The Role of *E. amylovora* small RNA ArcZ during fire blight disease development

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Research Proposal

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Abstract

Erwinia amylovora causes the devastating fire blight disease of apple and pear trees. Fire blight infects flowers leading to yield losses and also infects shoots and spreads systemically through the host tree. Conditions that favor disease can lead to death of the tree in a single season. Current control of the disease relies heavily on antibiotics, and in some regions resistance has emerged. *E. amylovora* coordinates regulation of flagellar motility to migrate to susceptible host tissues, produces exopolysaccharides and forms biofilms to build large populations, and expresses type III secretion to manipulate host cells. Through these virulence factors, *E. amylovora* overcomes host defenses, induces necrosis, and acquires the nutrients it needs to proliferate. The Hfq dependent small RNA ArcZ is involved in regulating each of these critical virulence factors and an *arcZ* mutant loses virulence. Although ArcZ is well conserved in enteric pathogens, it is not known how levels of the small RNA are regulated. Nor is it known with which specific mRNAs ArcZ interacts to affect virulence factors. To address these unknowns, I propose to characterize the role of ArcZ by determining how it is being regulated and how it regulates virulence. To determine how ArcZ is regulated, I will test for control at the transcriptional and post-transcriptional levels. To identify virulence factor associated mRNA targets, a combination of genetic screens and candidate gene approaches will be used. To place ArcZ regulation in context of disease development, I propose *in planta* transcriptomic analyses with spatio-temporal resolution by sampling different host tissues across time during disease development. It is anticipated that the findings of this work will directly contribute to current and future studies for development of novel fire blight control treatments.

Contents

Specific Aims

Erwinia amylovora causes fire blight of apple and pear trees, causing over 100 million dollars in damage in the USA each year (1). Like other enteric pathogens, *E. amylovora* utilizes several virulence factors to successfully colonize and infect hosts, including: flagellar motility, exopolysaccharide production and biofilm formation, and type III secretion (2). Navigating host defenses and different tissue types requires coordinated regulation of these virulence factors. Virulence can be regulated at the transcriptional level; however, rapid disease progression suggests that responses to changes in the environment faster than are explained by regulation by transcription factors alone are critical for *E. amylovora* success (3). This can be achieved at a low energy cost to the pathogen by regulating at a post-transcriptional level with small non-coding RNAs (sRNAs). One class of sRNAs is dependent on the chaperone protein Hfq and regulates trans-encoded target mRNAs through base-pairing interactions.

In *E. amylovora* the Hfq-dependent sRNA ArcZ has been shown to be essential for virulence, and it is involved in the regulation of several virulence factors (4). The ArcZ primary transcript is processed to form a mature species of ArcZ, which is well conserved in enterobacteriaceae. Although this processing step is known to occur, it is not known whether it is a regulated or constitutive event (5). Furthermore, regulation of ArcZ expression is not well characterized. Studies in other enteric pathogens have identified some direct targets of ArcZ, but these targets are not virulence associated (6). Because of the role *arcZ* must play in the post-transcriptional regulation of virulence factors to coordinate infection of a host, I propose to characterize regulation of ArcZ, as well as its regulation of virulence factors to determine the role this sRNA plays. Use of *in planta* studies will frame this work in the context of disease development and interactions with a host. I hypothesize that ArcZ is highly expressed during early stages of infection to activate motility and type III secretion, and that during later stages of infection, expression decreases, mediating the transition to biofilm formation in xylem vessels.

To test this hypothesis, I will pursue the following Specific Aims:

1. **Characterize the regulation of the ArcZ sRNA** by: a) identifying transcription factors and signals regulating its transcription, and b) determining post-transcriptional regulation of ArcZ via processing or degradation.
2. **Identify mRNA targets of ArcZ that regulate critical virulence traits including**: a) flagellar motility, b) exopolysaccharide production and biofilm formation, and c) type III secretion.
3. **Characterize the effect of ArcZ on the transcriptome during disease development** by generating a reference transcriptomic gene expression profile across host tissues and time points and determining the ArcZ regulon during disease progression on infected flowers.

Erwinia amylovora and fire blight

Erwinia amylovora is the causative agent of fire blight, the devastating disease of apple and pear trees (7). In 2003, annual estimates were placed at 100 million USD in losses in the United States to fire blight (1). However, changes toward high density production systems and consumer preference of varieties that are susceptible to fire blight since that estimate was made suggest that the contemporary losses are greater. Epidemics are often devastating to individual farmers and whole regions. For example, in 2000 an epidemic struck in Michigan where state-wide losses were estimated at 42 million USD, or about 36% of that year's production (8). Fire blight presents ongoing challenges to growers inside and outside of the United States.

E. amylovora is native to the northeastern United States where it infects a variety of native plants with low severity (9). Apples were introduced to the United States from Europe and lack coevolved defenses, leaving them susceptible to infection by *E. amylovora*. Since fire blight was first described in 1780 in New York, *E. amylovora* has spread throughout North America, and over the past century, throughout the globe (10). Infected plants can be unknowingly transferred from a nursery to be planted in an orchard anywhere facilitating the spread of this pathogen.

Although long-distance transfer is primarily facilitated by human activities, short range dissemination of the pathogen from host to host is primarily through insects, wind, and rain (11). Insects deposit bacteria on the flower stigma (**Figure 1B**, **1D**), where *E. amylovora* grows, consuming plant exudates to build populations that migrate down to the nectar (12). Once the bacteria have reached the nectar, they must utilize flagellar motility to swim down to the hypanthium, or base of the nectary, and there invade natural openings, the necarthodes (13). Once the bacteria have invaded the plant they transition to the environment of the apoplast, an acidic environment with reactive oxygen species produced by host defenses (14). *E. amylovora* continues to infect living cells, using type III secretion to translocate the effector protein DspE into host cells to suppress defenses and induce necrosis (15). The bacteria move deeper into plant tissues where oxygen levels decrease (16). Next, they migrate into xylem vessels, where the plant cells are dead, and form biofilms there (17). This provides a protected environment from host defenses, allowing the bacteria to grow to large populations and to re-program itself by turning off type III secretion genes and turning on exopolysaccharide production and biofilm formation genes.

