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# TN*5* MUTAGENESIS REVEALS POTENTIAL MECHANISMS OF METABOLIC PARASITISM OF APPLE FRUIT BY *ERWINIA AMYLOVORA*

A Thesis in

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# ABSTRACT

The gram-negative bacterium *Erwinia amylovora* is the causal agent of fire blight, a destructive disease of apples, pears, and other Rosaceae species. This study seeks to further elucidate the trophic aspects of the host-pathogen parasitic interaction and which metabolic pathways are required for pathogenicity. Auxotrophic mutants of *E. amylovora* were generated via Tn*5* transposon mutagenesis followed by plating mutagenized bacterial cells on a selective minimal media on which auxotrophs could not grow. Forty-seven confirmed auxotrophic mutants were then inoculated onto immature 'Gala' apple fruits in order to evaluate their pathogenicity. The mutated genes were identified by Sanger sequencing of *E. amylovora* DNA flanking the Tn*5* insertion in each auxotrophic mutant. Characterization of transposon insertion sites showed that the following biosynthetic pathways or cellular functions were disrupted: amino acid biosynthesis (19), nucleotide biosynthesis (12), sulfur metabolism (5), nitrogen metabolism (2), survival protein biosynthesis (2), exopolysaccharide biosynthesis (3), and a selection of uncharacterized proteins (4) (the number of mutants of each type is listed in parentheses). We hypothesize that if an auxotrophic mutant is able to cause disease, the mutant must be able to derive the missing metabolites from host tissues. If an auxotrophic mutant is not able to cause disease, this suggests that it cannot derive the missing metabolites from their host. It was determined that the disruption of amino acid biosynthesis, such as for the production of arginine, leucine, and methionine, and nucleotide biosynthesis, such as for the production of purines and pyrimidines, resulted in reduction or elimination of pathogenicity. These two groups of mutants are unable to obtain sufficient amounts of the missing metabolic products from the host tissue in order to complement their metabolism and grow normally. Conversely, mutants with disrupted sulfur metabolism remained pathogenic, indicating that these mutants were able to obtain sufficient amounts of sulfur and sulfur metabolites from the host tissue. In addition, mutants

defective in several survival proteins and exopolysaccharide biosynthesis were identified during screening as possible auxotrophs, and they displayed reduced or absent disease expression. The question of why these mutants are auxotrophs is still being investigated. In summary, this genetic study revealed new details of the profile of pathogen-accessible metabolites in colonizing the host tissues and furthered understanding of which metabolic pathways are needed for disease development.

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# Chapter 1

# Introduction

## 1.1 Rationale and Objectives

*Erwinia amylovora* is the causal agent of fire blight, a bacterial disease of plants in the order Rosaceae that causes significant and ongoing economic losses, particularly in the apple and pear industries. *Erwinia amylovora* was the first bacterial pathogen of plants to be identified over 200 years ago, and was the first plant pathogenic bacterium demonstrated to be transmitted by insects (Baker, 1971; Burrill, 1880). From the time of its first description in the late 18th century until its 1919 discovery in New Zealand, the disease was only found in North America (Jones and Aldwinckle, 1990). Since then fire blight has been described in nearly every major apple producing region across the globe.

Economic losses caused by fire blight are difficult to track, as yearly losses are not reported if they are not on epidemic levels. However economic losses are often greater than meets the eye, as the impact can last up to seven years if trees are a total loss. This is the typical amount of time for newly planted trees to grow to full production level (Bonn and Van der Zwet, 2000). Losses due to fire blight incidences can be measured both as direct perennial crop losses, as well as all costs related to the raising of trees to the age of productivity.

Yearly costs due to fire blight and its control can amount to upwards of $100 million in the U.S. (Norelli et al, 2003). A notably severe epidemic in Michigan in 2000 led to an estimated total economic loss of $42 million for the entire region and losses of 350,000 to 450,000 trees (Longstroth, 2000). *Erwinia amylovora* is an incredibly difficult pathogen to predict and control due to its spread by wind, rain and insects, especially bees and other pollinator species (Miller and Schroth, 1972). Genetics approaches to the control of *Erwinia amylovora* may be a great

solution as current control techniques, such as antibiotics, lose their efficacy or are no longer permitted due to rising environmental and health concerns (Loper et al. 1991; Kümmerer, 2008).

