sRNA chaperone Hfq

The RNA chaperone Hfq facilitates efficient base pairing between the sRNA and mRNA (Waters & Storz, 2009), an important role due to the limited complementarity between sRNAs and mRNAs (Aiba, 2007; Valentin-Hansen *et al*., 2004). Although the exact mechanism is unknown, it is thought that Hfq serves as a platform to allow sRNAs and mRNAs to test their complementarity, which increases the likelihood of base pairing. The Hfq protein is a ring-shaped hexamer with two distinct surfaces, the proximal side and the distal side, which bind specific sequences in sRNAs and mRNAs (Schumacher *et al.*, 2002; Link *et al.*, 2009). The proximal side binds U-rich sequences often contained in sRNAs, while the distal side binds mRNAs. It is not known, however, if all mRNAs must bind Hfq to be regulated by Hfq (Biesel & Storz, 2011). Each Hfq hexamer binds only one sRNA and one mRNA at one time (Updegrove *et al.*, 2011). Over 100 sRNAs have been identified in *E. coli,* and all trans-encoded

sRNAs require Hfq to regulate a target mRNA; sRNAs completely lose regulation of their target mRNAs in the absence of Hfq, as the chaperone protects unpaired sRNAs from RNase attack (Vogel & Luisi, 2011).

sRNAs as regulators of sugar metabolism in *E. coli*

The ability to rapidly coordinate regulatory networks is crucial for bacteria to take advantage of energy sources present in an environment. In *E. coli,* several sRNAs are known to control sugar uptake and metabolism, including Spot 42 and SgrS. The former is a 109 nucleotide-long sRNA encoded by the *spf* (spot 42) gene, and is broadly conserved within the *Enterobacteriaceae* family. Under *in vitro* conditions, null mutants of *spf* are viable, indicating that Spot 42 is non-essential (Hatfull & Joyce, 1986). In *E. coli,* Spot 42 plays an important role as a regulator of carbohydrate metabolism and uptake. The sRNA works synergistically with catabolite repression as part of a feedforward loop with the catabolite repressor protein (CRP), a central regulator in catabolite repression.

When a variety of carbon sources are present in an environment, catabolite repression allows bacteria to prioritize the consumption of specific sugars. Energetically efficient monosaccharides such as glucose are typically utilized first. In *E. coli,* the Spot 42 sRNA contributes to catabolite repression by accelerating repression of secondary carbohydrate utilization genes when glucose is present. Spot 42 reduces leaky expression of certain secondary sugar utilization genes, which helps to divert metabolic energy and resources towards the consumption of glucose.

Spot 42 was found to accumulate in the presence of glucose and decrease in cells grown in secondary carbon sources (Sahagan & Dahlberg, 1979). This direct response to glucose is due to the repression of *spf* by the cAMP-CRP complex under low glucose conditions (Polayes *et al.*,

1988). When glucose is present, levels of the second messenger cyclic AMP (cAMP) are low and *spf* is not repressed. When secondary carbon sources are predominant (no glucose), the cAMP-CRP complex binds to the *spf* promoter, which negatively regulates transcription of Spot 42 (Bækkedal & Haugen, 2015).

Authors Biesel and Stroz demonstrated through reporter fusions and microarray analysis that at least fourteen operons are regulated by Spot 42 (2011). A number of these operons contain genes implicated in the utilization of non-preferred carbohydrates. The Spot 42 sRNA was found to repress levels of the *srlA* mRNA, which is the first gene of the sorbitol operon and responsible for sorbitol uptake. Over-expression of Spot 42 limited growth on medium containing sorbitol (Biesel & Storz, 2011).

sRNAs in *E. amylovora*

Approximately 40 Hfq-dependent sRNAs were identified in *E. amylovora* via RNA-seq analysis and a Rho-independent terminator search (Zeng & Sundin, 2014). Analysis of *hfq* deletion mutants of *E. amylovora* indicate that Hfq and the sRNAs it regulates function as regulators of virulence traits like biofilm production, type III secretion and motility (Zeng *et al.*, 2013). Of the total Hfq-dependent sRNAs expressed at 12 hrs in Hrp-inducing minimal medium, 10% of the total sRNA reads were the Spot 42 sRNA (Zeng & Sundin, 2014). In *E. amylovora*, the Spot 42 deletion mutant was not affected in motility, amylovoran production, biofilm formation, nor hypersensitive response on tobacco (Zeng & Sundin, 2014). As detailed above, the Spot 42 sRNA in *E. coli* is known to contribute to catabolite repression. It is not known, however, whether Spot 42 regulates sugar utilization in *E. amylovora*.

Objectives

The objectives of my Masters research are 1) to characterize the sorbitol utilization genes in *E. amylovora* and to determine their effect on virulence traits 2) to evaluate the role of Spot 42 and other regulatory sRNAs on sugar utilization as *E. amylovora* infects the apple host and 3) to determine if sorbitol utilization is an additional factor in host specificity of the *Rubus*-infecting strains.

REFERENCES

REFERENCES

Aiba, H. (2007) Mechanism of RNA silencing by Hfq-binding small RNAs. *Current Opinion in Microbiology*, 10, 134-139.

Asselin, J. E., Bonasera, J. M., Kim, J. F., Oh, C.-S., and Beer, S. V. (2011) Eop1 from a *Rubus* strain of *Erwinia amylovora* functions as a host-range limiting factor. *Phytopathology,* 101, 935-944.

Aldridge, P., Metzger, M. and Geider, K. (1997) Genetics of sorbitol metabolism in *Erwinia amylovora* and its influence on bacterial virulence. *Molecular and General Genetics,* 256, 611-619.

Bækkedal, C. and Haugen, P. (2015) The Spot 42 RNA: A regulatory small RNA with roles in the central metabolism. *RNA Biology*, 12, 1071-1077.

Bayot, R.G., and Ries, S.M. (1987) Role of motility in apple blossom infection by *Erwinia amylovora* and studies of fire blight control with attractant and repellant compounds. *Phytopathology,* 76, 441-445.

Beisel, C. L. and Storz, G. (2011) The base-pairing RNA spot 42 participates in a multioutput feedforward loop to help enact catabolite repression in Escherichia coli. *Molecular Cell*, 41, 286-297.

Bellemann, P. and Geider, K. (1992) Localization of transposon insertions in pathogenicity mutants of *Erwinia amylovora* and their biochemical characterization. *Journal of General Microbiology,* 138, 931-940.

Bereswill, S., Jock, S., Bellemann, P. and Geider, K. (1998) Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. *Plant Disease,* 82, 158-164.

Bieleski, R.L. (1977) Accumulation of sorbitol and glucose by leaf slices of Rosaceae. *Aust. J. Plant Physiology.* 4, 11–24.

Bonn, W. G. and Van der Zwet, T. (2000) Distribution and economic importance of fire blight. In: *Fire blight: the disease and its causative agent, Erwinia amylovora.* (Vanneste, J., ed.). New York: CABI Publishing, pp. 37-53.

Braun, P. G. and Hildebrand, P. D. (2005) Infection, carbohydrate utilization, and protein profiles of apple, pear, and raspberry isolates of *Erwinia amylovora. Canadian Journal of Plant Pathology*, 27, 338-346.

De Lay, N., Schu, D. J. and Gottesman, S. (2013) Bacterial small RNA-based negative regulation: Hfq and its accomplices. *Journal of Biological Chemistry*, 288, 7996-8003.

Eden-Green, S. J. and Billing, E. (1974) Fireblight. *Review of Plant Pathology*, 53, 353-365.
Gottesman, S. (2005) Micros for microbes: Non-coding regulatory RNAs in bacteria. *Trends in Genetics*. 21, 399–404.

Hatfull, G. F. and Joyce, C. M. (1986) Deletion of the *spf* (spot 42 RNA) gene of *Escherichia coli*. *Journal of Bacteriology*, 166, 746-750.

Kawamoto, H., Koide, Y., Morita, T., and Aiba, H. (2006) Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Molecular Microbiology*. 61, 1013–1022.

Koczan, J. M., Lenneman, B. R., McGrath, M. J. and Sundin, G. W. (2011) Cell surface attachment structures contribute to biofilm formation and xylem colonization by *Erwinia amylovora*. *Applied and Environmental Microbiology*, 77, 7031-7039.

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

Link T.M., Valentin-Hansen P., Brennan R.G. (2009) Structure of Escherichia coli Hfq bound to polyriboadenylate RNA. *Proceedings of the National Academy of Sciences USA*, 106, 19292–19297.

Long, T. *et al*. (2009) Quantifying the integration of quorum-sensing signals with single-cell resolution. *PLoS Biology*, 7, 640649.

Longstroth, M. (2001) The 2001 fire blight epidemic in southwest Michigan orchards. *The Compact Fruit Tree*, 31, 16-19.

Maes, M., *et al* (2001) Influence of amylovoran production on virulence of Erwinia amylovora and a different amylovoran structure in E. amylovora isolates from *Rubus*. *European Journal of Plant Pathology*, 107, 839-844.

Malnoy, M., *et al*. (2012) Fire blight: applied genomic insights of the pathogen and host. *Annual Review of Phytopathology*, 50, 475-494.

Mann, R. A., Smits, T. H., Bühlmann, A., Blom, J., Goesmann, A., *et al*. (2013) Comparative genomics of 12 strains of *Erwinia amylovora* identifies a pan-genome with a large conserved core. *PLoS ONE*, doi:10.1371/journal.pone.0055644.

Massé, E., Escorcia, F. E. and Gottesman, S. (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes & Development*, 17, 2374-2383.

Momol M.T., Aldwinckle H.S. (2000) Genetic diversity and host range of *Erwinia amylovora*. In: Vanneste J.L., editor. Fire Blight: The Disease and its Causative Agent. Wallingford, UK: CAB International. 55–72.

Myung, I. S., *et al* (2016) Fire blight of apple, caused by Erwinia amylovora, a new disease in Korea. *Plant Disease*, 100(8), 1774-1774.

Nimtz, M., *et al* (1996) Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. *Carbohydrate Research*, 287:59-76.

Norelli, J. L., Jones, A. L. and Aldwinckle, H. S. (2003) Fire blight management in the twenty-first century: using new technologies that enhance host resistance in apple. *Plant Disease*, 87, 756-765.

Oh, C. and Beer, S. V. (2005) Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. *FEMS Microbiology Lett*ers, 253, 185-192.

Papenfort, K. and Vogel, J. (2011) Sweet business: Spot42 RNA networks with CRP to modulate catabolite repression. *Molecular Cell*, 41, 245-246.

Polayes, D. A., Rice, P. W., Garner, M. M. and Dahlberg, J. E. (1988) Cyclic AMP-cyclic AMP receptor protein as a repressor of transcription of the *spf* gene of *Escherichia coli*. *Journal of bacteriology*, 170, 3110-3114.

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

Pusey, P. L., Rudell, D. R., Curry, E. A. and Mattheis, J. P. (2008) Characterization of stigma exudates in aqueous extracts from apple and pear flowers. *HortScience*, 43, 1471-1478.

Rappaport, F. and Henig, E. (1952) Media for the Isolation and Differentiation of Pathogenic Bacteria *E. coli* (Serotypes O 111 and O 55). *Journal of Clinical Pathology*, 5, 361.

Raymundo, A.K., and Ries, S.M. (1981) Motility of *Erwinia amylovora.* *Phytopathology*, 71, 45-49.

Ries, S. M. and Otterbacher, A. G. (1977) Occurrence of fire blight on thornless blackberry in Illinois. *Plant Disease Report*, 61, 232-235.

Sahagan, B. G. and Dahlberg, J. E. (1979) A small, unstable RNA molecule of *Escherichia coli*: spot 42 RNA: Nucleotide sequence analysis. *Journal of Molecular Biology*, 131, 573-592.

Schroth, M. N., Thompson, S. V., Hildebrand, D. C., and Moller, W. J. (1974) Epidemiology and control of fire blight. *Annual Review of Phytopathology*, 12, 389-412.

Schumacher M.A., Pearson R.F., Muller T., Valentin-Hansen P., Brennan R.G. (2002) Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J* 21: 3546–3556.

Sharma, C. M., Darfeuille, F., Plantinga, T. H., and Vogel, J. (2007) A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes and Development.* 21, 2804–2817.

Sjulin, T. M. and Beer, S. V. (1978) Mechanism of wilt induction by amylovorin in cotoneaster shoots and its relation to wilting of shoots infected by *Erwinia amylovora*. *Phytopathology*, 68, 89-94.

Slack, S. M., *et al.*, (2017) Microbiological examination of *Erwinia amylovora* exopolysaccharide ooze. *Phytopathology*, *107*, 403-411.

Storz, G., Vogel, J. and Wassarman, K. M. (2011) Regulation by small RNAs in bacteria: expanding frontiers. *Molecular Cell*, 43, 880-891.

Thomson, S. V. (2000) Epidemiology of Fire Blight In: Fire blight: the disease and its causative agent, Erwinia amylovora. (Vanneste, J., ed.). New York: CABI publishing, pp. 9 - 36.

Triplett, L. R., Melotto, M. and Sundin, G. W. (2009) Functional Analysis of the N Terminus of the *Erwinia amylovora* Secreted Effector DspA/E Reveals Features Required for Secretion, Translocation, and Binding to the Chaperone DspB/F. *Molecular Plant-Microbe Interact*ions, 22, 1282-1292.

Triplett, L. R., Zhao, Y., and Sundin, G. W. (2006) Genetic differences between blight-causing *Erwinia* species with differing host specificities, identified by suppression subtractive hybridization. *Applied and Environmental Microbiology*, 72, 7359-7364.

Updegrove, T. B., Correia, J. J., Chen, Y., Terry, C. and Wartell, R. M. (2011) The stoichiometry of the Escherichia coli Hfq protein bound to RNA. *RNA*, 17, 489–500.

Valentin-Hansen, P., Eriksen, M., and Udesen, C. (2004) The bacterial Sm-like protein Hfq: A key player in RNA transactions. *Molecular Microbiol*ogy 51, 1525–1533.

Vanneste, J. (2000) What is fire blight? Who is *Erwinia amylovora*? How to control it? In: Fire blight: the disease and its causative agent, *Erwinia amylovora*. (Vanneste, J., ed.). New York: CABI Publishing, pp. 1-6.

Vogel J. and Luisi B.F. (2011) Hfq and its constellation of RNA. *Nature Reviews Microbiol*ogy, 9, 578–589.

Wang D., Korban S.S., Zhao Y. (2009) The Rcs phosphorelay system is essential for pathogenicity in *Erwinia amylovora*. *Molecular Plant Pathology*, 10, 277–290.

Waters, L. S. and Storz, G. (2009). Regulatory RNAs in bacteria. *Cell*, 136, 615-628.

Yamada, M. and Saier, M. H. (1987) Glucitol-specific enzymes of the phosphotransferase system in *Escherichia coli*. Nucleotide sequence of the gut operon. *Journal of Biological Chemistry*, 262, 5455-5463.

Zeng, Q. and Sundin, G. W. (2014) Genome-wide identification of Hfq-regulated small RNAs in the fire blight pathogen *Erwinia amylovora* discovered small RNAs with virulence regulatory function. *BMC Genomics*, 15, 414.

Zeng, Q. McNally, R. R., & Sundin, G. W. (2013) Global small RNA chaperone Hfq and regulatory small RNAs are important virulence regulators in *Erwinia amylovora. Journal of Bacteriology*, 195, 1706-1717.

Zhao, Y., Blumer, S. E. and Sundin, G. W. (2005) Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. *Journal of Bacteriology*, 187, 8088-8103.

Zhao, Y., Sundin, G. W. and Wang, D. (2009) Construction and analysis of pathogenicity island deletion mutants of *Erwinia amylovora. Canadian Journal of Microbiology*, 55, 457-464.

Chapter 2. Sorbitol utilization by *E. amylovora*

Abstract

Fire blight, caused by the gram-negative bacterium *Erwinia amylovora*, is a destructive disease of apple and pear trees worldwide. A unique aspect of apple and pear physiology is the use of sorbitol rather than sucrose as the predominant translocation carbohydrate. Mutants of *E. amylovora*, with deletions of one or more of the sorbitol utilization (*srl*) genes, are unable to cause significant fire blight symptoms on apple shoots. It is unknown, however, whether sorbitol utilization influences the production of other virulence factors in *E. amylovora* such as amylovoran expolysaccharide (EPS) or other virulence traits such as biofilm formation. In this study, deletion mutants were generated of *srlA*, *srlAEBDMR* and *srlMR* in *E. amylovora* Ea1189, and the ability of each mutant to cause symptoms in apple shoots and immature pear fruit was examined; the ability of each mutant to produce amylovoran EPS and to form biofilms was also examined. The findings of this study indicate that the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants are unable to obtain the energy base needed to infect apple shoots and immature pear fruit. Amylovoran production was reduced in the *srl* mutants, while biofilm formation was unaffected. The Ea1189Δ*srlMR* mutant, which had lost regulation of the *srl* operon, could infect apple shoots and immature pear fruit, although virulence was reduced compared to the wild type. This intermediate phenotype was also observed in amylovoran production, with reduced amylovoran levels compared to the wild type. As with the other *srl* mutants, the Ea1189Δ*srlMR* strain was unaffected in biofilm formation.