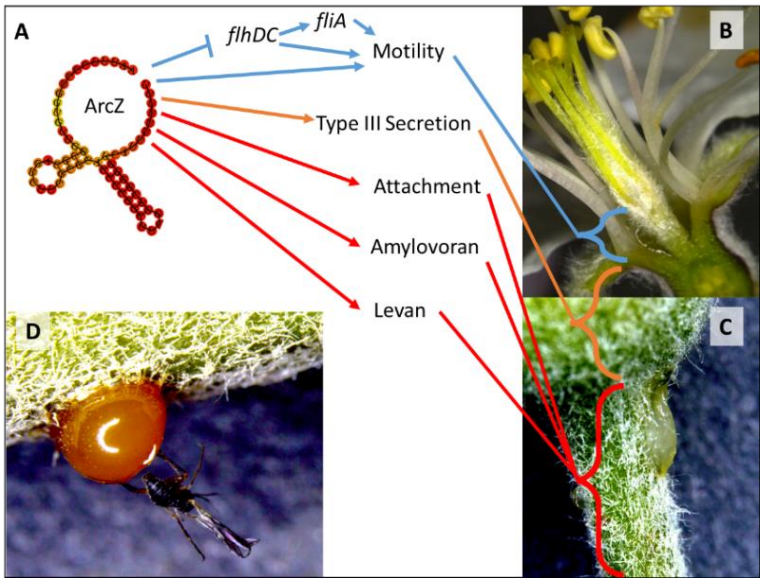


Figure 1: ArcZ regulates virulence factors in *E. amylovora*. A) What is known about ArcZ regulation of virulence, and indication of in which plant tissues each virulence factor has greatest importance. B) Cross section of an apple flower. C) Base of flower and flower pedicel exuding ooze. D) An insect stuck to an exuded ooze droplet of *E. amylovora*.

In order to disseminate to new hosts, the bacteria must emerge again from the host tissues. *E. amylovora* emerges from host tissues as ooze, a mass of exopolysaccharide and dense cells that seems to erupt out of host tissues by building turgor pressure until the host tissue tears (3). This is likely accomplished by accumulating a mass of the exopolysaccharides amylovoran and levan. The bacteria in the ooze are protected from host defenses and desiccation and are again exposed to atmospheric oxygen levels (18). This ooze is sticky and can be carried by insects (**Figure 1C, 1D**) or splashed by rain to new branches or new trees where the bacteria colonize and invade leaves using type III secretion and migrate into the host vascular system in the veins of leaves to repeat secondary cycles of the disease (11). To survive the winter, the bacteria will form cankers in woody host tissues. In the spring, the bacteria emerge from the cankers as ooze, which can serve as primary inoculum to infect young flowers and shoots.

Regulation of Virulence Factors

As a member of the enterobacteriaceae, *E. amylovora* utilizes many of the same virulence strategies as other enteric pathogens. *E. amylovora* requires flagellar motility for migration to natural openings in flowers, type III secretion and translocation of DspE to suppress host defenses, and exopolysaccharide production and biofilm formation to build populations in xylem vessels. Type III secretion and production of amylovoran, the primary exopolysaccharide of *E. amylovora*, are both pathogenicity determinants for the fire blight pathogen (2). Each of these critical virulence factors requires coordinated regulation of several genes to enable contributions to virulence. This regulation has been well characterized at the level of global transcription factors that regulate expression levels of virulence gene clusters (19). However, from the inoculation of a flower with *E. amylovora*, emergence of ooze from the flower pedicel can occur as rapidly as 48 hours (3). This suggests that for maximal virulence and fitness of *E. amylovora*, re-programming of gene expression needs to occur rapidly as the bacteria move from the floral environment to invade host tissues, then into the xylem where it builds populations, and re-emerges as ooze. To facilitate these rapid changes, post-transcriptional regulation likely plays a major role in disease development.

One mode of post-transcriptional regulation that can act rapidly and has low energetic costs to the bacteria is the use of small non-coding RNAs (sRNAs). One type of sRNAs includes the Csr system, in which sRNAs modulate the activity of a protein that interacts with target mRNAs (20). Another class is through cis-encoded anti-sense sRNAs where regulation is often through modulation of promoter strength between the mRNA and the sRNA (21). A third class is trans-encoded sRNAs that regulate specific mRNA targets by imperfect base-pairing between the two (22). These often require the chaperone protein, Hfq, which can serve to stabilize both the mRNA against degradation and the imperfect base-pair interactions.

High-throughput sequencing identified 42 Hfq-dependent sRNAs in *E. amylovora* (4). The ArcZ sRNA, identified both because of conservation in enterobacteriaceae and by high-throughput sequencing, is required for virulence in *E. amylovora* (23). Knock-out mutants of *arcZ* not only lose virulence, but are affected in many critical virulence traits. Loss of *arcZ* reduced motility, production of exopolysaccharides amylovoran and levan, and secretion and translocation of DspE by the type III secretion system.

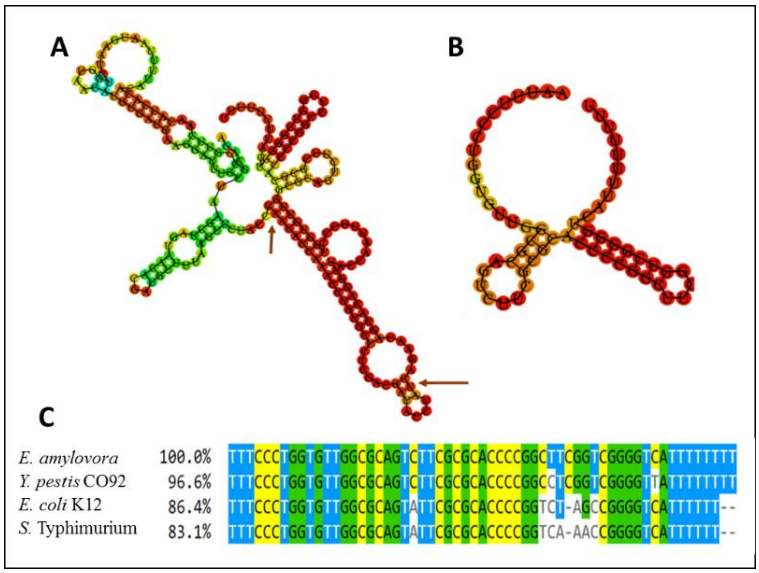


Figure 2: Structure and conservation of ArcZ. A) Structure of full length ArcZ, as predicted by mfold. Arrows indicate points of processing. B) Structure of mature ArcZ, as predicted by mfold. C) Conservation of ArcZ in enteric pathogens with percent identity to *E. amylovora arcZ* and multiple sequence alignment shown.