Throughout the 20th century, a great deal of research was conducted on the nutritional needs of *Erwinia amylovora* in laboratory *in vitro* settings (Holt et al., 1994; Starr and Mandel, 1950; Slade and Tiffin, 1978). While this information is integral to optimizing pathogen growth for laboratory research, it tells us very little about the nutritional needs of *Erwinia amylovora in vivo* and tells us nothing about the nature of the host-pathogen relationship in terms of nutrient acquisition and potential parasitism. Further research into the interactions between the pathogen and its apple host tissues *in situ* will enhance the understanding of how disease is shaped by the pathogen's ability to access the nutrient profile within the host. The purpose of this research is to further examine the parasitic relationship of *Erwinia amylovora* to its host plant by utilizing Tn*5* transposon mutagenesis to disrupt at random the biosynthetic pathways that are necessary for prototrophy in *E. amylovora*. This disruption will create auxotrophic mutant strains that may, or may not, be virulent in apple tissues. The predicted function of the disrupted gene(s) can then be used to identify which biosynthetic and functional pathways are affected in the auxotrophs. This information will allow deductions to be made about which metabolites and nutrients *E. amylovora* can acquire from host tissues, based on the virulence phenotype of the auxotroph. Auxotrophic mutants that are able to grow *in planta* are able to extract the missing metabolite(s) from the plant tissues, whereas auxotrophs that cannot grow *in planta* must not be able to extract sufficient quantities of the missing metabolite(s) from the host and must synthesize their own supply *de novo* (Ramos et al., 2015). This will provide insight into how the parasitic interaction of *E. amylovora* with its hosts is shaped by the available nutrients and molecules in the host tissue, and provide information about the physiological status of the plant tissue during infection.

This project aims to address the following major questions:

1. What bacterial biosynthetic and functional pathways must be intact for *E. amylovora* to cause disease in immature apple fruits?
2. What metabolites and nutrients can *E. amylovora* obtain from host tissues, and which metabolites and nutrients are unavailable or insufficiently available to the bacteria growing in the host?

## 1.2 Literature Review

### 1.2.1 Fire Blight

Fire blight, so named because of the burnt appearance of infected leaves and branches, affects plants in the Rosaceae family, including the economically important crops: apple, pear, raspberry, and quince. The host range includes more than 180 plant species across 39 genera within the rosaceous plants (Van der Zwet et al, 2012). The disease occurs in at least 27 countries globally, across four continents.

There are five distinct phases of disease caused by the fire blight pathogen, *Erwinia amylovora*. These types are: blossom blight, shoot blight, canker blight, trauma blight, and rootstock blight (Turechek and Biggs, 2014). The pathogen is able to enter the host plant interior through both wound sites and natural openings, including the nectarthodes, hydathodes, stomata, and lenticels (Heald, 1915; Rosen, 1929; Tullis, 1929).

Blossom blight is generally the earliest symptom to occur in the disease cycle. It is the direct infection of open, intact flowers leading to water-soaking of the petiole and possible infection of the entire blossom-bearing twig or branch, generally via the nectarthodes (Rosen, 1935; Hildebrand, 1937). Infected blossoms appear water-soaked and eventually wilt and turn dark. They may exude ooze from the peduncle under high humidity conditions (Thomson, 1986). Movement from the stigma (the site of initial colonization) to other flower parts is generally

facilitated by rain or dew accumulation, allowing *E. amylovora* to accumulate a relatively large population in the nectaries before spreading to the rest of the tree (Thomson, 1986). This characteristic is important in that it allows the pathogen to multiply on the stigma from a fairly small population up to numbers close to $10^{6-7}$ cells per flower within 1 to 2 days if growth-conducive temperatures occur (Johnson et al., 2006; Slack et al., 2017).

Shoot blight results from a direct infection of the young shoot tips from re-activated nearby overwintered cankers from the previous season or wounding events (Keil et al., 1966). Blighted shoots develop into necrotic "shepherd's crooks", an identifying feature of fire blight where shoots appear burnt and bent into a crook because of rapid collapse of parenchyma tissue. Shoot infections can be induced with surprisingly small numbers of cell. Crosse et al. determined that as few as 35-100 cells could induce cause infection under controlled conditions (Crosse et al. 1972).