I. Introduction

Erwinia amylovora, a gram-negative plant pathogenic bacterium, is the causal agent of fire blight in rosaceous species. The pathogen enters the host through flowers or natural openings in shoot tips and establishes systemic infections. Several pathogenicity and virulence factors of *E. amylovora* have been characterized, including amylovoran exopolysaccharide (EPS) production, biofilm formation, motility and type III secretion (Malnoy *et al*., 2012). Amylovoran EPS is a large component of ooze droplets, which protect bacterial cells against desiccation and are the primary mode of *E. amylovora* dispersal in orchards (Thomson, 2000). Additionally, amylovoran is required for the formation of biofilms, which physically block the movement of water in xylem vessels, leading to wilt symptoms (Sjulin & Beer, 1978). Motility, another virulence factor, facilitates flower infection by enabling migration down the floral stigma and into the nectarthodes, which serve as an entry point into the host. Another key pathogenicity determinant of *E. amylovora* is the type III secretion system, which is widespread in gram-negative bacterial pathogens and consists of a needle-like apparatus that delivers effector proteins into the host cytoplasm (Oh & Beer, 2005). DspE is one such effector, and its secretion is required for fire blight symptom development (Triplett *et al.,* 2009).

Sorbitol utilization mutants of *E. amylovora* are unable to establish disease in apple shoots (Aldridge *et al.,* 1997). In these mutants, symptom development is as equally hindered as in mutants of amylovoran EPS production, biofilm formation, motility and T3SS. Metabolism and carbohydrate utilization are not often considered virulence determinants; in rich medium, the sorbitol utilization operon is not essential. However, in nutrient-limited environments, such as found in apple shoots, sorbitol utilization is necessary for the survival and

spread of *E. amylovora*. In the host environment, sorbitol utilization can be considered a type of alternate virulence factor.

In this study, I further explored the role of the sorbitol utilization genes to determine whether loss of sorbitol utilization affects other virulence factors. I constructed sorbitol utilization operon mutants and measured their ability to produce amylovoran, form biofilms, and infect immature pears and apple shoots. Direct measurements of motility and type III secretion were not deemed relevant to sorbitol utilization. I hypothesized that 1) mutants deficient in sorbitol utilization would not establish infection in immature pear and apple shoots 2) amylovoran production would be reduced in the *srl* mutants, and 3) biofilm formation would either be disrupted or reduced compared to the wild type.

In addition, I conducted qRT-PCR analysis to measure expression levels of the sorbitol, sucrose and glucose uptake genes in minimal medium containing 1% sorbitol, 1% sucrose, or 0.5% glucose + 0.5% sorbitol. I hypothesized that the sorbitol uptake gene *srlA* would be highly expressed in sorbitol medium but not in the 0.5% glucose + 0.5% sorbitol medium due to catabolite repression. Additionally, I hypothesized that the glucose uptake gene *ptsG* would be highly expressed in all conditions.

II. Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study, and their relevant characteristics, are listed in Table 1.1. Unless otherwise noted, *E. amylovora* strain Ea1189 and sorbitol utilization mutants were grown in Luria-Bertani (LB) broth and plates at 28°C. Growth curves of relevant strains with sorbitol as the sole carbon source were conducted in 1% sorbitol minimal medium [per liter: 4.0 g L-Asparagine, 2.0 g K_2HPO_4, 0.2 g $MgSO_4$-$7H_2O$, 3.0 g NaCl, 0.2 g nicotinic acid, 0.2 g thiamin HCl, 10 g sorbitol] as previously described (Bereswill, 1998), and sorbitol fermentation analysis was conducted on MacConkey sorbitol indicator plates [per liter: 20 g peptone, 10 g sorbitol, 5.0 g NaCl, 0.03 g phenol red] as previously described (Rappaport & Henig, 1952). Amylovoran quantification assays were performed in MBMA medium [per liter: 0.03 g $MgSO_4$, 0.5 g citric acid, 1 g $(NH_4)_2SO_4$, 2 ml glycerol, 3 g KH_2PO_4,7g K_2HPO_4, 10 g sorbitol], and biofilm assays were conducted in 0.5xLB medium. Media were amended with chloramphenicol (Cm) at 20 µg/ml and ampicillin (Ap) at 50 µg/ml as needed.

Table 2.1. Bacterial strains, plasmids and primers used in Chapter 2

Strain or plasmid	**Characteristics**	**Source**
Ea1189	Wild type	(Burse *et al.*, 2004)
Ea1189Δ*srlA*	*srlA* deletion mutant	This study
Ea1189Δ*srlMR*	*srlMR* deletion mutant	This study
Ea1189Δ*srlAEBDMR*	*srlAEBDMR* deletion mutant	This study
Ea1189Δ*scrK*	*scrK* deletion mutant	This study
Ea1189Δ*ams*	*ams* operon deletion mutant	(Zhao *et al.*, 2009)
pKD3	Contains Cm cassette and flanking FRT sites; Cm^R	(Datsenko & Wanner, 2000)
pTL18	IPTG-inducible FLPase; Tet^R	(Long *et al.*, 2009)
pKD46	L-arabinose inducible lambda-red recombinase, Ap^R	(Datsenko & Wanner, 2000)
Primer	**Sequence**	**Source**
srlA mutagenesis F	5' –ATGATTGAAGCTATCACA CATGGGGCCGAATGGTTTATCG GTCTTTTCCAGTGTAGGCTGGAG CTGCTTC - 3'	This study
srlA mutagenesis R	5' – TTATAGATGC ACTGATTTATCAAGTTTG ATGCCCATCTTTTTCTCAAAAACATATGAATATCCTCCTTA – 3'	This study
srlMR mutagenesis F	5' –ATGGATGCAACG AATACGCTGATATTGCTGGCCG TGACGGCCTGGGTAGGGTGTAGGCTGGAGCTGCTTC – 3'	This study
srlMR mutagenesis R	5' –TCAGTCCTCTCCTGCAGT GATGACGCTGATATTC ATCCCTGACAGCTGTTCATATGAATATCCTCCTTA – 3'	This study
srlAEBDMR mutagenesis F	5' –ATGATTGAAGCTATCA CACATGGGGCCGAATG GTTTATCGGTCTTTTCCAGTGTAGGCTGGAGCTGCTTC – 3'	This study
srlAEBDMR mutagenesis R	5' –TCAGTCCTCTCCTGCAGTGATGACGCTGATAT TCATCCCTGACAGCTGTTCATATGAATATCCTCCTTA - 3'	This study
scrK mutagenesis F	5' –ATGAAAAAAAGAATCTG GGTGTTAGGTGATGCG GTGGTGGACTTGCTTCCGTGTAGGCTGGAGCTGCTTC – 3'	This study

Table 2.1. (cont'd)

scrK mutagenesis R	5' –CTACTGGCTGAAGCGGACGA GGTCTTTAGCGTAAGGCAGGGCGGTCATAGCATATGAATATCC TCCTTA - 3'	This study
ptsG qPCR F	5' – TGGCATACGGGATTATGGTT – 3'	This study
ptsG qPCR R	5' – GAAACGTTTACCCGCAAAAA – 3'	This study
scrK qPCR F	5' – GAGCTGGCAGACATCATCAA – 3'	This study
scrK qPCR R	5' – GGCACCGGTAGTATCCGTTA – 3'	This study
srlA qPCR F	5' – CGGAGAAATACAAGCCAAGC – 3'	This study
srlA qPCR R	5' – GTAGCTCACGGCAAGAGGTC – 3'	This study
srlABEDMR complement -ation F	5' –CCCGACTGGAAAGCGGGCA GTGGATTACGAATTTTGACAGGCTC – 3'	This study
srlABEDMR complement -ation R	5' - GTTGCGTCGCGGTGCATGG GAGGATGCTGAGTAGCGCTG - 3'	This study

Construction of chromosomal mutants

Deletion mutants of *scrK, srlA, srlMR* and *srlAEBDMR* were constructed using the λ Red recombinase system (Datesenko & Wanner, 2000). In short, the 1.1-kb chloramphenicol resistance (Cm^R) cassette was amplified from plasmid pKD3 using primers homologous to both 20 bp of the Cm^R cassette and to 50 bp upstream and downstream of the target gene. In pKD3, the Cm^R cassette is flanked by directly repeated flippase recognition target (FRT) sites, which facilitate site-directed recombination. The amplified regions were then purified and introduced into *E. amylovora* containing the pKD46 plasmid by electroporation. The pKD46 plasmid expresses the Red system (λ, ß, *exo* recombinase genes). Colonies were then selected on LB plates containing Cm and Ap, and mutants were confirmed by colony PCR using primers targeting regions 500 bp upstream and downstream of the mutation. To remove the Cm resistance cassette, the deletion mutants were transformed with the plasmid pTL18, which encodes an IPTG-inducible site-specific recombinase that prompts recombination between the FRT sites, leading to excision of the Cm resistance cassette. The loss of the cassette was tested via Cm sensitivity and colony PCR with the primers used to confirm the mutant.

CPC-binding assay for quantification of amylovoran production

Amylovoran production was quantified via a previously described method (Bellemann & Geider, 1992; Zhao *et al*., 2009). Briefly, a 3 ml overnight culture of *E. amylovora* was pelleted and washed with 0.5x PBS. The pellet was resuspended in 3 ml MBMA containing 1% sorbitol. The culture was incubated at 28°C for 48 hrs, after which 1 ml of the culture was removed and pelleted. A total of 800 µl of the supernatant was placed into a new tube, mixed with 40 µl of 50 mg/ml cetylpyridinium chloride (CPC), and shaken at 25°C for 10 min. The amylovoran concentration was determined by measuring the OD_{600} of the suspension normalized to the OD_{600}

of the original culture. The Ea1189Δ*ams* strain, which is unable to produce amylovoran, was used as a negative control (Zhao *et al*., 2009). The experiment was repeated at least three times.

Biofilm formation assay

Biofilm formation of each strain was quantified via a previously described method (Koczan *et al*., 2009). Glass coverslips were cut to size and placed into each well of a 24-well polystyrene microtiter plate after which 2 ml of 0.5xLB medium was added to each well, and then 100 µl of equilibrated bacterial culture was added. The plates were incubated at 28°C for 48 h, after which the glass coverslips were removed and stained with 10% crystal violet for 1 h. The stained coverslips were then washed three times with water and left to dry at 25°C for 1 h. The crystal violet stain was removed from the coverslip in a 200 µl 40% methanol/10% acetic acid mixture, and the OD_{595} of the resulting solution was measured. The experiment was repeated at least three times.

D**etermination of virulence using immature pear and apple shoot infection assays**

Virulence assays on immature Bartlett pears were performed as described previously (Zhao *et al*., 2005). Briefly, immature pears were surface sterilized with 10% bleach, rinsed with distilled water and air dried. Bacterial cultures were normalized to 1 x 10^4 CFU/ml in 0.5 x PBS. Each pear was stab-inoculated with 3 µl of the bacterial culture, and the pears were incubated at 28°C under high humidity. The resulting lesions were measured 3, 4, 5 and 6 days post inoculation (DPI). A total of 10 replicates were included in each assay, and the experiment was repeated at least three times.

Apple shoot infection assays were conducted as previously described (Koczan *et al*., 2011). In short, cultures were suspended in 0.5xPBS at 2x10^8 CFU/ml. Young apple shoots (*Malus* X domestica cv. Gala) were inoculated by dipping scissors in the bacterial suspension

and using the dipped scissors to make a diagonal cut between the leaf veins of the youngest leaf. Necrosis was measured from 4 DPI to 10 DPI. The experiment was repeated twice with at least four replicates per experiment.

Growth in sorbitol-containing minimal medium

Bacterial growth rates were measured via automatic OD_{600} measurements on a Tecan Spark® microplate reader (Mannedorf, CH). Cultures were normalized to an OD_{600} of 0.2 in LB or 1% sorbitol minimal medium, and 100 µl of this suspension was deposited into each well of a 96-well plate. The microplate reader was set to measure the OD_{600} every 30 min, and the cultures were shaken prior to each reading. The temperature remained at approximately 25°C over the course of the experiment. Each experiment was repeated at least 3 times.

Analysis of sorbitol fermentation ability using MacConkey medium

MacConkey indicator plates with 1% sorbitol were used to qualitatively analyze the ability of the mutant strains to ferment sorbitol. This medium is commonly used for the identification of *E. coli* 0157:H7, which is unable to ferment sorbitol. Phenol Red in the medium serves as a pH indicator; sorbitol fermentation is signaled by color change from red (> ~7.5) to yellow (< ~6.8) with intermediate shades of orange.

qRT-PCR

cDNA was synthesized from 1 µg of purified RNA using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was performed using the StepOnePlus Real-Time PCR system with SYBR® green master mix (Applied Biosystems). Thermal cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min, followed by a final melting curve analysis step. Gene expression levels were analyzed using the relative quantification ΔΔCt method. Expression levels of *srlA*, *scrK* and *ptsG* were

determined in each of three conditions: minimal medium containing 1% sorbitol, 1% sucrose, or 0.5% glucose + 0.5% sorbitol. All analyses were performed with strain *E. amylovora* Ea1189. Cultures were grown overnight in % sorbitol, 1% sucrose, or 0.5% glucose + 0.5% sorbitol medium. The following day, the cultures were diluted and grown to exponential phase before RNA was extracted. Expression of the *recA* gene in *E. amylovora* Ea1189 grown in glucose medium was used as the endogenous gene control.

III. Results

Sorbitol utilization mutants are reduced in growth in sorbitol-containing minimal medium

Growth curves of strains Ea1189, Ea1189Δ*srlA,* Ea1189Δ*srlMR* and Ea1189Δ*srlAEBDMR* were conducted to determine how the deletion of key sorbitol-utilization (*srl*) genes influenced growth under sorbitol conditions. As expected, growth of Ea1189 was basically unaffected in the 1% sorbitol minimal medium, with cultures reaching an OD_{600} of approximately 0.8 after 40 h (Fig 2.1). Mutant strains Ea1189Δ*srlA,* with a deletion of the sorbitol uptake gene, and Ea1189Δ*srlAEBDMR,* with a deletion of the entire sorbitol operon, were significantly reduced in growth in 1% sorbitol minimal medium compared to Ea1189. Ea1189Δ*srlMR,* which harbors a deletion of both regulatory genes of the sorbitol utilization operon, was also significantly reduced in growth compared to Ea1189, but to a lesser extent than Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* (Fig. 2.1).

On MacConkey medium amended with 1% sorbitol, growth of the WT strain Ea1189 resulted in the medium surrounding the bacterial cells turning yellow (pH < ~6.8), signaling the occurrence of sorbitol fermentation (Fig. 2.2). Additionally, Ea1189 cells growing on this medium are mucoid. In contrast, the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants displayed a non-mucoid phenotype, and the surrounding medium remained red (pH > ~7.5), indicating that sorbitol fermentation was not taking place. The Ea1189Δ*srlMR* mutant turned the surrounding medium orange, a phenotype intermediate to the Ea1189WT and the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants.

Figure 2.1. Growth of *E. amylovora* Ea1189 and Ea1189Δ*srl* gene mutants at RT in minimal medium containing 1% sorbitol.

Data represent three biological replicates for each strain, and error bars denote the standard error of the mean.