E. amylovora ArcZ is initially transcribed as a 204 base primary transcript, which is subsequently processed down to 112 bases and finally to a 60 base mature form, which is most abundant and most stable (4). See **Figure 2A** and **2B** for structures predicted by mfold (24). Similarly, *E. coli* ArcZ is initially transcribed as a 120 base primary transcript and has intermediate and mature forms (5). For *E. coli* and *E. amylovora*, all three forms; full-length, intermediate, and mature; can be detected by northern blot. Across enteric pathogens the mature form of ArcZ is highly conserved (**Figure 2C**), while the 5' portion that is not part of the mature form is far less conserved. The conservation of mature ArcZ and the conservation of the processing steps suggests that it may be important to function or may play a role in ArcZ abundance or activity regulation. Because other processed sRNAs are processed by ribonuclease E (RNAse E) (25), ArcZ processing may also be mediated by RNAse E.

Although ArcZ is required for virulence in *E. amylovora*, it has not been implicated in virulence in other enteric pathogens. In *E. coli* ArcZ directly interacts with RpoS mRNA to promote translation (5). Additional known targets of ArcZ include SdaCB mRNA (serine transport), Tpx mRNA (oxidative stress), and mRNA of a methyl-accepting chemotaxis gene in *S.* Typhimurium (6). During aerobic growth, ArcZ is at greatest abundance in stationary phase (5). In *E. coli*, ArcZ transcription is repressed under anaerobic conditions by the ArcBA two-component system, which senses environmental oxygen levels by sensing oxidative state of ubiquinone and menaquinone (5). Studies in *S.* Typhimurium (6) and *E. amylovora* (Zeng and Sundin, unpublished) have not found regulation of ArcZ by ArcBA, but these studies were only conducted under aerobic conditions, so ArcBA may regulate transcription of ArcZ under anaerobic conditions. The potential importance of oxygen sensing during fire blight disease development as the bacteria pass from atmospheric oxygen to reduced oxygen levels inside of the plant suggest that ArcBA repression of ArcZ under anaerobic conditions may be relevant to its role in disease development.

Although some ArcZ targets are known in other enteric bacteria, these targets are unable to explain ArcZ regulation of critical virulence factors. This suggests that although the same genes may be targets in *E. amylovora*, additional targets are the primary virulence regulators.

ArcZ and Flagellar Motility

Table 1: Motility associated phenotypes

Strain	*flhDC* mRNA levels	FlhDC translation	motility
wild-type	+	+	+
Δ*arcZ*	-	+++	-

In *E. amylovora*, flagellar biosynthesis is regulated by the FlhDC master regulators which are co-transcribed in a single operon (26). The FlhDC complex activates transcription of early flagellar biosynthesis genes, including *fliA*, the flagellar alternative sigma factor. FliA in turn is used to transcribe late flagellar biosynthesis genes, including the flagellin subunit FliC. Recent work found that expression of all tested flagellar genes is lower in the *arcZ* knock-out mutant compared to the wild-type (Zeng and Sundin, unpublished). This led to the hypothesis that the mRNA of the FlhDC master regulators is a direct target of ArcZ. FlhDC was found to be directly regulated by ArcZ at the post-transcriptional level, such that in the *arcZ* mutant FlhDC translation was high relative to wild-type. This indicates that ArcZ directly represses FlhDC translation, which is the opposite of the ArcZ effect on the motility phenotype (**Table 1**). This contradiction between the regulation of ArcZ on FlhDC translation and the motility phenotype suggests that at least one additional target of ArcZ regulates motility. The fact that even transcript levels of FlhDC were down in the *arcZ* knock-out mutant suggests that the additional target is upstream of FlhDC. Several global regulators, including H-NS, CsrA, Crp, OmpR, SdiA, LrhA, and UvrY, have been reported to regulate FlhDC expression in *E. coli* and could be targets of interest (27). However, additional regulators have also been reported, and this abundance of regulators of interest advocates for a broader screen approach rather than a systematic approach of testing individual known regulators of *flhDC* expression.

ArcZ and Exopolysaccharide Production and Biofilm Formation

There are three main types of exopolysaccharide produced by *E. amylovora*: amylovoran (28), levan (29), and cellulose (30). Each of these exopolysaccharides has been shown to contribute to biofilm formation as well as overall virulence. In the *arcZ* knock-out mutant, amylovoran and levan biosynthesis are greatly reduced relative to wild-type (4). To date, ArcZ has not been tested for influence on cellulose biosynthesis.

Amylovoran is a polysaccharide of branched pentasaccharide repeating units containing glucose and galactose (31). The twelve amylovoran biosynthesis genes are organized in an operon *amsGHIABCDEFJKL*. Although several two-component signaling systems affect amylovoran biosynthesis (32), the only known direct regulator of this operon is the Rcs phosphorelay (33). The Rcs phosphorelay is similar to a two-component system in that it senses environmental conditions to transduce a phosphorylation signal to a response regulator, RcsB. However, the Rcs phosphorelay involves two membrane bound kinases, RcsC and RcsD (34).

Levan is composed of repeating monosaccharide fructose units and is synthesized from sucrose by the levansucrase (Lsc) enzyme (29). Lsc both cleaves sucrose and attaches the fructose monomer to the growing chain. Evidence suggests that the Lsc enzyme is secreted and its

activity is manifest extracellularly (29). Expression of Lsc is regulated by three dedicated regulators: RlsA (35), RlsB (36), and RlsC (37). Additionally, Lsc is also a part of the Rcs regulon, with an RcsB binding site predicted in the promoter region of *lsc* (38).

Because ArcZ activates amylovoran and levan biosynthesis, and both are in the RcsB regulon, this suggests that some component of the Rcs phosphorelay may be a candidate target of ArcZ.