Canker blight symptoms develop at the margins of the previous year's cankers as overwintering bacteria re-activate at the start of a new season and serve as the primary inoculum source (Beer and Norelli, 1977). The first symptom to appear is water-soaking along old canker margins which then develop into darkened vascular tissues and eventually spread to nearby shoots and branches. Canker blight re-occurs every season in areas where fire blight is well established. When ooze occurs on the surface of cankers it can be carried to new inoculation sites by rain, ants, and, flies (Thomas and Ark, 1934; Eden-Green, 1972). This ooze, comprised of bacterial cells and exopolysaccharides, serves as primary and secondary inoculum and each droplet has been determined to carry an average of $10^8$ CFU/μl (colony forming units) of bacteria (Slack et al., 2017). Once ooze from the cankers has infected flowers, the pathogen is quickly dispersed to other host tree flowers by pollinators (Steiner, 2000).

Trauma blight is the development of blight on any host tissue caused by the entrance of bacteria to said tissue following trauma such as wind or hail damage, late or early frosts, and in-

season pruning (Turechek and Biggs, 2014). Populations of *E. amylovora* on the leaf tissue are able to opportunistically invade injured foliar tissue after trauma events up to approximately 48 hours post injury (Crosse et al., 1972). This occurrence can have huge potential for leading to epidemic spread. Trauma-induced infections are of particular importance because they are able to induce symptoms in more resistant varieties by circumventing cultivar resistance mechanisms (Steiner, 2000; Suleman, 1992).

Rootstock blight is the development of fire blight cankers just below the graft union after bacteria have moved systemically from the scion into a susceptible rootstock. This canker can eventually girdle and kill the tree. Bacteria reach the graft-union through three methods of transport: washing down the trunk from existing exterior infection sites, infection of suckers, or internal movement via the vascular tissues (Momol et al., 1998).

Fire blight is a polycyclic disease, meaning that the causal pathogen is capable of completing several infection cycles within one growing season. The disease cycle of fire blight can be broken down into four steps (CABI, 2016). First, bacteria arrive on the blossom and proliferate on the floral stigma, eventually entering the vascular tissues through the hypanthium (Hildebrand, 1937). The stigma is the only external site that *E. amylovora* is able to proliferate; it is therefore, not a true epiphyte (Thomson, 1986). Next, as the season progresses, the bacteria continue to enter and proliferate within the host via small wounds on young leaves and shoot tips caused by wind or insects or through natural openings (Crosse et al., 1972). Then, the internal infection continues to spread to healthy shoots, fruits, and branches from the initial sites of infection (Momol et al., 1998). The bacteria move through the intercellular spaces of parenchyma and, later in the disease cycle, probably via the xylem vessels (Van der Zwet and Keil, 1979). During this time the bacteria are also being transmitted to new trees via dispersal of ooze (Thomson, 2000). Finally, as the season comes to a close, the bacteria induce the development of

protective overwintering cankers in the woody tissues, from which primary inoculum emerges again the following season (Rosen, 1929).

The ability of *E. amylovora* to move throughout the various plant nutrient niches is a notable characteristic, one which makes it a fine candidate as a model system for the study of nutrient acquisition. This ability to migrate in the host tissues is not shared by many plant pathogenic bacteria. Other pathogens tend to be localized in parts of the plant, even when the entire plant appears to be affected (Vanneste and Eden-Green, 2000). During blossom infection bacteria tend to occupy mainly the intercellular spaces, and will tend to migrate through the xylem vessels after wound infection (Vanneste, 1995). GFP (green fluorescent protein)-labeled *E. amylovora* were utilized to better track the pathogen movement *in situ* through the xylem and into the parenchyma and root system (Bogs et al., 1998). Stem inoculations of three week old apple seedlings indicated that the bacteria were able to rapidly colonize at least part of the root system. Leaf inoculations led to detection of bacteria mainly in the leaf parenchyma, though some migrated to the xylem vessels. Movement from the vasculature into the parenchyma is not restricted to the site of inoculation. As the pathogen migrated through the vascular tissue, cells had a tendency to break out of the xylem vessels into the intercellular spaces of the parenchyma. Interestingly, it was also discovered that bacteria tended to aggregate around the base of root hairs in the leaf, which apparently helps to create new entryways for the pathogen. Some of this movement may be due to nutrient attractants. The xylem carries nutrients and amino acids, including aspartate, which has been described previously as an attractant for *E. amylovora*, as well as the organic acids, fumarate, malate, maleate, malonate, oxaloacetate, and succinate by the mechanism of positive chemotaxis (Raymundo and Ries, 1980). *Erwinia amylovora* has only one, highly specific chemoreceptor site for this purpose (Raymundo and Ries, 1980).