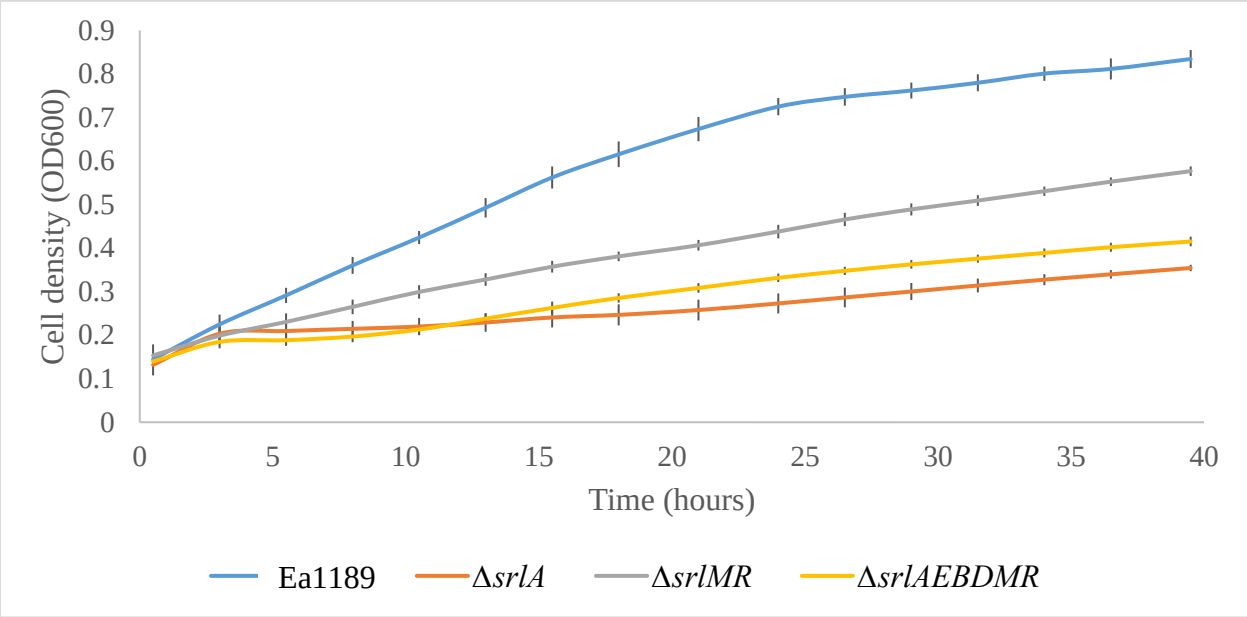
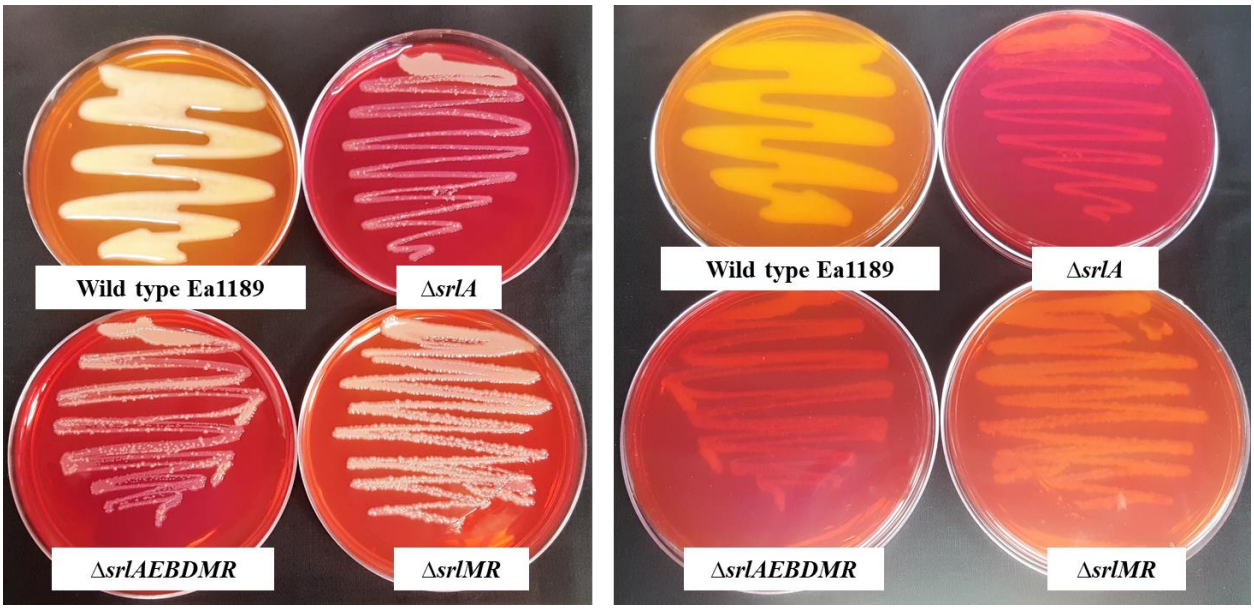


Figure 2.2. Growth of Ea1189 and Ea1189Δ*srl* gene mutants on MacConkey indicator plates containing 1% sorbitol.

Sorbitol fermentation is signaled by color change from red (> ~7.5) to yellow (< ~6.8) with intermediate shades of orange.

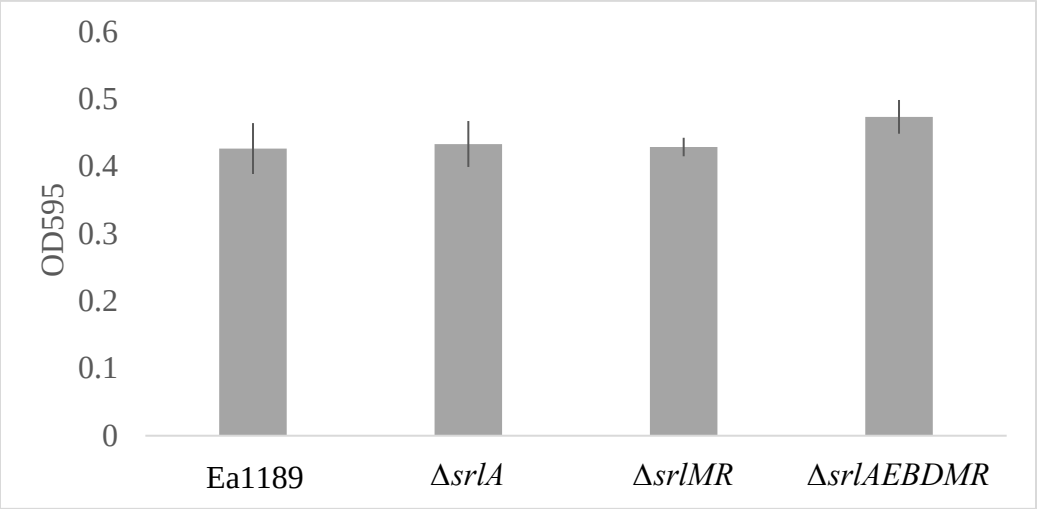


Biofilm formation is not affected in the sorbitol utilization mutants

To further explore the role of the sorbitol utilization genes in *E. amylovora*, I compared biofilm formation in Ea1189 to that of strains Ea1189Δ*srlA*, Ea1189Δ*srlMR* and Ea1189Δ*srlAEBDMR*. No significant differences were observed in biofilm formation of the sorbitol utilization mutants (Fig. 2.3). It is important to note, however, that biofilm formation was observed in 0.5 x LB medium. Although considered a nutrient-limiting environment, this medium likely exposes the sorbitol-utilization mutants to diverse carbohydrates, thereby reducing any effects of the *srl* gene mutations.

Figure 2.3. Biofilm formation by Ea1189 *srl* mutants in 0.5x LB medium.

Biofilm development was measured via quantification of crystal violet (CV) binding. Error bars signify standard errors of the mean; presence of the same letters above the bars indicate no statistically-significant difference observed ($P > 0.05$ by Student's *t* test).



Sorbitol utilization mutants are reduced in virulence on immature pears and apple shoots

To determine the impact of sorbitol utilization on the virulence of *E. amylovora*, I inoculated immature pears with Ea1189, Ea1189Δ*srlA*, Ea1189Δ*srlMR*, Ea1189Δ*srlAEBDMR* and the sucrose uptake mutant Ea1189Δ*scrK*. The latter mutant was included because sucrose concentrations are expected to be low in immature pear fruit. The resulting lesions produced by each strain were compared over the course of 6 DPI. In all strains, lesions began to form around the point of inoculation at 4 DPI (Fig. 2.4). As hypothesized, the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants exhibited significantly-reduced lesion sizes as compared to the WT. The sorbitol operon regulation mutant Ea1189Δ*srlMR* displayed an intermediate phenotype, producing larger lesions than the other *srl* mutants that were still reduced compared to the WT (Fig. 2.4). The sucrose uptake mutant, Ea1189Δ*scrK*, produced lesions similar in size to those of WT.

To determine if the sorbitol utilization mutants would be reduced in virulence on other tissues, I inoculated apple shoots with WT, Ea1189Δ*srlA*, Ea1189Δ*srlMR* and Ea1189Δ*srlAEBDMR*, and tracked the spread of infection over the course of 10 days. The results mirrored those of the immature pear assay, except the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants produced no symptoms aside from a very small area of necrosis around the inoculation site (Fig. 2.5). In contrast, the WT displayed severe tissue necrosis and migration through the central vein of the leaf over the course of the experiment. Again, the Ea1189Δ*srlMR* mutant displayed a phenotype intermediate to the WT and the other *srl* mutants.

Figure 2.4. Virulence of Ea1189 *srl* mutant strains on immature pears.

Diameters of lesions on immature pears inoculated with the indicated strains. Measurements taken day 3 to day 6 post inoculation. Error bars represent the standard errors. Different letters above the bars indicate statistically significant differences between the strains (P <0.05 by Student's t test).

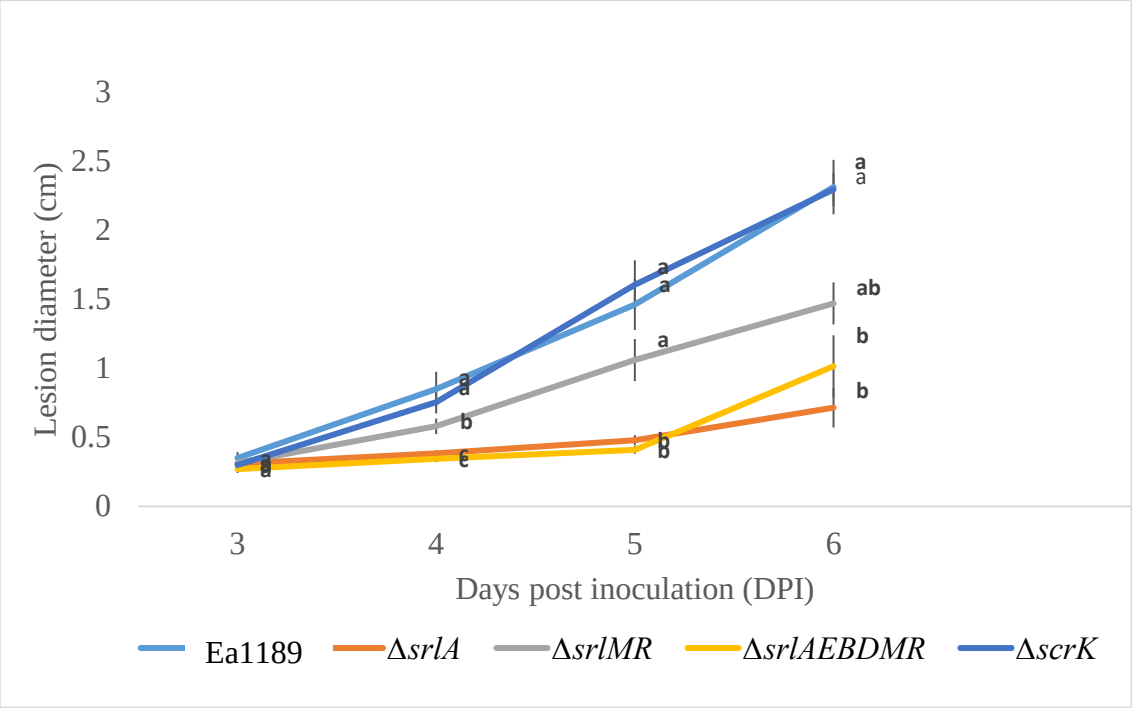
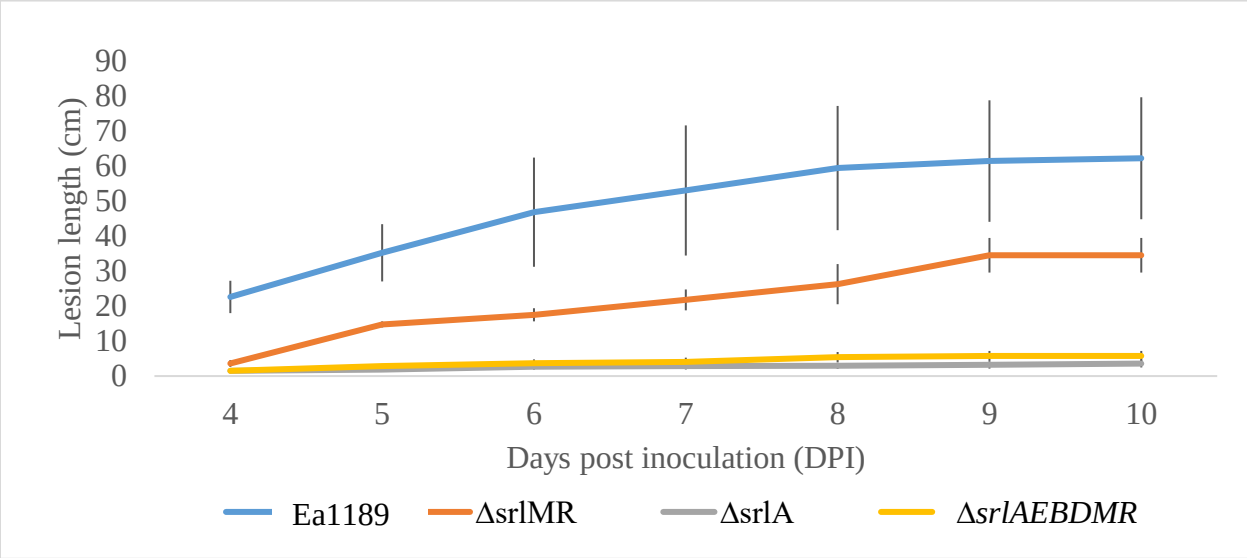


Figure 2.5. Virulence of Ea1189 *srl* mutant strains on apple shoots.

Lesion development on apple shoots from 4 to 10 days post inoculation. Error bars represent standard error of the mean.

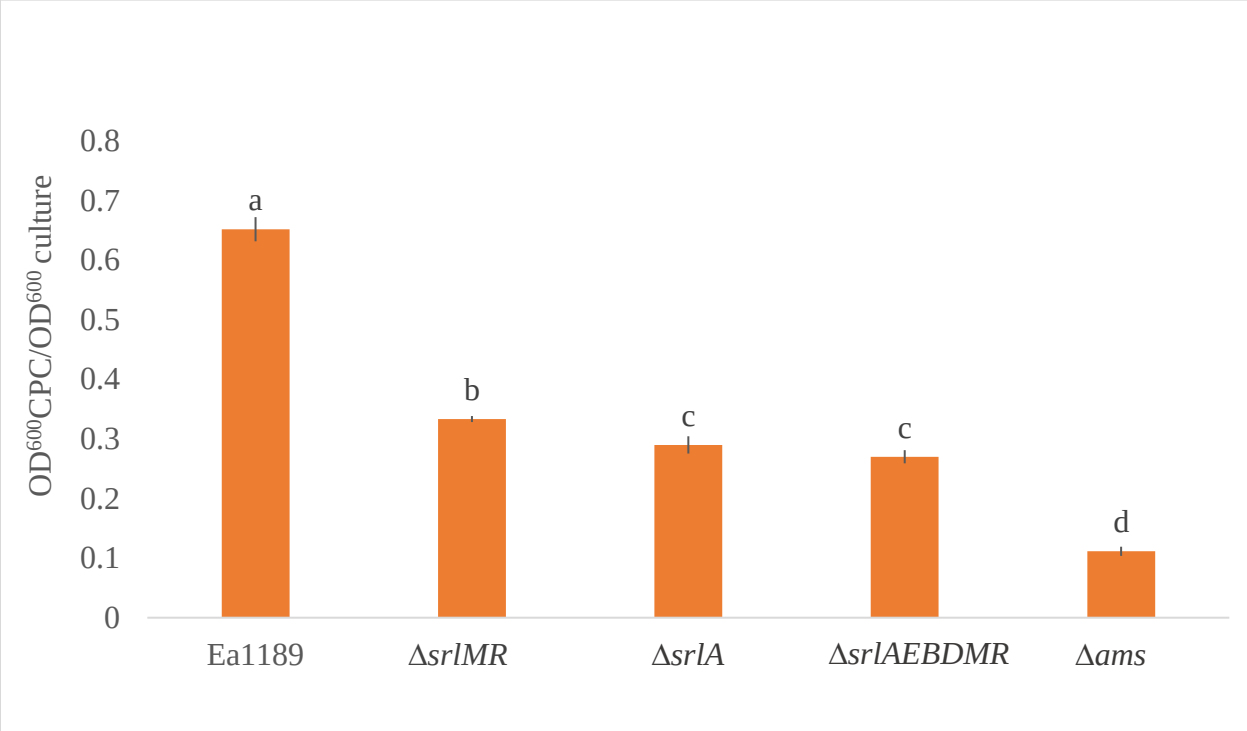


Amylovoran production is reduced in the sorbitol utilization mutants

The CPC-binding assay was used to determine whether sorbitol utilization played a role in amylovoran production. Compared to the WT, the sorbitol utilization mutants were significantly reduced in amylovoran production, although levels were not as low as in the Ea1189Δ*ams* mutant, which was deficient in amylovoran production (Fig. 2.6). Ea1189Δ*srlMR* amylovoran production was intermediate to that of Ea1189Δ*srA* and Ea1189Δ*srlAEBDMR*.