Cellulose is a polysaccharide of repeating glucose monomers. In *E. amylovora*, cellulose is synthesized from genes in the bacterial cellulose synthase (*bcs*) operon: *bcsOQABCDZ* (30). Regulation at the transcriptional level has not been characterized in *E. amylovora*, but BcsA binds to the second messenger cyclic-di-GMP to regulate its activity post-translationally (30).

In addition to exopolysaccharide production, attachment structures play a role in biofilm formation and may be regulated by ArcZ. In a traditional biofilm crystal violet staining assay, the ArcZ mutant gives high staining readings despite low exopolysaccharide levels (4). When analyzed by scanning electron microscopy, it was found that ArcZ cells were hyper-attaching to the provided surface, but were not aggregating in communities to form mature biofilms. This suggests that an attachment structure may be expressed at high levels in the *arcZ* knock-out mutant. Previous studies have identified putative attachment structures of *E. amylovora*, including: putative type I fimbriae gene clusters, putative type IV pili, and a putative invasin (39). Each of these was found to contribute to biofilm formation, suggesting that all are expressed during biofilm formation and one of them may be highly expressed in the *arcZ* knock-out mutant. Because there are few candidate genes, a systematic approach is likely to be successful in identifying the structure responsible for the hyper-attachment phenotype, which may or may not be a direct target of ArcZ.

ArcZ and Type III Secretion

In the *arcZ* knock-out mutant, type III secretion is reduced (23). Translocation of the effector DspE can be tested by infiltration of *E. amylovora* into the apoplast of *Nicotiana tabacum* (tobacco) leaves, a non-host (40). If the effector is translocated, the tobacco cells will recognize DspE as foreign and induce programmed cell death, leaving a region of dead cells on the leaf in what is known as the hypersensitive response. The *arcZ* knock-out mutant fails to elicit a hypersensitive response on tobacco, indicating that DspE is not being effectively translocated. Additionally, *in vitro* testing of the secretome of the *arcZ* knock-out mutant showed reduced secretion of DspE under conditions that induce type III secretion in wild-type cells (23).

The type III secretion system of *E. amylovora* is a pathogenicity determinant, and includes several layers of regulation (41). Initially, a two-component system, HrpXY, is activated and turns on transcription of *hrpS*. HrpS is a sigma-54 enhancer binding protein required for activation of *hrpL*. HrpL is an alternative sigma factor that is used to transcribe the type III secretion system structural components, as well as effectors, including DspE. Although ArcZ affects type III secretion, no candidate targets have been identified.

Significance of ArcZ in fire blight

Given the importance of ArcZ in regulating all critical virulence factors in *E. amylovora*, as well as its broad conservation in enteric pathogens, understanding the role of ArcZ in development of fire blight disease and identifying the major interactors will provide insights into disease biology that can inform future control practices. At present, the antibiotics streptomycin, oxytetracycline, and kasugamycin are the major chemical controls available for fire blight. However, concerns surrounding multi-drug resistant pathogens suggest that no new antibiotics will be developed or approved for control of fire blight. Streptomycin resistant *E. amylovora* was first identified in California in 1971 (42), and has since spread, although streptomycin is still widely used. In 2015, 5600 pounds of streptomycin were used in Michigan alone (USDA-NASS 2016). It is clear that novel control strategies will be needed in the future. One alternative to antibiotics is the use of treatments that inhibit virulence factors. Some studies have screened for chemical inhibitors of type III secretion in plant pathogens, and have found promising results (43, 44). Another approach is to use synthetic nucleotide analogs that permeate the cell membrane and hybridize by complimentary base pairing to knock down expression of a target gene in the bacteria, which is effective *in vitro* (45). Although initially proposed to target essential genes and be used to generate highly specific antimicrobials, this approach could also be an effective approach that targets virulence regulators, reducing selection pressure for resistance. Because of its role in regulating all critical virulence factors, the sRNA ArcZ is a potential target for such a virulence manipulation control strategy. This study will provide information that is critical to development of this type of control by characterizing the role of ArcZ during disease development by specifically identifying when and how it is being expressed, and regulating critical virulence factors.

Research Proposal

Aim 1 – How is ArcZ regulated?

Characterize the regulation of the ArcZ sRNA by: a) identifying transcription factors and signals regulating its transcription, and b) determining post-transcriptional regulation of ArcZ via processing or degradation.

Aim 1a – Transcription factors regulating ArcZ

To address which transcription factors regulate ArcZ expression, I hypothesize that ArcZ is regulated by the ArcBA two-component system under anaerobic growth conditions and that additional regulators exist to repress ArcZ during aerobic exponential growth.

Aim 1a Approach

The ArcBA two-component system will be tested for regulation of ArcZ. An *E. amylovora arcB* knock-out mutant was found to have no effect on ArcZ abundance at atmospheric oxygen levels (Zeng and Sundin, unpublished); however, this strain has not been tested under low oxygen conditions. Additionally, because ArcA is the response regulator, alternative kinases could result in its activation without ArcB (46). A knock-out mutant of *arcA*, and an *arcBA* double mutant

will be generated to test for ArcZ expression under atmospheric oxygen and low oxygen conditions. Knock-out mutants will be generated using a lambda-red recombinase system as previously described (47).

A promoter fusion of the 500 bp upstream of the ArcZ transcriptional start site and green fluorescent protein (*gfp*) will be generated to assess *arcZ* promoter activity. Transposon (Tn) mutagenesis will be carried out, and the mutant population will be assessed for fluorescence from GFP, as a proxy for *arcZ* promoter activity, with flow cytometry. Cell sorting will enable the flow cytometry to serve as a genetic screen for mutants with fluorescence levels deviating (higher or lower) from that of a control population of *E. amylovora* carrying the reporter plasmid. Cells separated in the screen will be plated to recover individual colonies with higher or lower levels of *arcZ* promoter activity. Preparation of mutant populations will be conducted both under atmospheric oxygen levels as well as under reduced oxygen levels because of the possibility that differences in *arcZ* regulation may only manifest themselves under one condition, as in *E. coli* (5). Tn insertion sites will be sequenced and identified using an arbitrary PCR approach, in which the region flanking the Tn in the genome is amplified using one primer specific to the Tn and another primer that has random bases at the 3' end and an arbitrary sequence at the 5' end (48). For any transcription factors identified in the screen, site-directed knockouts will be generated to confirm their regulation of *arcZ*. It is anticipated that flow cytometry will enable screening of enough mutants to exhaustively identify all regulators of ArcZ.