Fire blight is often spread by wind and rain or vectored by insects, in particular bees and other Hymenopterans. Because one cannot control the weather or suppress pollinator species

populations, the control of fire blight is mostly focused on preventative measures, chemical controls, and exclusion and quarantine methods. Control is further complicated by the fact that once *E. amylovora* has entered the vascular system, foliar application methods of control become ineffective (Psallidas and Tsiantos, 2000). Preventative measures are primarily best cultural practices to reduce the rate of infection, including pruning during the pathogen dormant period, disinfection of all pruning tools, and the prompt removal of infected tissues (Turechek and Biggs, 2014). The benefits of these practices, however, have not been able to compete with the increase in fire blight susceptibility in commercial orchards caused by other cultural practices adopted in the last century (Norelli et al., 2003). The apple and pear industries have shifted toward high-density planting systems, which improve fruit quality but increase fire blight incidence. Orchards are also primarily planted with the M.9 and M.26 rootstocks, which confer the dwarfing that makes high-density planting possible, but are highly susceptible to fire blight. And finally, the most desirable and delicious cultivars are also the most susceptible. Cultural controls must be supplemented with secondary control approaches in order to combat these issues.

Chemical controls generally come from one of four groups of products: antibiotics, copper solutions, growth regulators, and elicitors. These chemicals must be applied three times throughout the growing season in order to successfully control inoculum: when trees are dormant, during bloom, and post-bloom (Psallidas and Tsiantos, 2000). After fire blight has been established chemicals have no demonstrable effect because of the systemic distribution of the bacteria in the plant (Psallidas and Tsiantos, 2000). Antibiotics, most often streptomycin, have historically been the most successful chemical control method as they are less phytotoxic than copper/bordeaux mixtures; however in recent years resistances have rapidly developed to the most commonly used antibiotics such as streptomycin (Loper, 1991). Moreover, these chemicals are often prohibitively costly and have raised some environmental and health concerns (Aćimović et al, 2015).

Research on the use of the growth regulators, prohexadione-calcium and trinexapac-ethyl, has shown that their application can reduce migration of *E. amylovora* in the host tissues by suppressing gibberellin biosynthesis which inhibits vegetative growth, however they require a cost-prohibitively high dosage, lack reliability due to environmental influences, and must be applied prophylactically (Spinelli, et al., 2007).

Some success has also been found in biocontrol research with the application of antagonistic bacterial populations, primarily with strains of *Pseudomonas agglomerans, P. fluorescens,* and some *Pantoea* species (Johnson and Stockwell, 2000). These antagonistic populations are sprayed during bloom and interact with *E. amylovora* on the stigma and hypanthium in order to prevent blossom blight. The short persistence period of the pathogen in the flowers makes it a good candidate for the application of biological controls, since biocontrol populations need only occupy the stigma for around one week in order to be effective. However, this control approach does not always translate as well from the lab and greenhouse to field conditions (Beattie and Lindow, 1994). Antagonistic bacteria can be difficult to apply in the field, though once established have been shown to become partially self-sustaining (Nuclo et al., 1998). Biological control with avirulent strains of *E. amylovora* has been found to have some protective effect, depending on the virulence of the pathogen and the population concentration (Tharaud et al. 1997). The most recent research into biological control involved the use of 12 bacteriophage isolates from *E. amylovora*. These strains were each characterized and, in combination, were found to suppress pathogen growth in lab conditions (Schwarczinger et al., 2017). Over all, biological control approaches have a lot of potential for successful control, especially in combination with other methods.