Figure 2.6. Amylovoran production in *srl* gene mutants of *E. amylovora.*

Ea1189Δ*ams* was used as a negative control. Data represents 3 biological replicates, and error bars indicate the standard error of the means. Letters above each bar indicate statistically significant differences of the means (P <0.05 by Student's t test).



srlA is highly expressed in 1% sorbitol medium and 0.5% glucose + 0.5% sorbitol medium

Gene expression levels of *srlA*, *scrK* and *ptsG* in Ea1189 were analyzed in each of three conditions: 1% sorbitol, 1% sucrose or 0.5% sorbitol + 0.5% glucose. As anticipated, the sorbitol uptake gene *srlA* was highly expressed in the 1% sorbitol medium with a 65-fold increase as compared to the reference condition (Fig. 2.7A). Likewise, the sucrose uptake gene *scrK* was upregulated approximately 11-fold in 1% sucrose medium (Fig. 2.7B). I hypothesized that *srlA* would not be highly expressed in the 0.5% glucose + 0.5% sorbitol medium due to catabolite repression; however, I observed an approximately 10-fold increase of *srlA* as compared to the reference condition (Fig. 2.7B). The glucose uptake gene *ptsG* was constitutively expressed across all three conditions.

Figure 2.7. Expression of sorbitol, sucrose and glucose transporter genes (*srlA*, *scrK* and *ptsG*, respectively) of Ea1189 grown in sorbitol (A), sucrose (B) or 50% glucose, 50% sorbitol (C) medium.

Cultures were grown overnight in 1% sorbitol, 1% sucrose or 0.5% sorbitol + 0.5% glucose medium at 28C and then were diluted in fresh medium. RNA was extracted at exponential phase. Expression data were normalized to *recA* in glucose medium. Error bars denote standard deviation of the mean, and results represent two biological replicates.

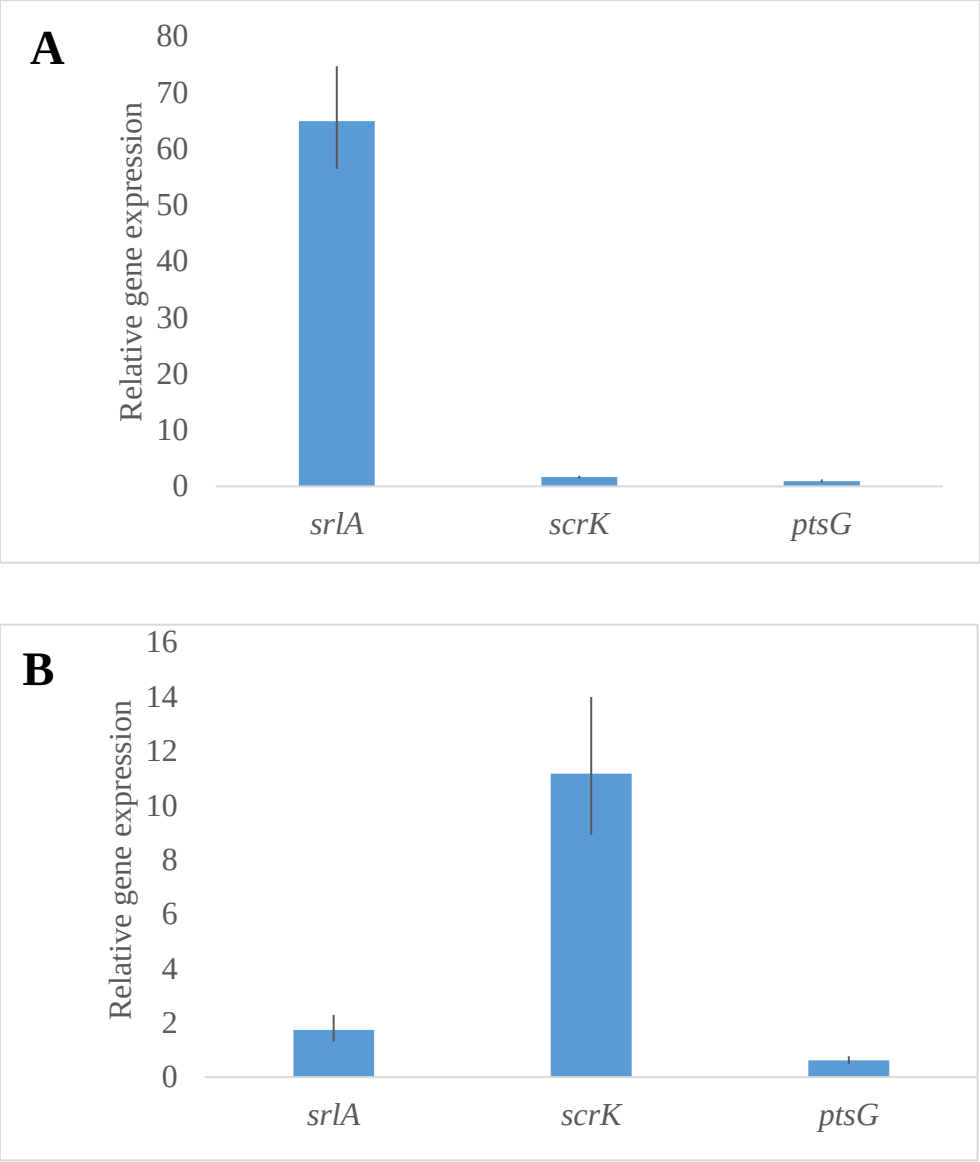
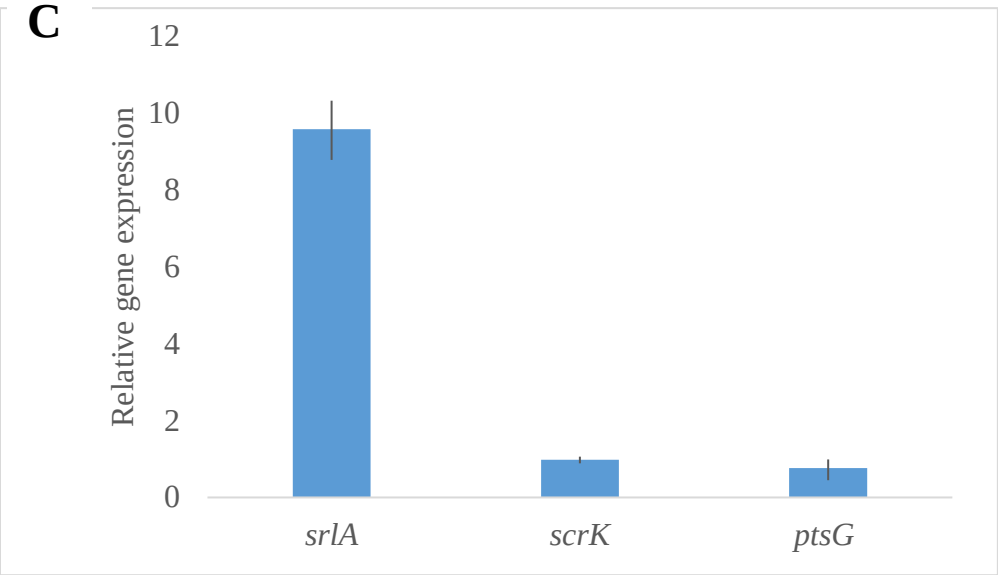


Figure 2.7. (cont'd)



IV. Discussion

The *in vitro* and *in planta* analyses demonstrated that the sorbitol utilization genes allow *E. amylovora* to obtain the energy base needed for infection most likely through enabling the synthesis of amylovoran, a critical pathogenicity factor that is required for infection. Ea1189Δ*srlA*, the deletion mutant of the sorbitol uptake gene and Ea1189Δ*srlAEBDMR*, the deletion of the entire sorbitol utilization operon and regulatory genes, exhibited dramatically reduced growth in minimal medium with sorbitol as the sole carbohydrate source. These mutants also displayed a "non-fermenting" phenotype on MacConkey sorbitol indicator plates, and bacterial growth of these strains was distinctly non-mucoid compared to the WT. The *in vitro* results were mirrored *in vivo*, where the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants were severely reduced in virulence on immature pear fruit and apple shoots. Since sorbitol predominates in both plant tissues, the results suggest that the mutants cannot obtain the energy required for further infection. The mutant of the sorbitol operon regulatory genes, Ea1189Δ*srlMR*, displayed an intermediate phenotype in both *in vitro* and *in vivo* tests. This indicates that loss of regulation of the operon interferes with the pathogen's ability to respond to available sorbitol through activation of the sorbitol utilization genes.

Both amylovoran production and biofilm formation are key virulence determinants in *E. amylovora* (Malnoy *et al*., 2012). The Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants exhibited significantly-reduced amylovoran production compared to the WT, while the Ea1189Δ*srlMR* strain produced amylovoran levels intermediate to the WT and other mutants.

Biofilm formation, however, was not significantly different in the WT and *srl* mutants. However, the half-strength LB medium used for the biofilm assay likely serves as an adequate source of nutrients, minimizing the negative effects of the defective sorbitol utilization operon. It is possible that biofilm formation by *E. amylovora srl* mutants would not be affected in leaf

xylem; however, since the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants were essentially nonpathogenic after inoculation into apple leaves, the biofilm formation phenotype would become irrelevant.

The sorbitol uptake gene *srlA* was highly expressed both in 1% sorbitol medium and in 0.5% glucose + 0.5% sorbitol medium. Because *srlA* gene activation is not inhibited by the presence of glucose, it is possible that sorbitol uptake in *E. amylovora* is not under catabolite repression. Glucose utilization does, however, seem to be critical for *E. amylovora*, because *ptsG* is constitutively expressed in all three media conditions. Future research could further explore the possibility that catabolite repression is absent in *E. amylovora* through additional qPCR studies evaluating conditions such as 0.5% glucose + 0.5% sucrose medium, or 0.5% sucrose + 0.5% sorbitol medium.

In conclusion, the type III secretion system and amylovoran production are known pathogenicity determinants, and motility and biofilm formation are known virulence determinants of *E. amylovora*. In the predominantly sorbitol-containing environment of the apple tree, the *srl* genes are necessary for full virulence and are thus an additional virulence factor. In this study, the Ea1189Δ*srlA* and Ea1189Δ*srlAEBDMR* mutants were growth-inhibited in sorbitol-containing minimal medium and significantly decreased in virulence on apple shoots and immature pear fruit. Loss of sorbitol utilization in a sorbitol environment was also found to reduce amylovoran formation. Although biofilm formation was not impaired in the *srl* mutants, it is likely that the 0.5 x LB medium used in the assay is not an accurate representation of the nutrient content of apple shoots. Future studies of biofilm formation in the *srl* mutants should amend the assay to mimic the high-sorbitol environment of the apple shoot.

REFERENCES

REFERENCES

Aldridge, P., Metzger, M. and Geider, K. (1997) Genetics of sorbitol metabolism in *Erwinia amylovora* and its influence on bacterial virulence. *Molecular and General Genetics*, 256, 611-619.

Bereswill, S., Jock, S., Bellemann, P. and Geider, K. (1998) Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. *Plant Disease*, 82, 158-164.

Datsenko, K. A. and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences USA*, 97, 6640-6645.

Koczan, J. M., Lenneman, B. R., McGrath, M. J. and Sundin, G. W. (2011) Cell surface attachment structures contribute to biofilm formation and xylem colonization by *Erwinia amylovora*. *Applied and Environmental Microbiology*, 77, 7031-7039.

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

Malnoy, M., *et al*. (2012) Fire blight: applied genomic insights of the pathogen and host. *Annual Review of Phytopathology*, 50, 475-494.

Oh, C. and Beer, S. V. (2005) Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. *FEMS Microbiology Lett*ers, 253, 185-192.

Rappaport, F. and Henig, E. (1952) Media for the Isolation and Differentiation of Pathogenic Bacteria *E. coli* (Serotypes O 111 and O 55). *Journal of Clinical Pathology*, 5, 361.

Sjulin, T. M. and Beer, S. V. (1978) Mechanism of wilt induction by amylovorin in cotoneaster shoots and its relation to wilting of shoots infected by *Erwinia amylovora*. *Phytopathology*, 68, 89-94.

Thomson, S. V. (2000) Epidemiology of Fire Blight In: Fire blight: the disease and its causative agent, Erwinia amylovora. (Vanneste, J., ed.). New York: CABI publishing, pp. 9 - 36.

Triplett, L. R., Melotto, M. and Sundin, G. W. (2009) Functional Analysis of the N Terminus of the *Erwinia amylovora* Secreted Effector DspA/E Reveals Features Required for Secretion, Translocation, and Binding to the Chaperone DspB/F. *Molecular Plant-Microbe Interact*ions, 22, 1282-1292.

Zhao, Y., Blumer, S. E. and Sundin, G. W. (2005) Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. *Journal of Bacteriology*, 187, 8088-8103.

Zhao, Y., Sundin, G. W. and Wang, D. (2009) Construction and analysis of pathogenicity island deletion mutants of *Erwinia amylovora. Canadian Journal of Microbiology*, 55, 457-464.

Chapter 3. The role of sorbitol in host specificity of *E. amylovora Rubus* isolates

Abstract

Erwinia amylovora, causal agent of fire blight, is divided into two host-specific groupings: Spiraeoideae-infecting strains, which infect apple, pear, and related species, and *Rubus*-infecting strains, which infect raspberry and blackberry. These two groups of plants differ in their carbohydrate content, with sorbitol the main translocation carbohydrate of Spiraeoideae plants, and sucrose predominating in *Rubus* plants. Spiraeoideae-infecting isolates of *E. amylovora* are capable of infecting *Rubus* plants, while *Rubus*-infecting isolates can only infect *Rubus* species and are avirulent in Spiraeoideae plants. The type III effector-encoding gene *eop1* has been determined to be an avirulence gene in *Rubus*-infecting isolates. Deletion of *eop1* enabled a *Rubus*-infecting isolate to infect immature pear fruit; this strain, however, was not an effective pathogen of apple shoots. In this study, strain MR1Δ*eop1*/*srlAEBDMR* was constructed, which is the *Rubus*-infecting isolate MR1 with both deletion of *eop1* and complemented with the sorbitol-utilization operon (*srlAEBDMR*) of the apple-infecting strain Ea1189. The ability of this complemented strain to grow in sorbitol, produce amylovoran and infect apple shoots was examined. The findings of this study indicate that the MR1Δ*eop1*/*srlAEBDMR* strain exhibits significantly increased amylovoran production and a minor increase in virulence in apple shoots compared to MR1Δ*eop1*. This strain, however, did not attain the virulence level of the apple-infecting isolate Ea1189 on apple shoots, indicating that additional host-specificity factors remain to be identified.

I. Introduction

At least two distinct groups of the fire blight pathogen *Erwinia amylovora* exist in nature. These groups are separated by host range into strains that infect the sub-family Spiraeoideae, including apple and pear, and strains that infect plants in the genus *Rubus*, including raspberry and blackberry (Mann *et al.,* 2013). *Rubus* strains of *E. amylovora* cause fire blight on raspberry, with symptoms identical to those that occur on apple, including wilted, necrotic shoots forming a "shepherd's crook", and the production of bacterial ooze (Braun *et al*., 2004). Interestingly, the raspberry-infecting strains only infect plants in the genus *Rubus,* while the Spiraeoideae-infecting strains can infect both apple and raspberry plants (Braun & Hildebrand, 2005; Ries & Otterbacher, 2005).

Previous researchers have identified differences in RNA expression, amylovoran structure and serological properties between the *Rubus* and apple-infecting *E. amylovora* strains (Braun & Hildebrand, 2005; Maes *et al.,* 2001; Mizuno *et al.,* 2002; Triplett *et al.,* 2006). In addition, there are distinct differences in genome sequence among Spiraeoideae and Rubus strains, as well as easily differentiable CRISPR genomic patterns that can be used for rapid strain discrimination (McGhee & Sundin, 2012; Mann *et al*., 2013). The structure of the exopolysaccharide amylovoran from *Rubus* strains is different from that of Spiraeoideae strains, as it lacks a glucose on residue F in the repeating subunit. In addition, there are subtle genetic differences in *Rubus* expolysaccharide and transporter genes, and antigens on the lipopolysaccharide (LPS) of *Rubus* isolates are distinct from antigens on Spiraeoideae LPS. It is not known whether any of these differences affect the host specificity of *Rubus* isolates.