Aim 1a Progress and Preliminary Results

A transcriptional fusion was generated containing 500 bp upstream of the *arcZ* transcriptional start site cloned upstream of *gfp* in the vector pPROBE-AT (49). This construct was transformed into *E. amylovora* strain Ea1189, confirmed to have fluorescence activity (**Figure 3**) and the sequence was confirmed by Sanger sequencing. This strain is ready for Tn mutagenesis.

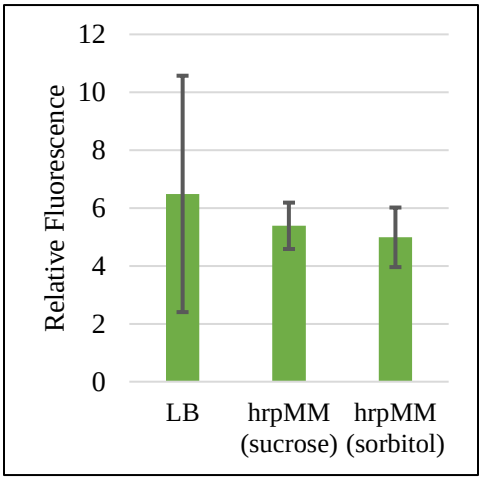


Figure 3: Confirmation of fluorescence of pPROBE-AT::arcZ-promoter construct. Fluorescence measured after 24 hours of growth in either LB or type III secretion inducing medium (hrpMM) with the indicated sugar as sole carbon source. Measurements normalized to cell density and expression of a *recA* control construct.

Aim 1a Potential Pitfalls and Solutions

Because Tn insertions could occur in the plasmid carrying the *arcZ-gfp* promoter fusion and ablate fluorescence by mutating *gfp* or the *arcZ* promoter, mutants with no fluorescence as compared to a population lacking the *arcZ-gfp* promoter fusion will be excluded from analysis, and only mutants with low levels of fluorescence will be retained. This may block detection of regulators required for *arcZ* transcription. If exclusion of non-fluorescent mutants prevents identification of candidate regulators, *E. amylovora* strain Ea1189 will first be mutagenized with Tn5-B20 and following selection of mutants. The mutant library will be transformed with the *arcZ-gfp* reporter fusion so that no Tn insertions can occur in the plasmid, allowing inclusion of complete loss of fluorescence cells during cell sorting.

Although this alternative approach overcomes the challenge of Tn insertions in the reporter plasmid, it is anticipated that this approach will generate a smaller pool of mutants owing to the challenges of transforming a mutant library. Transformation of the mutant library with the reporter plasmid will also be more likely to introduce bias because some mutants will be more readily competent than others. It is anticipated that many mutants isolated in this screen will either have multiple Tn insertions or insertions that cause genome instability. This will be overcome by screening very large numbers of mutants (~30,000) for each independent Tn mutagenesis. Several independent rounds of Tn mutagenesis and screening will be carried out to confirm that repeated hits in the screen are independent insertions and not due to cell division during selection before screening.

Aim 1b – Post-transcriptional Processing and Stability of ArcZ

I hypothesize that ArcZ processing is a regulated event that occurs differentially under distinct environmental conditions, but that degradation is constant.

Aim 1b Approach

To test ArcZ processing and stability, the quantitation of ArcZ will be carried out under a variety of growth conditions with combinations of variables such as growth phase, rich media (LB) or T3SS inducing conditions, anaerobic vs aerobic growth, and different carbon sources. To determine the half-life, *E. amylovora* strain Ea1189 will be grown under the specified conditions and then rifampicin will be added to inhibit additional RNA synthesis. Samples will be collected immediately at time zero, and then subsequent samples will be taken every 5 minutes for 30 minutes. RNA will be isolated immediately as described previously (50). Because of the small size of mature ArcZ and the shared regions between the different ArcZ species, qPCR has proven to be ineffective at distinguishing abundance of the three species. Thus, northern blotting and densitometry will provide a means to address these questions by enabling quantification of full length and processed ArcZ. These abundances will then be used for comparison across growth conditions and determination of half-life under each growth condition. If my hypothesis that processing is regulated is correct I expect to see different ratios of the different ArcZ species at time zero when grown under different conditions. If my hypothesis that degradation is constant is correct, I expect the half-life of ArcZ to remain constant regardless of growth conditions. Additionally, this approach to determine the half-life of ArcZ may provide beneficial insights to the kinetics of ArcZ processing.

Aim 1b Potential Pitfalls and Solutions

Although northern blotting may require experience and optimization to be successful, no major experimental challenges are anticipated. However, a major limitation of this approach is that it is exploratory, and no difference in processing or turnover may exist under different environmental conditions. Additionally, this approach only addresses only ArcZ abundance. Post-transcriptional regulation of ArcZ could also promote or prevent interaction with the chaperone Hfq to modulate its activity. If results do not indicate that ArcZ is post-transcriptionally regulated, it will magnify the importance of transcription factors found in Aim 1a.

Aim 1 Expected Outcomes

It is anticipated that completion of this aim will provide an understanding of how ArcZ is being regulated at the molecular level. Specifically, transcription factors that regulate expression of ArcZ will be identified, and the role, if any, of post-transcriptional regulation in regulating ArcZ levels will be determined. If the hypothesis that the ArcBA two-component system represses ArcZ under anaerobic conditions is true, it will provide that the impact of ArcZ in infection is greatest in tissues with access to high levels of oxygen, like in flowers, leaves, and exuding ooze.

Aim 2 – How is ArcZ regulating virulence?

Identify mRNA targets of ArcZ that regulate critical virulence traits including: a) flagellar motility, b) exopolysaccharide production and biofilm formation, and c) type III secretion.

Aim 2a – ArcZ regulation of flagellar motility

Because ArcZ directly represses *flhDC* but activates motility, I hypothesize that another global regulator upstream of *flhDC* is a direct target of ArcZ.