Genetic controls, including both traditional breeding methods and genetic engineering approaches are at the forefront of fire blight research. Developing plant resistances in fruits is a particularly difficult task for several reasons. First, orchard crops have a long production life,

throughout which pathogen populations can build to destructive levels each season or over the course of many seasons (Lespinasse and Aldwinckle, 2000). Disease resistances must be incredibly stable and durable to combat this, an issue which is not present in other non-perennial crops. Second, *E. amylovora* is able to occupy nearly every possible niche in the host plant. Although certain organs are more strategic for disease prevention, such as the blossom, this ability still presents a challenge in terms of resistance gene expression (Bogs et al., 1998). And last, genetic engineering approaches are not well received by the public. Despite engineering approaches possibly having a higher potential for control than conventional breeding, the public is distrustful of these products and consequently, producers may resist adopting engineered cultivars, even if they are resistant to fire blight (Emeriewen et al., 2017; Hallman et al., 2003).

As of this year, only one functionally proven gene for resistance has been demonstrated in the *Malus* species, although several quantitative loci have been identified (Emeriewen et al., 2017). The more aggressive naturally-occurring strains of *E. amylovora* have already successfully overcome this gene, so a more pyramided approach leading to more durable resistance is needed. Research identifying more quantitative loci from various Malus species is essential for determining the resistance mechanisms that exist in some of these species (Emeriewen et al., 2017; Durel et al., 2009; Le Roux et al., 2010).

Expression of a viral depolymerase gene for degradation of the exopolysaccharide capsule in transgenic apple seedlings resulted in lower levels of colonization in preliminary screens (Hanke et al., 2002). Yet another novel genetic approach involved the development of a cisgenic resistant apple line, where a cisgene from wild apple (*Malus ×robusta)* is inserted into a cultivated apple gene via *Agrobacterium tumefaciens*-mediated transformation (Kost et al., 2015). A cisgenic plant is defined as a plant that has been genetically modified with one or more genes isolated from a crossable donor plant, and contains no transgenes (Schouten et al., 2006).

These novel genetic engineering approaches have only been demonstrated in small-scale laboratory conditions. It remains to be seen how they will perform in mature apple trees in commercial orchard production. These approaches can all be improved as they develop by a better understanding of how *E. amylovora* and the host are interacting on a genetic and metabolic level. Greater knowledge of the *in planta* interaction between *E. amylovora* and its apple hosts will be invaluable to developing genetic approaches for use in the field.

### 1.2.2 *Erwinia amylovora*

*E. amylovora* is the type species of its genus, described as members of the Enterobacteriaceae that are gram-negative, mobile, aerobic to facultative anaerobic, non-sporulating, and associated with plants (Brenner, 1984). The taxonomy of *Erwinia amylovora* is as follows:

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Erwinia*
Species: *amylovora*

The *Erwinia amylovora* bacterium is gram negative, rod-shaped and approximately 0.3 x 1.3 μM in size. Each cell is covered in 2-7 peritrichous flagella. When grown on sucrose nutrient media the colonies are mucoid, domed, and circular (Billing et al, 1960). *E. amylovora* has an obligate requirement for nicotinic acid, to such a degree that it has been proposed as a biochemical test for its identification from other *Erwinia* species (Holt et al., 1994). *Erwinia amylovora* is differentiated from other species within the genus by positive attributes described by Holt et al.: motility, weak anaerobic growth, mucoid growth, reducing substance from sucrose,

production of acetoin (in shaken culture) and liquefaction of gelatin. Though *E. amylovora* can grow utilizing a variety of carbon sources, it prefers sorbitol and grows optimally at 28°C. A great effort was made through the course of the 20th century to describe in detail the biochemical, serological, metabolic, and nutritional characteristics of *E. amylovora in vitro*, and yet its trophic relationship to the host largely remains a mystery. To quote Jean-Paul Paulin: "*E. amylovora* can be considered both as a well-known bacterial species and as a poorly known bacterial plant pathogen" (Paulin, 2000).