However, a host specificity determinant between the two strain groups was found in the *hrp* pathogenicity island, a region encoding the type III secretion system, effectors and

regulatory components. The gene *eop1*, which encodes a type III effector protein, is divergent in the *Rubus*-infecting strains (Asselin *et al.,* 2011). The Eop1 protein is conserved in *Rubus* strains and was determined to be the host-range-limiting factor, as an *eop1* deletion in the raspberry-infecting strain exhibited a gain-of-virulence phenotype in immature pear fruit (Asselin *et al.,* 2011). The *eop1* deletion mutant, however, did not change the *Rubus* strain into an aggressive pathogen of apple shoots, leading the authors to suggest that other host specificity factors may be at play.

The carbohydrate contents of apple and raspberry are different, with sucrose the primary photosynthate of raspberry and sorbitol the primary photosynthate of apple, except for the sucrose-containing flower nectar (Bieleski, 1977; Aldridge, 1997). Bogs and Geider (2000) presented correlational evidence that carbohydrate use is a host specificity factor in *E. amylovora*. Braun and Hildebrand (2005), however, rejected this hypothesis on the basis that *Rubus* strains are not able to infect the sucrose-rich nectary of apple flowers. Their study, however, predated the Eop1 findings by Asselin *et al.* (2011), so the authors were not able to take into consideration the role of this avirulence protein.

I hypothesized that differences in the sorbitol utilization operon, in addition to differences in *eop1*, limit infection of apple shoots by *Rubus* strains of *E. amylovora*. In this study, I deleted *eop1* in the *Rubus*-infecting strain *E. amylovora* MR1 and further complemented this mutant strain with the sorbitol-utilization genes *srlAEBDMR* from a Spiraeoideae-infecting *E. amylovora* strain. The MR1Δ*eop1*/*srlAEBDMR* strain was then inoculated into apple shoots to determine if Ea1189-level virulence was restored.

II. Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study, and their relevant characteristics, are listed in Table 2.1. Unless otherwise noted, *E. amylovora* strain Spiraeoideae-infecting strain Ea1189 and *E. amylovora Rubus* strains were grown in Luria-Bertani (LB) broth and plates at 28°C. Growth curves under sorbitol conditions were conducted in 1% sorbitol minimal medium [per liter 4.0 g L-Asparagine, 2.0 g K_2HPO_4, 0.2 g $MgSO_4$-$7H_2O$, 3.0 g NaCl, 0.2 g nicotinic acid, 0.2 g thiamin HCl, 10 g sorbitol] as previously described (Bereswill, 1998), and sorbitol fermentation analysis was conducted on MacConkey sorbitol indicator plates [per liter 20 g peptone, 10 g sorbitol, 5.0 g NaCl, 0.03 g phenol red] as previously described (Rappaport & Henig, 1952). Media were amended as needed with chloramphenicol (Cm) at 20 µg/ml, kanamycin (Km) at 30 µg/ml, rifampicin (Rif) at 50 µg/ml and ampicillin (Ap) at 50 µg/ml.

Table 3.1. Bacterial strains, plasmids and primers used in Chapter 3

Strain or plasmid	**Characteristics**	**Source**
Ea1189	Wild type Spirioidiae-infecting strain of *Erwinia amylovora*; Ap^R	(Burse *et al.*, 2004)
Ea1189Δ*ams*	*ams* operon deletion mutant; Ap^R	(Zhao *et al.*, 2009)
MR1	*Rubus*-infecting isolate of *Erwinia amylovora*; Ea574	Michigan, USA
MR1Δ*eop1*	Deletion mutant of *eop1*, Cm^R	(Asselin *et al.*, 2011)
E. coli TG1	Fast-growing *E. coli* with high transformation efficiency	Lucigen Corp.
E.coli DH5α (pRK2013)	Contains helper plasmid pRK2013 for mobilization of non-self-transmissible plasmids; Km^R	Clontech Corp.
pKD3	Contains Cm cassette and flanking FRT sites; Cm^R	(Datsenko & Wanner, 2000)
pTL18	IPTG-inducible FLPase; Tet^R	(Long *et al.*, 2009)
pKD46	L-arabinose inducible lambda-red recombinase, Ap^R	(Datsenko & Wanner, 2000)
pES1	pBBR1 containing sorbitol-utilization operon (*srlAEBDMR*) of Ea1189; Kan^R	This study
MR1Δ*eop1*/pES1	MR1Δ*eop1* complemented with pES1; Km^R and Cm^R	This study
Primer	**Sequence**	**Source**
eop1 mutagenesis F	5' – ATGAATATATCTGGTCTGAGAGGC GGGTACAAAAGCCAGGCACAGCAGGCGTGTAGG CTGGAGCTGCTTC – 3'	This study
eop1 mutagenesis R	5' – CTAACTTTTGCGATTTTGCGCGGA CAGAAACGCACCCGCACGCTGAATTT – 3'	This study
srlABEDMR complementation F	5' –CCCGACTGGAAAGCGGGCA GTGGATTACGAATTTTGACAGGCTC – 3'	This study
srlABEDMR complementation R	5' - GTTGCGTCGCGGTGCATGG GAGGATGCTGAGTAGCGCTG - 3'	This study

Construction of chromosomal mutants

A deletion mutant of *eop1* was constructed via the λ Red recombinase system (Datesenko & Wanner, 2000). In short, the 1.1 kb chloramphenicol resistance (Cm^R) cassette was amplified from plasmid pKD3 using primers homologous to both 20 bp of the Cm^R cassette and to 50 bp upstream and downstream of the target gene. In pKD3, the Cm^R cassette is flanked by directly repeated flippase recognition target (FRT) sites, which facilitate site-directed recombination. The amplified regions were then purified and electroporated into *E. amylovora* MR1 containing the pKD46 plasmid, which expresses the Red system (λ, ß, *exo* recombinase genes) (Datsenko & Wanner, 2000). Colonies were then selected on LB plates containing Cm and Ap, and mutants were confirmed by colony PCR using primers targeting regions 500 bp upstream and downstream of the mutation. To remove the Cm^R cassette, the deletion mutants were transformed with the plasmid pTL18, which encodes an IPTG-inducible site-specific recombinase that prompts recombination between the FRT sites, leading to excision of the Cm resistance cassette. The loss of the cassette was tested via Cm sensitivity, and colony PCR with the primers used to confirm the mutant.

Transfer of the sorbitol-utilization genes into *E. amylovora* MR1

Plasmid pES1 was constructed by cloning *srlAEBDMR* into pBBR1 via the FastCloning method (Li *et al.,* 2011). pES1 was transferred to MR1Δ*eop1* through triparental mating, and the resulting strain was named MR1Δ*eop1*/pES1, or MR1Δ*eop1/srlAEBDMR*. The triparental mating was conducted as follows: *Escherichia coli* TG1 carrying pES1 (Km^R) was combined with helper strain *E.coli*/pRK2013 and recipient strain MR1Δ*eop1* (Rf^R and Ap^R) in a ratio of 8:1:1. This mixture was plated overnight onto LB medium and incubated at 28◦C. The following day, the cells were scraped off and plated onto LB amended with rifampicin, ampicillin and

kanamycin to select for *E. amylovora* MR1Δ*eop1/srlAEBDMR*. pES1 was determined to be stable without antibiotics in *E. amylovora* MR1 for 10 days (data not shown).

Determination of virulence using immature pears

Virulence assays on immature Bartlett pears were performed as described previously (Zhao *et al*., 2005). Briefly, immature pears were surface sterilized with 10% bleach, rinsed with distilled water and air dried. Bacterial cultures were normalized to 1 x 10^4 CFU/ml in 0.5 x PBS. Each pear was stab-inoculated with 3 µl of the bacterial culture, and the pears were incubated at 28°C under high humidity. The resulting lesions were measured 3, 4, 5 and 6 days post inoculation (DPI). Ten replicates were included in each assay, and the experiment was repeated at least 3 times.

Apple shoot infection assay

Apple shoot infection assays were conducted as previously described (Koczan *et al*., 2011). In short, cultures were suspended in 0.5xPBS at 2x10^8 CFU/ml. Young actively-growing apple shoots (*Malus* X domestica cv. Gala) were inoculated by dipping scissors in bacterial suspension and making a diagonal cut between the leaf veins of the youngest leaf. Necrosis was measured from 4 dpi to 10 dpi. The experiment was repeated twice with at least four replicates per experiment.

Analysis of sorbitol fermentation ability using MacConkey medium

To analyze the ability of the mutant strains to use sorbitol, MacConkey indicator plates with 1% sorbitol were used. This medium is commonly used for the identification of *E. coli* 0157:H7, which is unable to ferment sorbitol. Phenol Red in this medium serves as a pH indicator; sorbitol fermentation is signaled by color change from red (> ~7.5) to yellow (< ~6.8) with intermediate shades of orange sometimes observed.

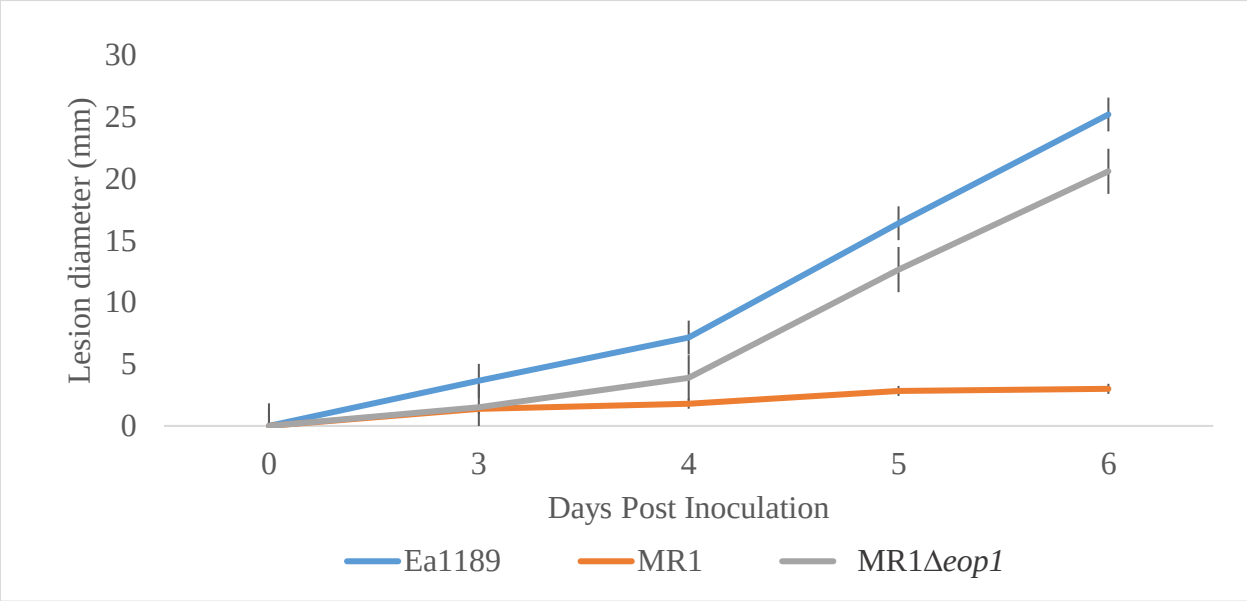
III. Results

Rubus strain *E. amylovora* MR1Δ*eop1* is virulent on immature pear fruit

Virulence of the *Rubus* strains *E. amylovora* MR1 and MR1Δ*eop1* and Spiraeoideae strain *E. amylovora* Ea1189 was tested by measuring lesion development on immature pears over the course of 6 days the apple strain E. amylovora Ea1189 developed visible lesions at 3 DPI, before lesions developed with either of the *Rubus* strains. Differences between the three strains were apparent at 4 DPI and remained until 6 DPI, with *E. amylovora* Ea1189 lesions averaging ~25 mm in diameter, while lesions produced by E. amylovora MR1Δ*eop1* were ~20 mm in diameter and MR1 lesions were only ~3 mm in diameter (Fig. 3.1).

Figure 3.1. Virulence of *E. amylovora* strains Ea1189 (apple-infecting), MR1 (*Rubus*-infecting), and MR1Δ*eop1* on immature pear fruit

Diameters of lesions were measured on immature pears inoculated with the indicated strains. Measurements were taken day 3 to day 6 post inoculation. Error bars represent the standard error of the mean.



Erwinia amylovora MR1Δ*eop1*/*srlAEBDMR* exhibited improved growth in sorbitol medium and was more mucoid than MR1Δ*eop1*

Growth of strains *E. amylovora* Ea1189, MR1 and MR1Δ*eop1* /*srlAEBDMR* was analyzed in 1% sorbitol minimal medium. In this medium, growth of sorbitol utilization (*srl*) gene mutants is significantly reduced as compared to wild type Ea1189 (data not shown). Since the *Rubus* strains do not infect sorbitol-containing apple shoots, I hypothesized that the MR1 strain would not grow well in the sorbitol medium, and that the MR1Δ*eop1*/*srlAEBDMR* strain would have improved growth with sorbitol as the sole carbon source. As expected, growth of the *Rubus*-infecting strain MR1Δ*eop1* was reduced as compared to the Spiraeoideae-infecting strain Ea1189 (Fig. 3.1). After 15 h, the OD_{600} of the Ea1189 culture reached ~1.4, while the OD_{600} of the MR1 culture was only ~0.5. Growth of MR1/*srlAEBDMR,* which is complemented with the apple-infecting *srl* operon, was improved compared to the MR1 strain, to an OD_{600} of ~0.8 after 15 h.

Strains were streaked onto MacConkey medium amended with 1% sorbitol to determine whether sorbitol fermentation is occurring in the *Rubus* strains and *srl* complement. On this medium, growth of the Spiraeoideae-infecting strain Ea1189 is typically mucoid and turns the surrounding medium from the original red color to yellow or orange, indicating sorbitol fermentation (Fig. 3.2b). An *srl* mutant is non-mucoid on this medium, and the surrounding color remains red (data not shown). On the MacConkey medium, the *Rubus*-infecting strain MR1Δ*eop1* was non-mucoid, and the surrounding medium remained red in color (Fig. 3.2a). In the *srl* complement MR1Δ*eop1* /*srlAEBDMR*, however, bacterial growth was visibly mucoid, and the surrounding medium turned orange, although the color change was not as drastic as by Ea1189.

Figure 3.2. Growth of *E. amylovora* Ea1189 (Spiraeoideae-infecting), MR1Δ*eop1* (*Rubus*-infecting) and MR1Δ*eop*1 /*srlAEBDMR* (Rubus-infecting strain complemented with apple-infecting *srl* operon) in minimal medium containing 1% sorbitol.

Growth trend observed 3 times.

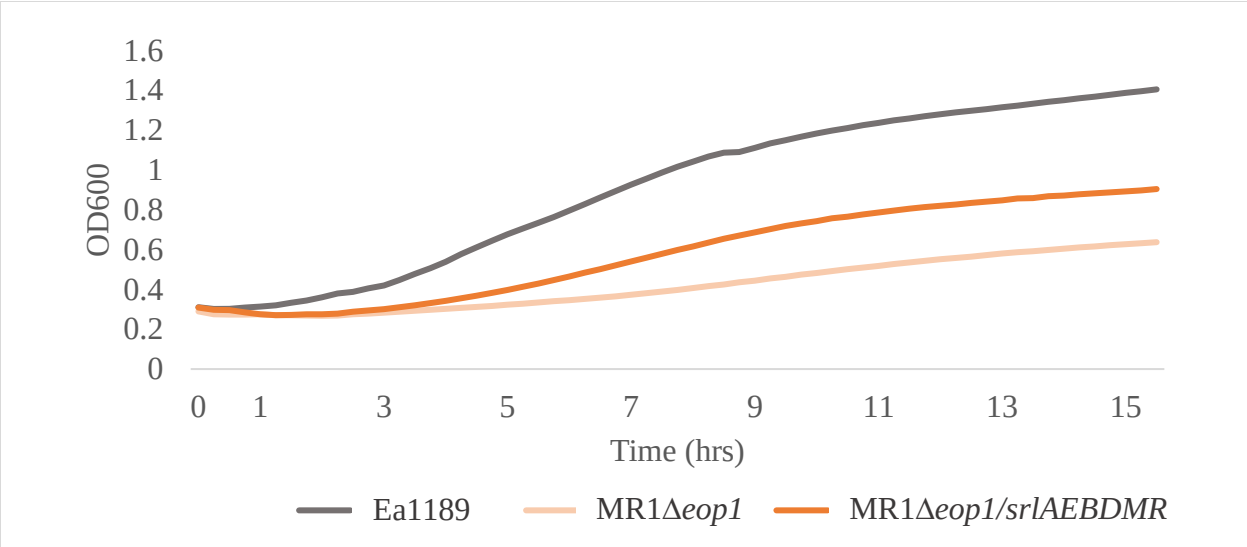
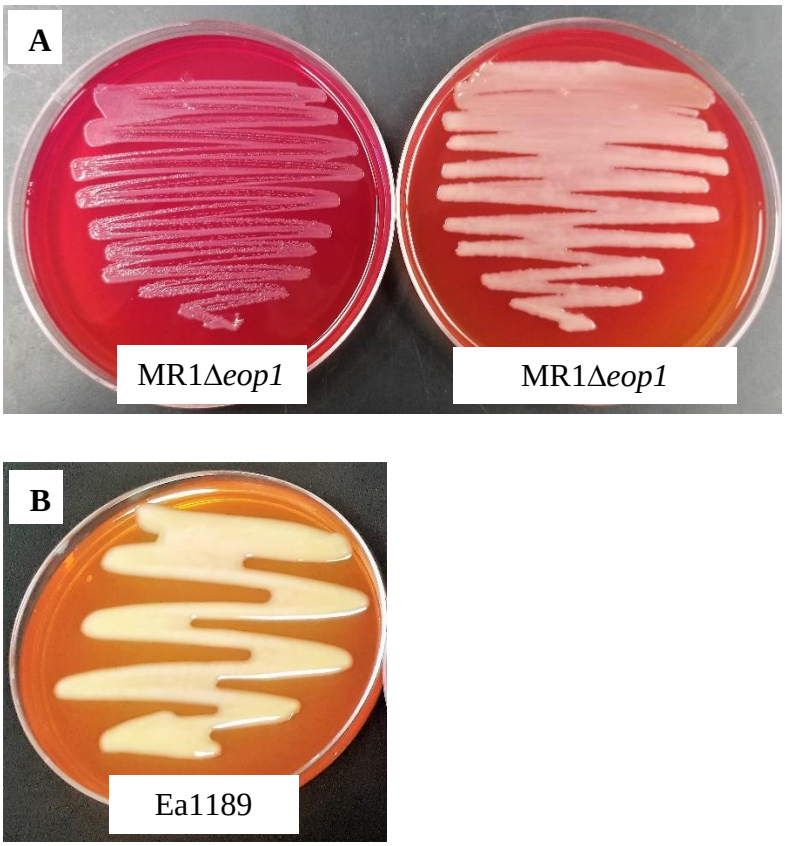


Figure 3.3. Growth of *Rubus*-infecting strains *E. amylovora* MR1Δ*eop1* and MR1Δ*eop1* /*srlAEBDMR* (complemented with *srlAEBDMR* from the Spiraeoideae-infecting strain Ea1189) on MacConkey indicator plates containing 1% sorbitol.