Aim 2a Approach

It has previously been shown that ArcZ directly binds to *flhDC* mRNA to repress translation (Zeng and Sundin, unpublished). In spite of this, the phenotypic effect of deleting *arcZ* is a loss of motility rather than an increase. This suggests that additional regulators are acting downstream of ArcZ to regulate flagellar motility. To identify regulators that act between *arcZ* and flagellar motility, a suppressor screen will be conducted. In this screen, Tn mutagenesis will be conducted in the *arcZ* mutant background, and the resulting mutants will be screened for re-gain of flagellar motility by stab inoculation to soft agar plates (4). For mutants that suppress the *arcZ* loss of motility, Tn insertion sites will be identified by arbitrary PCR (48). Identified genes will be knocked out by site-directed mutagenesis to confirm the role of the gene into which the insertion was made. The resulting candidate targets of ArcZ will be tested for direct interaction with and regulation by ArcZ by generating translational fusions for those genes using an established protocol (51). In the translational fusions, the 5' UTR and start of the gene of interest are fused in frame to *gfp*. Expression of the fusion is under control of a constitutive promoter, such that variations in fluorescence between strains are due to differential translational control on the 5' UTR. If the translational fusion of a candidate is regulated by ArcZ, and point mutations to *arcZ* ablate regulation of the construct, and the regulation is restored by introducing compensatory mutations in the construct, this will constitute evidence for direct interaction between ArcZ and a target. The point mutations will be made in predicted interaction regions between ArcZ and the target mRNA as predicted by RNAHybrid (52). It is anticipated that this approach will identify at least one additional direct target of ArcZ that regulates flagellar motility explaining the contradiction in the regulation of *flhDC* and the motility phenotype.

Aim 2a Progress and Preliminary Results

Tn mutagenesis has been conducted in the *arcZ* mutant background, and 18,000 Tn mutants have been screened for ability to suppress the loss of motility phenotype. Twenty-seven suppressor mutants have been isolated. Of these, when insertion sites were determined, 17 had single insertions without chromosomal rearrangements. The Tn insertion sites are outlined in **Table 2**.

Table 2: Motility Suppressor Mutants of ArcZ

Locus	Gene Name	# hits	Putative function
EAM_1328	*lrp*	6	transcriptional regulator
EAM_0564	*edcB*	4	diguanylate cyclase
EAM_3388	*bcsQ*	2	cellulose synthase operon
EAM_0546	-	1	hypothetical protein
EAM_0609	-	1	putative acyltransferase
EAM_1800	-	1	putative phage protein
EAM_2064	*fliZ*	1	regulator of *fliA*
EAM_2651	-	1	hypothetical protein

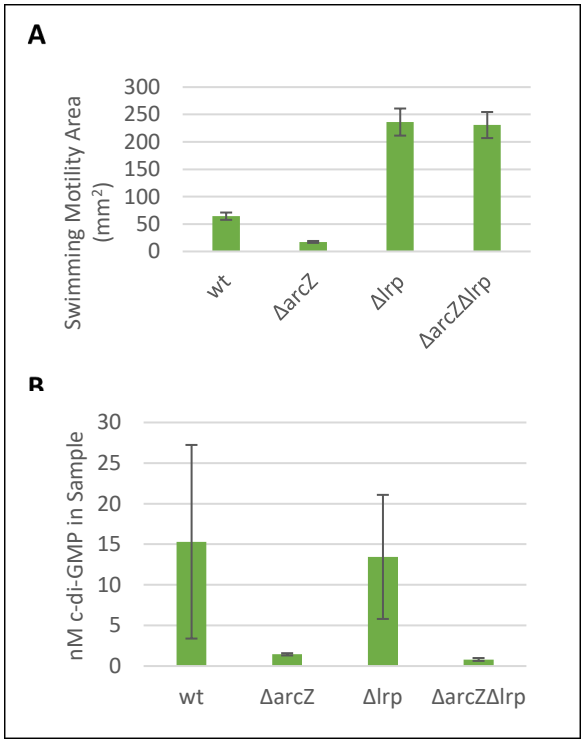


Figure 4: *lrp* is epistatic to *arcZ* for motility but not cyclic-di-GMP levels. A) Swimming motility area after 20h incubation at 28 C. B) Intracellular cyclic-di-GMP levels of cells grown overnight in LB at 28 C.

The gene coding for the leucine responsive regulator protein (*lrp*) was the most common site of Tn insertion in the suppressor mutants (**Table 2**). A reported role for Lrp regulation of *flhDC* supports this result (53). Site-directed knock-out mutants of *lrp* have been generated in the wild-type and *arcZ* mutant backgrounds, and its role in repressing swimming motility epistatic to *arcZ* has been confirmed (**Figure 4A**). Rather than simply revert to wild-type levels of motility, however, the *lrp* mutants are hyper-motile. I am generating of a translational fusion of *lrp* to *gfp* which will be used to test for direct regulation by ArcZ. The *edcB* and *bcsQ* hits are likely in a single pathway, as *bcsQ* is part of the cellulose biosynthesis operon, where cellulose synthase is post-translationally activated by cyclic-di-GMP through the EdcB diguanylate cyclase (30). Initial testing suggests that *arcZ* impacts intracellular cyclic-di-GMP levels, but *lrp* does not (**Figure 4B**). This suggests that these are two distinct pathways epistatic to *arcZ* that are affecting motility; however, this experiment needs to be repeated to confirm the results.

Aim 2a Potential Pitfalls and Solutions

It is possible that none of the candidates from the suppressor screen are direct targets of ArcZ. They may be indirectly regulated, or they may be false positives. To reduce the number of false positive candidates generated by multiple Tn insertions or genomic re-arrangements after Tn insertion, Tn insertion sites will be sequenced from each side of the Tn, and only those mutants in which both sides of the Tn indicate the same site of insertion will be included in analysis. Additionally, each arbitrary PCR determination of a Tn insertion site will be conducted twice, each time with a different arbitrary primer to reduce bias introduced by the sequence of the

arbitrary primer. To resolve cases of indirect regulation, results from sequencing approaches in Aim 2c will help to guide identification of candidate genes that may act between ArcZ and Lrp.

Aim 2b – ArcZ regulating exopolysaccharide production and biofilm formation

Because of its role in regulating both amylovoran and levan biosynthesis in *E. amylovora*, I hypothesize that ArcZ regulates these exopolysaccharides by directly regulating the Rcs phosphorelay. I further hypothesize that the hyper-attachment phenotype in the *arcZ* mutant is due to high expression of type I fimbriae.