Sorbitol fermentation is signaled by color change from red (> ~7.5) to yellow (< ~6.8) with intermediate shades of orange. Mucoid appearance results from increased exopolysaccharide production.

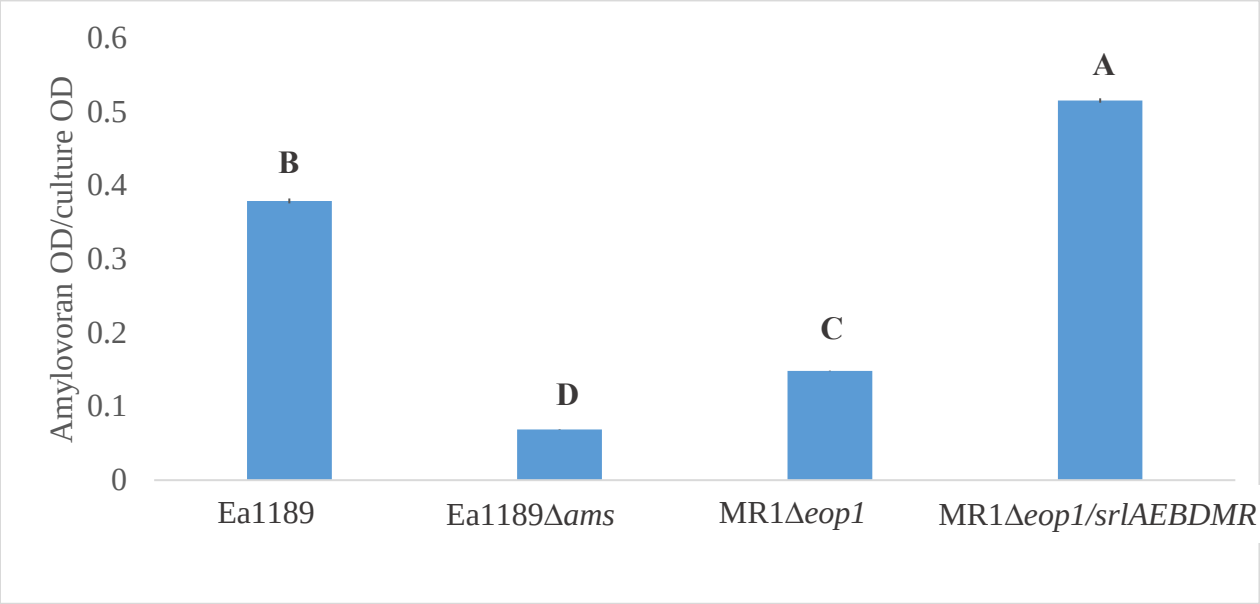


Erwinia amylovora MR1Δ*eop1*/*srlAEBDMR* exhibits increased amylovoran production

The *E. amylovora* MR1Δ*eop1*/*srlAEBDMR* strain was visibly mucoid on sorbitol medium, and so I used the CPC-binding assay to quantify amylovoran production of this strain as compared to *E. amylovora* Ea1189 and MR1Δ*eop1*. The amylovoran-deficient Ea1189Δ*ams* mutant was used as a control. Compared to Ea1189, the *Rubus*-infecting strain MR1Δ*eop1* mutant had low amylovoran yields, with levels comparable to those produced by the amylovoran-deficient Δ*ams* mutant (Fig. 3.3). The complemented strain MR1Δ*eop1*/*srlAEBDMR*, however, has significantly increased amylovoran production as compared to MR1Δ*eop1*.

Figure 3.4. Amylovoran production of *E. amylovora* Spiraeoideae-infecting strain Ea1189, *Rubus*-infecting strain MR1Δ*eop1* and MR1Δ*eop1* complemented with *srlAEBDMR* from Ea1189 (MR1Δ*eop1*/*srlAEBDMR*).

Ea1189Δ*ams* was used as a negative control. Data represents 3 biological replicates, and error bars indicate the standard error of the means.



Erwinia amylovora MR1Δ*eop1*/*srlAEBDMR* is slightly more virulent on apple shoots than MR1Δ*eop1*

Apple shoots were inoculated with strains *E. amylovora* Ea1189, MR1Δ*eop1* and MR1Δ*eop1*/*srlAEBDMR,* and the spread of infection was tracked over the course of 10 days. As expected, strain MR1Δ*eop1* produced no symptoms in apple shoots at 10 dpi, while Ea1189 displayed severe necrosis, wilting and emergence of ooze (Fig. 3.5). Strain MR1Δ*eop1*/*srlAEBDMR* developed a small necrotic lesion that halted at the main vein of the leaf and did not spread into the shoot. Interestingly, the inoculated leaves curled over in both Ea1189 and MR1Δ*eop1*/*srlAEBDMR* infections, while leaves infected with MR1 remained flat.

Figure 3.5. Virulence of *E. amylovora* Spiraeoideae-infecting strain Ea1189, *Rubus*-infecting strain MR1Δ*eop1* and MR1Δ*eop1* complemented with *srlAEBDMR* from Ea1189 (MR1Δ*eop1*/ *srlAEBDMR*).

Symptom development on apple shoots at 10 DPI is shown.

Ea1189 MR1Δ*eop1* MR1Δ*eop1*/*srlAEBDMR*

IV. Discussion

The effector Eop1 from *Rubus*-infecting isolates functions as a host specificity determinant in *E. amylovora*, as *Rubus* strains harboring a wild type copy of *eop1* are avirulent when inoculated into immature pear fruit or apple shoots (Asselin *et al.,* 2011). Deletion of *eop1* in a *Rubus* isolate, however, does not create a virulent pathogen of apple shoots, and so the question remains as to which other determinants are involved in limiting host range. The potential involvement of sorbitol in affecting host range was hypothesized by Bogs and Geider (2000), but this hypothesis was not supported by Braun and Hildebrand (2005), who noted that *Rubus* isolates cannot infect the sucrose-containing flowers of apple. Because the latter were not aware of the involvement of Eop1 in host specificity, I hypothesized that sorbitol utilization, in conjunction with Eop1, both contribute to host range in *E. amylovora.*

To test this hypothesis, I created strain MR1Δ*eop1/srlAEBDMR* with deletion of the host specificity factor Eop1 and addition of sorbitol utilization genes from Spiraeoideae isolate Ea1189. I examined growth of this strain on 1% sorbitol MacConkey plates to determine whether addition of *srlAEBDMR* from Spiraeoideae allows the *Rubus* isolate to ferment sorbitol. MR1Δ*eop1/srlAEBDMR* did not display a strong "fermenting" phenotype like Ea1189; however, the strain was visibly mucoid compared to MR1Δ*eop1*. The CPC-binding assay confirmed that MR1Δ*eop1/srlAEBDMR* has significantly increased amylovoran production, on average greater quantities than Ea1189. Because amylovoran protects the bacteria from host defenses and is required for biofilm formation (Koczan *et al.,* 2009), I hypothesized that the ability to partially utilize sorbitol and to produce increased amounts of amylovoran would convert strain MR1Δ*eop1/srlAEBDMR* into a virulent pathogen of apple.

When inoculated into apple shoots, MR1Δ*eop1/srlAEBDMR* developed small necrotic lesions that stopped at the main vein of each leaf. Thus, strain MR1Δ*eop1/srlAEBDMR* was able

to cause initial infection, but could not proceed into the next stage of pathogenesis in the xylem. I hypothesized that MR1Δ*eop1/srlAEBDMR* bacteria used type III secretion to initiate infection and amylovoran to evade host detection, but that cells were unable to establish biofilms in the xylem. There are several possible reasons why MR1Δ*eop1/srlAEBDMR* could not produce a biofilm, including differences in apple and raspberry plant xylem structure, differences in amylovoran structure between *Rubus* and Spiraeoideae isolates, and differences in cyclic-di-GMP synthesizing enzymes. The next steps to understanding the MR1Δ*eop1/srlAEBDMR* phenotype are to conduct an analysis of biofilm formation *in vitro* and to quantify bacterial populations in the apple shoot. Additionally, further studies of amylovoran structure could explore whether differences between *Rubus* and Spiraeoideae amylovoran affect biofilms. Compared to amylovoran in Spiraeoideae, amylovoran in *Rubus* strains is missing residue F ((1→6)-β-D-glucopyranosyl) (Maes *et al.*, 2001), but it is unknown whether this affects the overall function of amylovoran from *E. amylovora Rubus* strains or is involved in host specificity. Previous research has shown that the ability to synthesize amylovoran can be transferred between *Erwinia* species by cosmid clones carrying the *ams* gene cluster (Bernhard *et al.*, 1996). Cross-complementation of the *ams* gene cluster between Spiraeoideae and *Rubus* strains could provide insight into the relevance of amylovoran structure to biofilm formation and host specificity.

Levan EPS is another major component of *E. amylovora* biofilms, and *lsc* mutants are weak pathogens of apple shoots (Nimtz *et al.*, 1996; Zhang & Geider, 1999; Koczan *et al.*, 2009). A recent study by Borruso *et al.* (2017), has determined that *rlsA*, a regulator of levan production, is absent in the *Rubus* isolate MR1. Future studies could introduce Spiraeoideae *lsc*

into MR1Δ*eop1/srlAEBDMR* to determine if infection by the double-complemented strain can progress further in apple shoots.

Additional host specificity determinants remain, and an investigation of the protein profiles of *Rubus* and apple strains has identified potential targets (Braun & Hildebrand, 2005). Differences in outer membrane protein OmpA, flagellin proteins, heat-shock protein Hsp70, and a periplasmic ABC transporter were found between the two host groupings. Investigation of these proteins, in conjunction with Eop1, *srlAEBDMR,* amylovoran, and levan production may yield greater insight into the host divide of *E. amylovora.*

REFERENCES

REFERENCES

Aldridge, P., Metzger, M. and Geider, K. (1997) Genetics of sorbitol metabolism in *Erwinia amylovora* and its influence on bacterial virulence. *Molecular and General Genetics*, 256, 611-619.

Asselin, J. E., Bonasera, J. M., Kim, J. F., Oh, C.-S., and Beer, S. V. (2011) Eop1 from a *Rubus* strain of *Erwinia amylovora* functions as a host-range limiting factor. *Phytopathology*, 101, 935-944.

Bereswill, S., Jock, S., Bellemann, P. and Geider, K. (1998) Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. *Plant Disease*, 82, 158-164.

Bieleski, R.L. (1977) Accumulation of sorbitol and glucose by leaf slices of Rosaceae. *Aust. J. Plant Physiology.* 4, 11–24.

Datsenko, K. A. and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences USA*, 97, 6640-6645.

Koczan, J. M., Lenneman, B. R., McGrath, M. J. and Sundin, G. W. (2011) Cell surface attachment structures contribute to biofilm formation and xylem colonization by *Erwinia amylovora*. *Applied and Environmental Microbiology*, 77, 7031-7039.

Maes, M., *et al* (2001) Influence of amylovoran production on virulence of *Erwinia amylovora* and a different amylovoran structure in *E. amylovora* isolates from *Rubus*. *European Journal of Plant Pathology*, 107, 839-844.

Mann, R. *et al* (2013) Comparative genomics of 12 strains of *Erwinia amylovora* identifies a pan-genome with a large conserved core. *PLoS ONE*, 8, e55644.

McGhee, G. C., & Sundin, G. W. (2012) *Erwinia amylovora* CRISPR elements provide new tools for evaluating strain diversity and for microbial source tracking. *PLoS ONE*, 7, e41706.

Mizuno, A. (2002). Serological differences among *Erwinia amylovora* biovars. *Journal of General Plant Pathology*,68, 350-355.

Ries, S. M. and Otterbacher, A. G. (1977) Occurrence of fire blight on thornless blackberry in Illinois. *Plant Disease Reporter*, 61, 232-235.

Triplett, L. R., Zhao, Y., and Sundin, G. W. (2006) Genetic differences between blight-causing Erwinia species with differing host specificities, identified by suppression subtractive hybridization. *Applied and Environmental. Microbiol*ogy 72, 7359-7364.

Chapter 4. sRNA regulation of carbohydrate utilization in *Erwinia amylovora*

Abstract

Fire blight, caused by the gram-negative bacterium *Erwinia amylovora*, is a destructive disease of apple and pear trees worldwide. A unique aspect of flower infection by *E. amylovora* is the progression of the organism through three different carbohydrate zones: from the glucose-containing stigma surface, to the high sucrose environment of the nectary, then to leaves and shoots where sorbitol is most abundant. It is not yet known how the sugar-utilization genes of *E. amylovora* are regulated in response to these host changes. However, in *Escherichia coli*, small RNAs (sRNAs) dependent upon the chaperone protein Hfq for stability and function, have been implicated in the metabolic regulation of uptake and utilization of non-preferred sugars such as sucrose and sorbitol. These sRNAs act by base-pairing with target mRNAs to affect translation or stability. One such sRNA is called Spot 42 and is known to target a sorbitol uptake gene (*srlA*) in *E. coli*; this sRNA has recently been identified in *E. amylovora*. In this study, it was hypothesized that the Spot 42 sRNA is involved in regulating *E. amylovora* carbohydrate utilization. To test this hypothesis, knock-out mutants of *hfq* and the Spot 42 gene *spf* were generated. Using these strains, a translational fusion was constructed of Gfp to *srlA*. Significantly increased SrlA translation was found in the Δ*hfq* mutant; however, it was determined that Spot 42 is not the sRNA involved in sorbitol regulation in *E. amylovora*. Here, the hypothesis is presented that *E. amylovora* has evolved to evade Spot 42 regulation in order to adapt to the high-sorbitol content of apple and pear hosts.

I. Introduction

Flower infection by *Erwinia amylovora* progresses from the stigma to the nectary and finally into the pedicel. In these three stages of flower infection, the bacteria encounter glucose, sucrose and sorbitol, respectively (Pusey *et al.*, 2008; Aldridge *et al.*, 1997). In flower stigma exudates, glucose and fructose predominate, and *E. amylovora* cells can quickly consume these monosaccharides to facilitate high population growth before spreading into the floral nectary. Sucrose is the major component of nectar while largely absent in stigma exudates. As the bacteria migrate through the nectarthodes and into the vascular system of the tree, sucrose is replaced by sorbitol as the predominant carbohydrate (Aldridge *et al.*, 1997). It is unknown how *E. amylovora* regulates the transition between these different sugar environments.

Small regulatory RNAs (sRNAs) are used by many gram-negative bacteria to quickly adjust to environmental changes, including changes in nutrient availability. sRNAs are non-coding RNAs, approximately 50-350 nt, that base pair with mRNAs at the ribosome binding site (RBS) to control translation and stability of the mRNA (Sharma *et al.*, 2007). In *E. coli*, all trans-encoded sRNAs require the chaperone protein Hfq to regulate the target mRNA, and these sRNAs lose regulation of their target mRNAs in the absence of Hfq (Vogel & Luisi, 2011). Approximately 40 Hfq-dependent sRNAs have been identified in *E. amylovora*, several of which are regulators of pathogenicity and virulence traits such as type III secretion, biofilm production, and motility (Zeng *et al.*, 2013).

In *E. amylovora*, the sRNA Spot 42 comprises approximately 10% of the total sRNA profile expressed at 12 hrs in Hrp-inducing minimal medium (Zeng & Sundin, 2014). This sRNA is known in *E. coli* to regulate carbohydrate metabolism as part of a feedforward loop with the catabolite repressor protein (CRP) (Hatfull & Joyce, 1986). When *E. coli* is exposed to glucose, an energetically efficient monosaccharide, Spot 42 accelerates the repression of secondary

carbohydrate utilization genes. Spot 42 also reduces the leaky expression of certain secondary sugar utilization genes, which diverts metabolic energy and resources towards glucose consumption. Notably, the gene responsible for sorbitol uptake, *srlA*, is a known target of Spot 42 in *E. coli* (Beisel & Storz, 2011).

I hypothesized that the Spot 42 sRNA in *E. amylovora* negatively regulates the sorbitol utilization gene *srlA.* Regulation of this gene would ensure that the bacteria first consume the energetically efficient glucose on the stigma, thereby establishing significant populations for successful infection. Preliminary microarray analyses have found that *srlA* transcripts are significantly increased in the Δ*hfq* strain (Quan Zeng, unpublished). To further investigate this result, I measured SrlA translation in Ea1189, Ea1189Δ*hfq* and Ea1189Δ*spf* (Spot 42 gene mutant) through a translational fusion of *srlA* to green fluorescent protein (Gfp). Additional screening of the Ea1189Δ*hfq* strain was completed on media with various carbohydrates to discover if sRNAs regulate use of other sugars.

II. Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study, and their relevant characteristics, are listed in Table 3.1. Minimal medium with 1% or 2.5% added carbohydrate [per liter 4.0 g L-Asparagine, 2.0 g K_2HPO_4, 0.2 g $MgSO_4$-$7H_2O$, 3.0 g NaCl, 0.2 g nicotinic acid, 0.2 g thiamin HCl, 10 g or 25 g carbohydrate] was made as previously described (Bereswill, 1998). Bacterial growth rates were measured via OD_{600} measurements using a Tecan Spark® microplate reader (Mannedorf, CH). Cultures were normalized to an OD_{600} of 0.2, and 100 µl of this suspension was deposited into each well of a 96-well plate. The microplate reader measured the OD_{600} every 30 minutes, and the cultures were shaken prior to each reading. The growth temperature remained at approximately 25◦C over the course of the experiment. Each experiment was repeated at least 3 times.

Table 4.1. Bacterial strains and plasmids used in Chapter 4

Strain or plasmid	**Characteristics**	**Source**
Ea1189	Wild type Spirioidiae-infecting strain of *Erwinia amylovora*	(Burse *et al.*, 2004)
Ea1189Δ*ams*	*ams* operon deletion mutant	(Zhao *et al.*, 2009)
Ea1189Δ*hfq*	*hfq* deletion mutant, ApR	(Zeng *et al,* 2013)
Ea1189Δ*spf*	*spf* deletion mutant, CmR and ApR	(Zeng *et al,* 2013)
pXG-20	Contains constitutive promoter pLtet; transcription starts at the mapped +1 sight	(Urban & Vogel, 2007)
pXG::*srlA*-GFP	Includes the 5' UTR of *srlA* (-720 nt from the *srlA* start codon), the first 40 amino acids of *srlA* and green fluorescent protein (GFP)	This study.
pKD3	Contains Cm cassette and flanking FRT sites; Cm^R	(Datsenko & Wanner, 2000)
pTL18	IPTG-inducible FLPase; Tc^R	(Long *et al.*, 2009)
pKD46	L-arabinose inducible lambda-red recombinase, Ap^R	(Datsenko & Wanner, 2000)
Primer	**Sequence**	**Source**
srlA translational fusion F	5' –GAGATTGACATC CCTATCAGTGATAGAGAT ACTGAGCACA GCTACCTGTTAGTTAAGGGC GGC – 3'	This study
srlA translational fusion R	5' – AGTTCTTCTC CTTTGCTCATGAATT CGCCA GAACCGGTCACCAT CCCGACAAAAAC - 3'	This study

Construction of translational fusion and fluorescence readings

A translational fusion was constructed in pXG-20, which contains the constitutive promoter PLtet, using the FastCloning method (Li *et al.*, 2011). The construct includes the 5' UTR of *srlA* (-720 nt from the *srlA* start codon), the first 40 amino acids of *srlA* and green fluorescent protein (GFP). Fluorescence was measured on the Tecan Spark® microplate reader (Mannedorf, CH) using an excitation wavelength of 480 nm and an emission wavelength of 520 nm. All fluorescence readings were normalized relative to fluorescence of Ea1189 + pXG:20-*srlA*-GFP.

III. Results

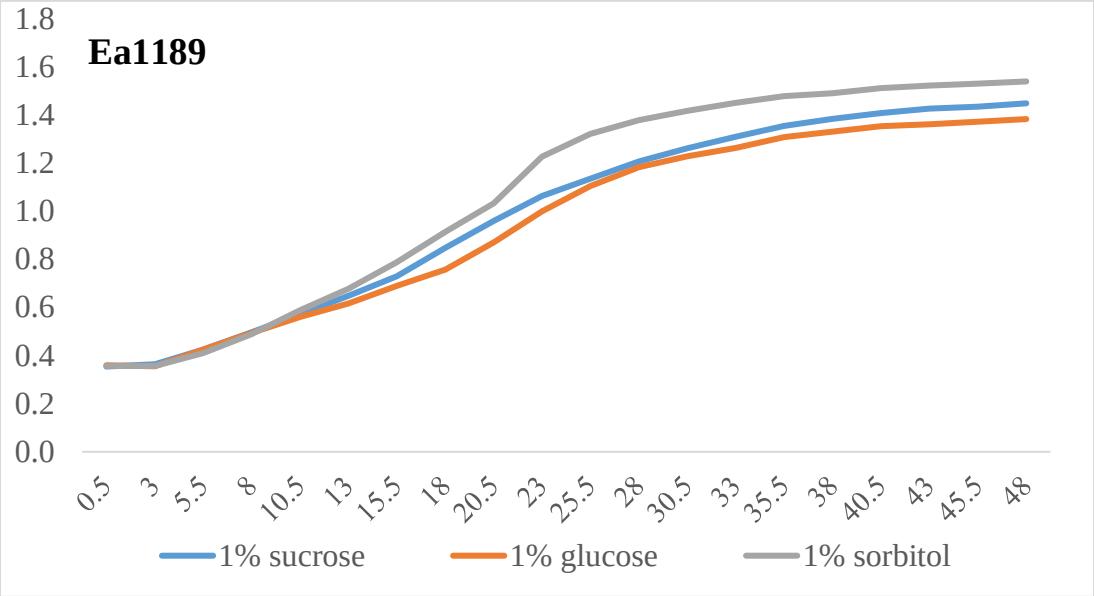
Ea1189Δ*spf* does not exhibit improved growth in sorbitol minimal medium

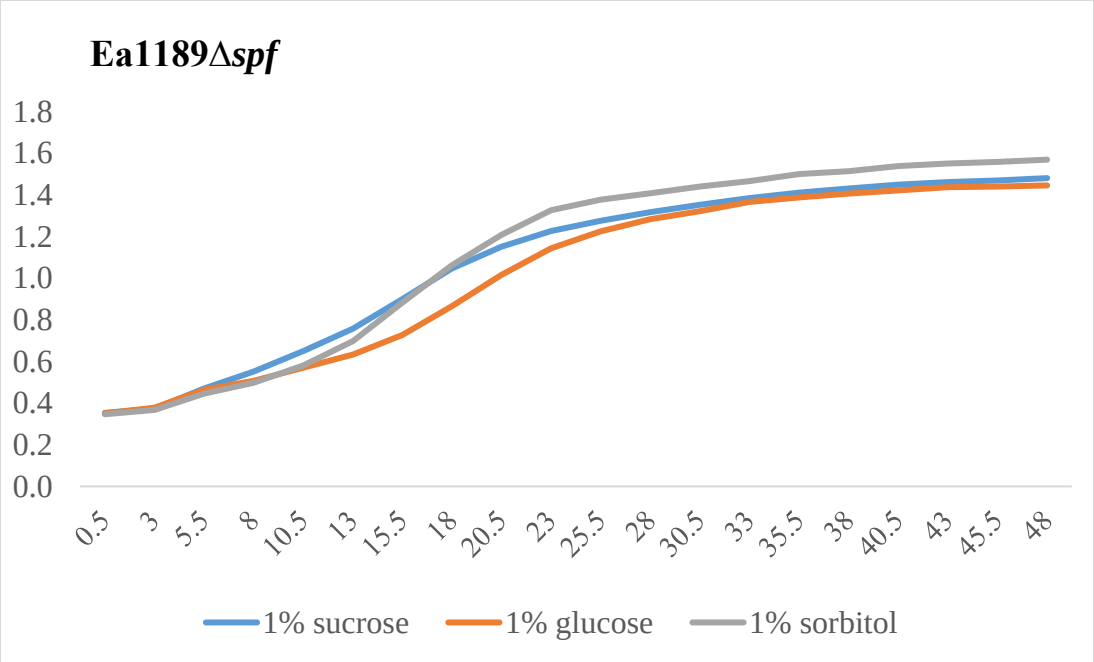
Strains Ea1189 and Ea1189Δ*spf* were grown overnight in 1% glucose minimal medium and then transferred to either 1% sorbitol, 1% sucrose or 1% glucose minimal medium. The OD_{600} of each culture was measured over the course of 48 hrs. I hypothesized that in the Ea1189Δ*spf* mutant, which lacks the Spot 42 sRNA, binding to *srlA* would not occur, and therefore growth in sorbitol would be improved compared to the wild type Ea1189. I hypothesized that growth of the Ea1189Δ*spf* mutant would be similar to Ea1189 in sucrose and glucose. Growth of the Ea1189Δ*hfq* strain was not compared in this study, as this mutant is already growth impaired due to absence of several key sRNAs.

The results indicated that strain Ea1189Δ*spf* was not improved in growth in sorbitol medium compared to Ea1189. At 48 h, the OD_{600} of Ea1189 was 1.54 (Fig. 4.1a), whereas the OD_{600} of Ea1189Δ*spf* was not significantly different at 1.57 (Fig. 4.2b). In addition, no growth differences were observed between the strains grown in sucrose or glucose medium.

Figure 4.1. Growth comparison of strains Ea1189 (A) and Ea1189Δ*spf* (B) in 1% glucose, 1% sucrose and 1% sorbitol minimal medium.

Growth analyses repeated 3 times.





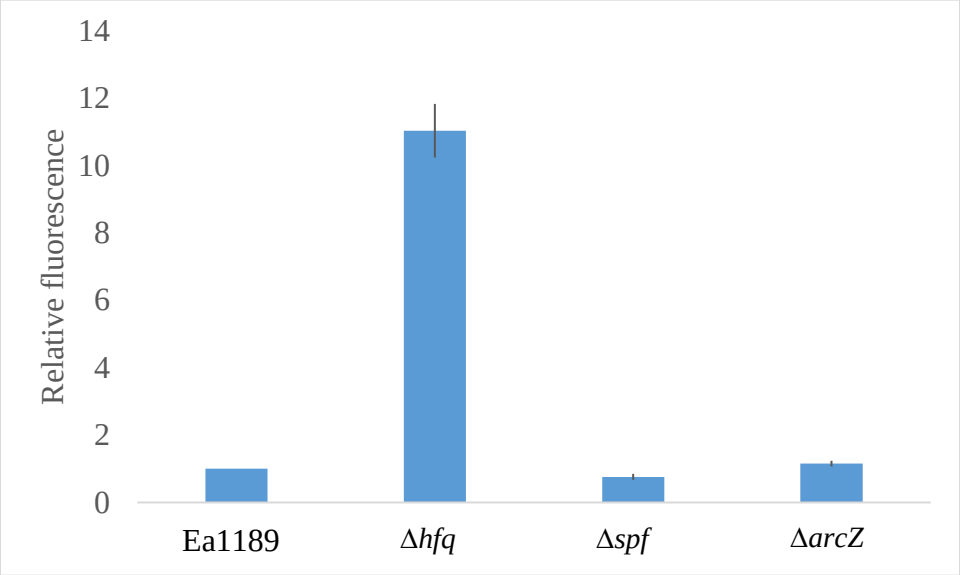
SrlA translation is increased in Ea1189Δ*hfq* but not in Ea1189Δ*spf*.

I constructed a translational fusion of SrlA to GFP, and electroporated this construct into *E. amylovora* Ea1189, Ea1189Δ*hfq* and Ea1189Δ*spf* to determine if SrlA translation is increased in the mutants compared to the wild type. Previous microarray data showed a significant increase in *srlA* transcription in Ea1189Δ*hfq* compared to Ea1189 (Quan Zeng, unpublished), which indicates that in wild type conditions, a sRNA is binding to the *srlA* mRNA. I hypothesized that SrlA translation would be significantly increased in the Ea1189Δ*hfq* strain compared to Ea1189. Because Spot 42 binds to *srlA* in *E. coli*, I hypothesized that the Ea1189Δ*spf* mutant, which does not produce Spot 42, would have increased SrlA translation.

GFP fluorescence was 11-fold greater in the Ea1189Δ*hfq* mutant compared to Ea1189 (Fig. 4.2). This implies that a sRNA is repressing *srlA*, and that deletion of the sRNA chaperone removes this regulation. However, I did not observe an increase in SrlA translation in Ea1189Δ*spf*, which indicates that an alternate sRNA must be repressing *srlA*. I also tested SrlA translation in a strain lacking the sRNA ArcZ, which regulates many virulence traits in *E. amylovora* (Zeng & Sundin, 2014). However, increased SrlA translation was not observed.

Figure 4.2. Translation of SrlA in Ea1189, Ea1189Δ*spf*, Ea1189Δ*hfq* and Ea1189Δ*arcZ* in 2.5% sorbitol minimal medium.

GFP fluorescence relative to Ea1189 (set at 1). Excitation wavelength 480 nm and emission wavelength 520 nm.



Spot 42-*srlA* binding site sequences are only 40% similar in *E. amylovora* and *E. coli*

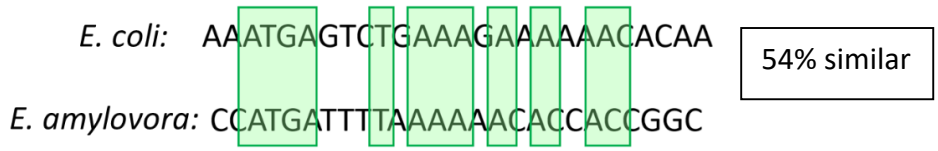
The evidence presented above indicates that Spot 42 in *E. amylovora* does not repress SrlA translation as it does in *E. coli,* although the sequence of Spot 42 is identical in both pathogens. I sought to determine whether the Spot 42 binding site in the 5'-untranslated region of *srlA* is different in *E. coli* and *E. amylovora,* and whether other Spot42-mRNA binding sites are different or the same in the two pathogens.

I performed a nucleotide BLAST comparing five different Spot 42 binding sites in target genes that are present in both pathogens. These binding sites were previously identified using the folding algorithm NUPACK and site-directed mutations in Spot 42 (Beisel & Storz, 2011). Interestingly, the Spot 42-*srlA* binding site was only 40% similar between *E. coli* and *E. amylovora*, while the four additional binding sites compared, located in *galK, sucC, sthA* and *gltA,* exhibited between 54 – 100% sequence similarity (Fig. 4.3).

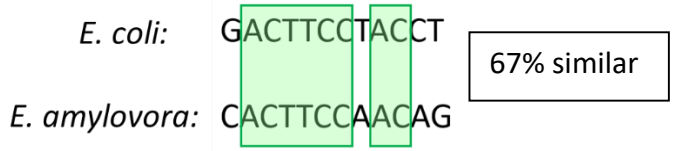
Figure 4.3. Comparison of five Spot 42-mRNA binding sites in *E. coli* and *E. amylovora*. Nucleotide comparison performed using NCBI Basic Local Alignment Search Tool (BLAST).