Aim 2b Approach

To identify a direct target of ArcZ that regulates exopolysaccharide production and biofilm formation, a candidate gene approach will be used. In this approach, translational fusions to genes in the Rcs phosphorelay will be generated and tested for regulation by ArcZ. If Rcs genes are found to be regulated post-transcriptionally by ArcZ, then mutation analysis will be conducted to test whether the regulation is due to a direct or indirect interaction.

To identify the attachment structure that is responsible for the hyper-attachment phenotype of the *arcZ* mutant, genes for putative type I fimbriae (EAM_0230–EAM_0237; EAM_1825–EAM_1828; EAM_2948–EAM_2952), putative type IV pili (EAM_0729–EAM_0731), and a putative invasin (EAM_0309) will be knocked out in the *arcZ* mutant background and the resulting double mutants will be screened for loss of hyper-attachment by a crystal violet staining assay (4). Scanning electron microscopy of the arcZ mutant and double mutant lacking hyper-attachment will be used to confirm loss of the attachment structure and to contribute to characterization of the attachment structure.

Aim 2b Progress and Preliminary Results

Translational fusions of *rcsB* and *rcsC* have been generated as described (51), and confirmed both by Sanger sequencing and GFP fluorescence testing. Comparison of fluorescence in wild-type and the *arcZ* knock-out mutant shows that ArcZ represses translation of RcsB, but not RcsC. A putative interaction region between ArcZ and the RcsB mRNA has been predicted using RNAhybrid, and work has begun to generate site-directed mutants of the interaction site to confirm a direct interaction.

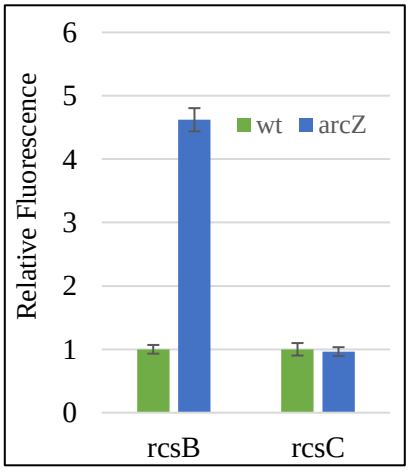


Figure 5: ArcZ regulates translation of RcsB but not RcsC. Fluorescence of strains normalized to wild-type cells after 24h growth in LB at 28 C.

Aim 2b Potential Pitfalls and Solutions

It is possible that RcsB is not a direct target of ArcZ, and that the regulation is indirect. If this is the case, a shotgun proteomic approach, with liquid chromatography separation and tandem mass spectrometry (LC-MS/MS) detection of peptides (54), will be utilized to directly quantify differential protein levels between wild-type and the *arcZ* mutant. This approach has technical limitations that prevent coverage of all proteins in the proteome. However, ArcZ regulates direct targets post-

transcriptionally, so proteomics may be able to detect targets by directly quantifying protein levels instead of mRNA abundance.

Aim 2c – ArcZ regulating type III secretion

I hypothesize that ArcZ regulates core type III secretion factors and secreted effectors.

Aim 2c Approach

To determine T3SS direct targets of ArcZ, high-throughput sequencing approaches will be used to identify candidate targets. An initial determination of how different T3SS genes are regulated by *arcZ*, differential RNAseq will be conducted comparing the *in vitro* transcriptome of wild-type, *arcZ* mutant, and an *arcZ* overexpression strain, all under conditions that normally induce type III secretion. This initial experiment will indicate if any genes are being regulated differently from the rest of the T3SS genes by ArcZ, suggesting those genes as potential targets. If there is no difference across T3SS genes, it may suggest that the regulation is at the level of a global regulator, such as the alternative sigma factor, HrpL. This RNAseq approach will be supplemented with another *in vivo* high-throughput sequencing approach for Global small non-coding RNA target Identification by Ligation and sequencing (GRIL-seq) (55). In this experiment, T4 RNA ligase is co-expressed with the sRNA, and chimeras are formed between base-pairing RNAs in the cell. Total RNA is isolated and enriched via pull-down for chimeras that contain the sRNA sequence. These enriched chimeric RNAs are then sequenced. In the analysis stage, the sRNA sequence is trimmed away and the reads are mapped to the genome, giving signal peaks at the locations where the sRNA consistently was ligated to another interacting RNA. Thus, this approach not only provides candidates for subsequent confirmation, but also provides evidence for a direct interaction between the sRNA and the targets *in vivo*.

Any candidate type III secretion proteins identified either from *in vitro* RNAseq analysis or GRIL-seq analysis will be used to generate translational fusions to confirm post-transcriptional regulation by ArcZ. Point mutation analysis will be used to confirm the interacting sequences.

Aim 2c Progress and Preliminary Results

RNA samples for *in vitro* RNAseq experiments have been prepared, quality tested, and submitted to the MSU genomics core for library preparation and sequencing. Data processing and differential gene expression analysis will begin once data is received from the sequencing core.

Aim 2c Potential Pitfalls and Solutions

It is anticipated that a simple differential RNAseq experiment may not successfully identify direct targets of ArcZ because it regulates targets post-transcriptionally. This limitation is overcome by the GRIL-seq approach which provides evidence for *in vivo* interactions between sRNAs and their targets. GRIL-seq identified interacting RNAs may represent RNAs that target ArcZ for sequestration or degradation or spurious relationships due to high RNA abundance. To overcome these false-positive results, the *in vitro* ArcZ transcriptome experiment will

supplement the GRIL-seq analysis to verify that ArcZ regulates the identified targets. In this way, these two high-throughput approaches are complementary.

Aim 2 Expected Outcomes

The combined results of Aim 2 will identify several novel targets of ArcZ, each of which regulates a critical virulence factor of *E. amylovora*. For each of these direct targets, confirmation of direct interaction will identify the sequences that interact between ArcZ and the target mRNA. Because the role of each of these virulence factors is known during disease development, identification of the ArcZ target mRNAs regulating motility, exopolysaccharide and biofilm formation and type III secretion will provide insights to the stages of disease development at which ArcZ exerts its regulatory effects on these factors to contribute to virulence.

Aim 3 – What is ArcZ doing inside of a host?