Sorbitol-specific enzyme II (*srlA*):

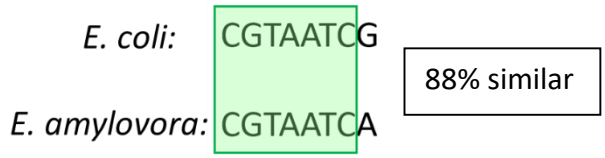
Galactokinase (*galK*):



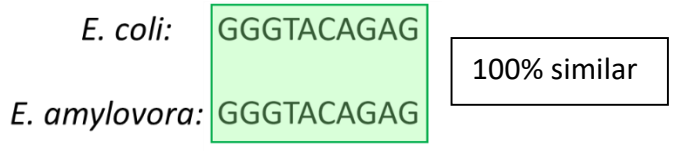
Succinyl-CoA synthetase (*sucC*):



Pyridine nucleotide transhydrogenase (*sthA*):



Citrate synthase (*gltA*):

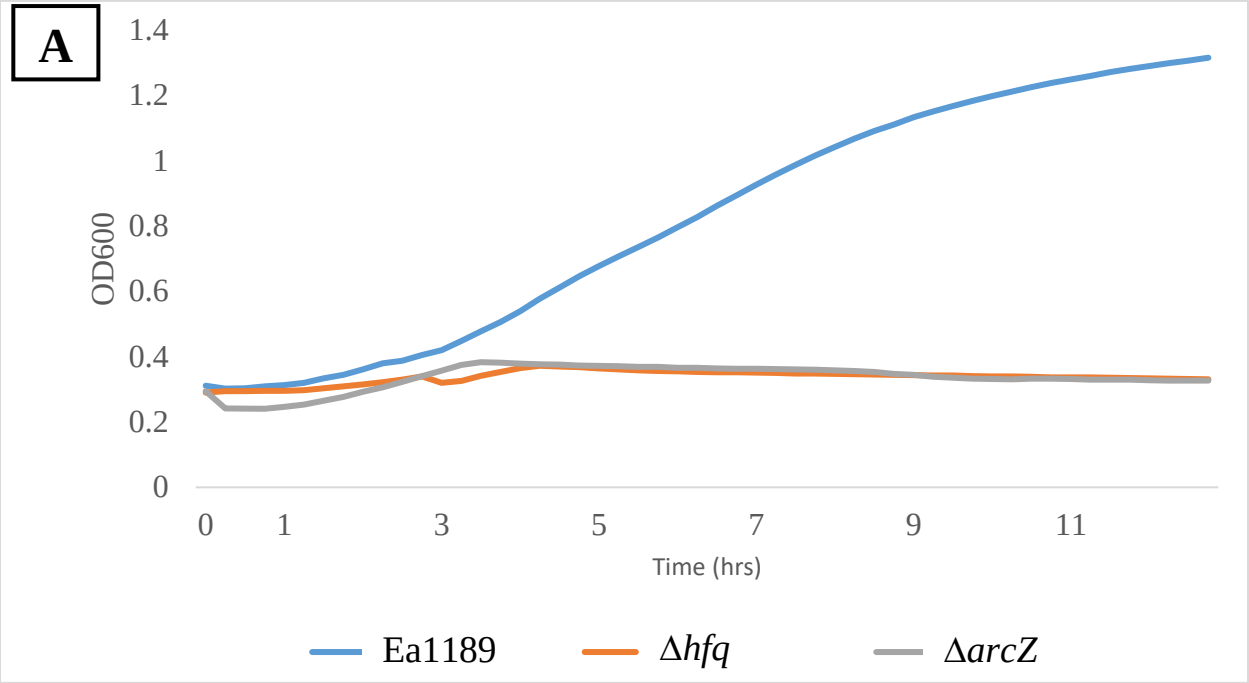


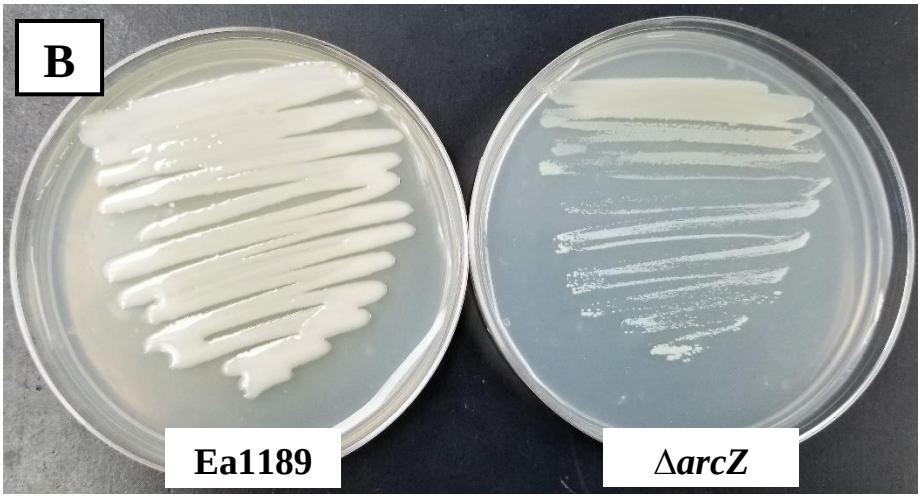
sRNAs regulate glucose utilization in *E. amylovora*

To determine if sRNA regulation affects the utilization of other sugars in *E. amylovora*, I screened the Ea1189Δ*hfq* strain on 1% glucose, 1% sorbitol, 1% sucrose or 1% fructose-containing minimal medium and searched for variable growth phenotypes. Compared to Ea1189, Ea1189Δ*hfq* was significantly growth-impaired on glucose medium (Fig. 4.4A). To determine which specific sRNA is activating glucose utilization in wild type condition, I screened approximately 40 sRNAs for reduced ability to grow on glucose. The sRNA mutant Ea1189Δ*arcZ* was found to be equally growth-impaired in glucose liquid (Fig. 4.4A) and solid (Fig. 4.4B) medium, indicating that it is the Hfq-dependent sRNA that is responsible for reduced glucose utilization phenotype of Ea1189Δ*hfq*.

Figure 4.4. Growth of Ea1189, Ea1189Δ*arcZ* and Ea1189Δ*hfq* on 1% glucose liquid (A) and solid (B) minimal medium.

Growth trend in minimal medium observed 3 times.





IV. Discussion

In this study, I determined that Ea1189Δ*hfq*, a mutant of the sRNA chaperone Hfq, has an 11-fold increase in SrlA translation as compared to Ea1189. This signifies that an sRNA is repressing *srlA* translation in the wild type strain. Because of the role of Spot 42 in *E. coli*, and the known presence of this sRNA in *E. amylovora*, I hypothesized that Spot 42 was the specific sRNA involved in suppressing *srlA* translation. In *E. coli*, the Spot 42 sRNA binds to *srlA* and blocks its translation when glucose is present. This reinforces catabolite repression and prevents leaky expression of *srlA* when glucose utilization is prioritized.

The results indicate that in *E. amylovora*, the Spot 42 mutant is neither improved in growth on sorbitol medium, nor does it have increased translation of SrlA. Interestingly, the Spot 42-*srlA* binding sites are only 40% similar between *E. amylovora* and *E. coli*. As the Spot 42 sRNA sequences are identical in the two organisms, I wanted to know whether its binding sites to other genes were also changed in *E. amylovora*. The binding site sequences of four other genes in *E. amylovora* were compared, and they ranged 54-100% similarity to *E. coli*. I hypothesized that Spot 42 is no longer a regulator of sorbitol utilization in *E. amylovora*, because *srlA* repression could be a hindrance in the high-sorbitol environment of the apple host. Because SrlA translation is significantly increased in the Ea1189Δ*hfq* mutant, I can conclude that an sRNA other than Spot 42 has co-opted sorbitol regulation. I hypothesize that this unknown sRNA evolved to be a more targeted regulator of sorbitol-utilization, needed to fine-tune the response of *E. amylovora* to the rapidly changing sugar sources of the apple tree.

In the glucose, sucrose and sorbitol growth analyses, I observed that Ea1189 growth in sorbitol is typically improved compared to growth in the other sugars. This trend has been observed in multiple independent growth assays. Additionally, the sorbitol-utilization (*srl)* genes

are still highly expressed under 0.5% glucose + 0.5% sorbitol medium (Fig. 2.7 in Chapter 2). Because sorbitol seems to be favored over glucose, and *srl* gene expression is not inhibited in the presence of glucose, I hypothesize that *E. amylovora* may not undergo catabolite repression in the same way as *E. coli.* This hypothesis is consistent with the mutation of the Spot 42-*srlA* binding site in *E. amylovora* and implies that *E. amylovora* has adapted to take advantage of the high-sorbitol environment of apple and pear trees.

To find the sRNA involved in sorbitol utilization, future research should screen sRNA mutants in *E. amylovora* with the pXG::*srlA*-GFP translational fusion plasmid. Ea1189Δ*hfq* levels of fluorescence in sorbitol medium would indicate that the sRNA under wild type conditions is repressing SrlA translation.

Additional analyses of Ea1189Δ*hfq* on various carbohydrate sources found that the strain is significantly reduced in growth on glucose as compared to Ea1189. Screening of approximately 30 sRNA mutants identified Ea1189Δ*arcZ* as the sRNA responsible for the Ea1189Δ*hfq* phenotype on glucose. I hypothesize that when *E. amylovora* cells land on the stigma surface, ArcZ activates glucose utilization. ArcZ is an important regulator of virulence traits in *E. amylovora*, such as type III secretion, biofilm formation, amylovoran EPS production and motility (Zeng & Sundin, 2014). The bacterial cells typically emerge as bacterial ooze from cankers in stem tissue, a high-sorbitol environment, prior to landing on the stigma, so glucose activation could provide an advantage. Future studies could focus on identifying the glucose-related mRNA target that ArcZ appears to activate. Several possibilities include genes of the glucose phosphotransferase system (PTS) and catabolite repressor protein (CRP).

My understanding of carbohydrate gene regulation in flower infection is beginning to take shape, although many questions remain. As flies and pollinators transfer *E. amylovora* cells

to the flower stigma, the bacteria must rapidly adjust to a new environment high in glucose and fructose. Here I hypothesize that the sRNA ArcZ is activating glucose utilization, while a sRNA is blocking SrlA translation to focus metabolic efforts on glucose consumption. As the bacteria progress down into the nectary, I suspect that sRNAs are involved in the regulation of sucrose utilization. As the cells enter the pedicel and shoots of the tree, sRNA repression of *srlA* is likely lifted so that sorbitol utilization can commence.

REFERENCES

REFERENCES

Aldridge, P., Metzger, M. and Geider, K. (1997) Genetics of sorbitol metabolism in *Erwinia amylovora* and its influence on bacterial virulence. *Molecular and General Genetics*, 256, 611-619.

Beisel, C. L. and Storz, G. (2011) The base-pairing RNA spot 42 participates in a multioutput feedforward loop to help enact catabolite repression in Escherichia coli. *Molecular Cell*, 41, 286-297.

Datsenko, K. A. and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences USA*, 97, 6640-6645.

Hatfull, G. F. and Joyce, C. M. (1986) Deletion of the *spf* (spot 42 RNA) gene of *Escherichia coli*. *Journal of Bacteriology*, 166, 746-750.

Li, C., *et al* (2011) FastCloning: a highly simplified, purification-free, sequence-and ligation-independent PCR cloning method. *BMC Biotechnology*, 11, 92.

Pusey, P. L., Rudell, D. R., Curry, E. A. and Mattheis, J. P. (2008) Characterization of stigma exudates in aqueous extracts from apple and pear flowers. *HortScience*, 43, 1471-1478.

Sharma, C. M., Darfeuille, F., Plantinga, T. H., and Vogel, J. (2007) A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes & Development.* 21, 2804–2817.

Urban, J. H., & Vogel, J. (2007) Translational control and target recognition by *Escherichia coli* small RNAs in vivo. *Nucleic Acids Research*, 35, 1018-1037.

Zeng, Q. and Sundin, G. W. (2014) Genome-wide identification of Hfq-regulated small RNAs in the fire blight pathogen *Erwinia amylovora* discovered small RNAs with virulence regulatory function. *BMC Genomics*, 15, 41

Chapter 5. Conclusions and Future Directions

I. Summary of Work

During host infection, *E. amylovora* cells encounter sorbitol in the leaves and shoots, glucose on the flower stigma surface and sucrose in the flower nectary. The cells must finely tune the expression of carbohydrate utilization genes to adjust to these changing nutrient environments. However, it has not been determined how these genes are regulated, and it is unknown how carbohydrate utilization genes, in particular sorbitol utilization genes, impact virulence. This Master's thesis explored carbohydrate utilization in relation to virulence, regulatory small RNAs (sRNAs), other virulence factors and host specificity. `

Previous research determined that in apple shoots, mutants of *E. amylovora*, with deletions of one or more of the sorbitol utilization (*srl*) genes, are unable to cause significant fire blight symptoms. The aim of Chapter 2 was to determine whether absence of the *srl* genes affects virulence factors such as amylovoran EPS production and biofilm formation, and ability to infect apple shoots and immature pear fruit. The results suggest that the *srl* mutants are amylovoran-deficient, and they are unable to obtain the energy base needed to infect apple shoots and immature pear fruits. Thus, the sorbitol utilization genes are necessary for full virulence of *E. amylovora* in the apple host.

In Chapter 3, I explored whether the *srl* genes are a host specificity factor for apple-infecting isolates of *E. amylovora.* I hypothesized that the ability to partially utilize sorbitol and produce increased levels of amylovoran would convert raspberry-infecting strain MR1Δ*eop1/srlAEBDMR* into a virulent pathogen of apple. MR1Δ*eop1/srlAEBDMR* developed small necrotic lesions when inoculated into apple shoots, but these lesions stopped at the main vein of each leaf. Thus, the strain was able to initiate infection, but was not able to continue infection into the xylem. I hypothesized that that MR1Δ*eop1/srlAEBDMR* bacteria used type III

secretion to establish infection and amylovoran to evade host detection, but that cells were unable to form biofilms in the xylem.

Previous research has characterized the involvement of the Spot 42 sRNA in suppressing *srlA* in *E. coli*. In Chapter 4, I hypothesized that Spot 42 in *E. amylovora* would likewise suppress *srlA.* To test this hypothesis, I measured translation of SrlA in Ea1189, Ea1189Δ*hfq* and Ea1189Δ*spf* mutant backgrounds. In the Ea1189Δ*hfq* mutant, I observed significantly increased SrlA translation compared to Ea1189; the Ea1189Δ*spf* mutant, however, did not have increased translation of SrlA In addition, I found that the Spot42-*srlA* binding sites are only 40% similar in *E. amylovora* and *E. coli*. From these results, I hypothesized that Spot 42 is no longer a regulator of sorbitol utilization in *E. amylovora*. It is possible that repression of *srlA* could be a hindrance to the pathogen in the high-sorbitol environment of the apple host. The high SrlA translation of Ea1189Δ*hfq* indicates that an unknown sRNA may have evolved to be a more targeted regulator of sorbitol utilization.

II. Future Directions

This thesis begins to investigate various roles of carbohydrate utilization in *E. amylovora*; however, additional questions remain. In Chapter 2, the *srl* gene mutants were found to have decreased amylovoran production, but biofilm formation was not successfully measured in these mutants. Future efforts should repeat the biofilm assay in a growth medium that better mimics the nutrient content of the xylem. This will help to determine if *E. amylovora* cells that lack *srl* genes can produce biofilms in host tissues where sorbitol predominates.

In Chapter 3, the *Rubus*-infecting mutant MR1Δ*eop1/srlAEBDMR* was able to initiate apple shoot infection but could not proceed into the next stage of pathogenesis in the xylem. I hypothesize that this strain is unable to construct biofilms, and continued research could determine what factors are missing for biofilm production. Further investigation could begin by conducting an analysis of biofilm formation *in vitro* to quantify bacterial populations in the apple shoots. Additionally, cross-complementation of the *ams* gene cluster between the Spiraeoidiae and *Rubus* strains could determine whether differences in amylovoran lead to abnormal biofilm formation. Recent research has found that *rlsA*, a regulator of levan production, is absent in the *Rubus* isolate MR1. Future studies could introduce Spiraeoideae *lsc* into MR1Δ*eop1/srlAEBDMR* to determine if levan production is a host specificity factor.

With the translational fusion constructed in Chapter 4, I found that Spot 42 does not suppress *srlA* in *E. amylovora* as it does in *E. coli.* However, significant SrlA translation was found to occur in the Ea1189Δ*hfq* mutant, indicating that an sRNA is indeed involved in regulating *srlA* under wild-type conditions. Continued screenings of sRNA mutants with the pXG::*srlA*-GFP translational fusion plasmid could identify the sRNA involved in *srlA* regulation.

In addition to differences in SrlA translation, the Ea1189Δ*hfq* mutant was found to be significantly reduced in growth on glucose. This implies that an sRNA, under wild-type

conditions, is activating glucose utilization. The sRNA ArcZ was determined to be this sRNA, as Ea1189Δ*arcZ* mirrored the phenotype of Ea1189Δ*hfq*. Future studies could focus on identifying the glucose-related mRNA target that ArcZ appears to activate.