Characterize the effect of ArcZ on the transcriptome during disease development in apple flowers and shoots

This aim addresses both the timing of *arcZ* and ArcZ regulon expression during disease development. I hypothesize that ArcZ expression is highest during early infection of living cells in flowers or leaves, leading to the greatest gene expression differences between wild-type and *arcZ* mutant transcriptomes.

Table 3: 2016 *in planta* transcriptomics samples collected

Flower Samples						
	6h	12h	24h	48h		
stigma		X	X			
flower base	X		X	X		
Strains Included in growth chamber: Ea1189, arcZ, Ea110. Strains Included in field samples: Ea110 on McIntosh and Gala cultivars						
Shoot Samples						
	24h	48h	72h	96h	120h	168h
Leaf	X	X	X	X	X	X
Vein	X	X	X	X	X	X
Pedicel		X	X	X	X	X
Stem				X	X	X
Shoot samples are all strain Ea110 on Gala apple trees.						

Aim 3 Approach

Two separate *in planta* RNAseq experiments will be conducted to characterize the role of ArcZ during infection, both with spatio-temporal resolution. First, wild-type *E. amylovora* strain Ea110 (native to Michigan) will be inoculated onto apple trees under field conditions. These inoculations will be made to both flowers and shoots. Samples will be taken of the stigma, the hypanthium (flower base), and the pedicel of infected flowers at indicated time points (**Table 3**). From inoculated shoots, the following tissues will be sampled: leaf, vein, petiole, and stem (**Table 3)**. Analysis will provide a reference transcriptome during disease development under field conditions that can serve as a baseline not only for this study, but for many future studies. This dataset will be probed for ArcZ expression in all samples and analysis will be made of genes that are co-expressed.

The second *in planta* experiment will be conducted in a controlled environment growth chamber on apple flowers blooming on detached branches maintained in water. This will involve

inoculation of flowers with either wild-type or *arcZ* mutant. This experiment will characterize an *arcZ* regulon that is relevant under disease development conditions in the apple tissues that are most affected by fire blight.

For both *in planta* experiments, triplicates of each sample will be collected. Total RNA will be isolated from samples using a plant total RNA extraction kit (OMEGA Bio-Tek, Georgia), depleted of host RNA using a new kit available for enrichment of microbial RNA (MICROBEnrich Kit; Thermo Scientific, Maryland), and then used for library preparation and sequencing on the Illumina HiSeq 4000 platform.

Combining ArcZ expression data with that of co-expressed genes as well as data on how ArcZ is regulated (Aim 1) and what it is regulating (Aim 2) will provide a thorough characterization of the role of ArcZ in disease development and how the virulence traits it regulates become limiting in the *arcZ* knock-out mutant.

Aim 3 Progress and Preliminary Results

RNA samples for several flower and shoot samples have been collected (**Table 3**), extracted, and quality checked. Twenty-nine samples passed quality checking and were submitted to the MSU genomics core for library preparation and sequencing. Results from these samples will guide modifications to time-points and inoculum concentrations for 2017 experimentation.

Aim 3 Potential Pitfalls and Solutions

Initial extractions did not extract sufficient quantities of good quality RNA from most flower stigmas as well as from leaf, vein, and petiole samples. This year (2017), additional samples will be taken of each tissue at each time point to be able to pool multiple tissue samples in each biological replicate prior to RNA extraction. In the first round of field inoculations, environmental conditions were very dry and therefore not conducive to disease development, which limited the number of samples that could be taken for pooling. If environmental conditions this spring and summer are also unfavorable to disease development, the shoot experiments may be conducted in a growth chamber to enable completion of the experiment, even though field conditions are ideal. A major challenge to this aim will be sampling at appropriate time points to resolve disease developments which occur ahead of visual symptoms. In this way, initial testing of quality samples from 2016 will guide modifications to the time scale of sampling. Additionally, from the 2016 data set, marker genes will be selected to enable rapid qPCR testing to guide sampling in future years to mitigate this challenge.

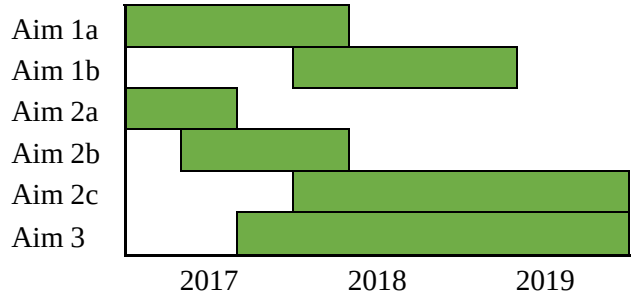
Aim 3 Expected Outcomes

Completion of Aim 3 will provide reference transcriptomic expression data with spatio-temporal resolution of *E. amylovora* gene expression during disease development, both for assessment of the role of ArcZ, but also providing a broad resource to guide future experiments. Additionally, the ArcZ regulon *in planta* will be determined with spatio-temporal resolution. This will enable biological characterization of the role ArcZ is playing *in planta*, and will determine if ArcZ or one of its regulators or interacting mRNAs may be appropriate to target for development of novel control strategies.

Research Summary

The objective of the proposed research is to provide in-depth characterization of the role of the ArcZ sRNA in *E. amylovora*. Aim 1 is targeted at understanding how the sRNA is being regulated under different environmental conditions at both the transcriptional and post-transcriptional level. Aim 2 seeks to identify direct targets of ArcZ that act as regulators of critical virulence traits in *E. amylovora*: flagellar motility, exopolysaccharide production and biofilm formation, and type III secretion. Aim 3 is directed at providing an *in planta* framework for the findings of Aims 1 and 2 that can serve to guide future studies to build off these results to develop novel fire blight control strategies. ArcZ is critical for virulence, and this study will determine the role it plays in interaction with the host through sensing the host environment and mediating regulation of several virulence traits.

Proposed Timeline



Potential Funding Sources

The Sundin lab is currently funded by a USDA-NIFA grant for work outlined in Aim 1 and Aim 2. An MSU Project GREEEN grant was recently obtained to provide funding for the high-throughput sequencing experiments in Aim 2c and Aim 3. It is anticipated that additional funding through renewal or an additional grant can be obtained from USDA-NIFA as this project addresses its goals of researching persistent pests and developing alternative pest management strategies.

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