### 1.2.3 Bacterial Access to Plant Nutrient Niches

An essential aspect of plant-pathogen interactions is the parasitic nature of the relationship, wherein the pathogen must acquire various biological molecules and nutrients from the host tissues in order to survive and induce disease (Beattie and Lindow, 1995; Fatima and Senthil-Kumar, 2015). All plant pathogenic bacteria are chemoheterotrophs, meaning they must obtain their energy from the metabolism of carbohydrates, amino acids, or other carbon compounds because they are unable to utilize carbon dioxide to form their own organic compounds (Goto, 1992). The plant cell is a nutrient dense environment, therefore one can hypothesize that nutrient availability and the specific nutrient profile of a plant tissue will have a strong influence on bacterial pathogen population dynamics and disease incidence. Nutrients are acquired by: 1) the use of transporters to uptake available nutrients, 2) modifying less available nutrients with extracellular enzymes, 3) secreting effectors via the type III secretion system to modify host cell metabolism, and 4) secreting cell wall degrading enzymes (Delmotte et al., 2009; Jacobs et al., 2012; Gohre and Robatzek, 2008; Barras et al., 1994).

Plants and pathogens are engaged in an evolutionary struggle, with the plant limiting accessibility of nutrients and initiating immune responses, and the pathogen adapting in order to better access nutrients and overcome or escape harsh conditions and immune responses (Rico and

Preston, 2008; Fatima and Senthil-Kumar, 2015). Because nutrients are distributed unevenly throughout plant tissues and within cells, pathogens have further adapted to gain entry to host nutrient niches (Beattie and Lindow, 1995). Initial pathogen presence on the host begins by populating the phyllosphere and rhizosphere, obtaining nutrients from the surface (Crosse, 1959; Bonkowski et al., 2009). The phyllosphere is a site of inhomogeneous nutrient availability, with deposits of nutrients available via leakage from stomata, hydathodes, trichomes or wounds, or deposition of honeydew, pollen, or microbial debris, and the passive leakage of small amounts of metabolites (Leben, 1988; Mew and Vera Cruz, 1986). Because of this, bacteria are more likely to be found in crevices between epidermal cells, near the base of trichomes, in the proximity of stomata, and along veins (Bashan et al., 1981; Blakeman, 1985; Mansvelt and Hattingh, 1987). Eventually, populations may then invade the interior spaces and vascular tissues in search of greater nutrient accessibility and asylum from harsh and variable phyllosphere environmental conditions (Wilson et al. 1999; Mercier and Lindow, 2000).

The primary site of bacterial invasion is the apoplast due to relatively high nutrient availability, though some species will penetrate further into the vascular tissues (Sattelmacher and Horst, 2007; Rico and Preston, 2008). Though nutrients are in greater abundance in this space, they are not as readily available as they are in the phyllosphere and bacteria must adopt strategies to release them from their binding in the region of the cell wall (Rico and Preston, 2008; Rico, 2009; Thornton and Macklon, 1989; Ae and Otani, 1997). Furthermore, the apoplast is slightly acidic and has less water availability compared to the phyllosphere, reducing the ability of bacteria to multiply (Wright and Beattie, 2004; Yu et al., 2013). Some species, such as *Pseudomonas syringae* pv. *syringae*, promote alkalinization in order to boost their ability to multiply in the space. This is accomplished by activating a host plasma membrane $K^+$ efflux/$H^+$ influx exchange, resulting in a pH increase from 5.5 to 7.5 (Atkinson and Baker 1987; Hutchison, 1995). Bacterial pathogens can express specific nutrient utilization pathways to utilize the most

abundant nutrient present in the apoplast. For example, *P. syringae* pv. *tomato* inhabiting the tomato (*Solanum lycopersicum*) apoplast uses GABA (gamma-aminobutyric acid), the most abundant amino acid present in tomato apoplast (Rico and Preston, 2008).

The two vascular tissues, the phloem and xylem, are incredibly different nutrient niches for bacterial invasion. Sugars are loaded from the mesophyll into the apoplast, then from the apoplast into the phloem companion cells and sieve elements (Riesmeier et al., 1994). This phloem sap is an abundant source of sugars and sugar alcohols, organic acids, amino acids, and some minerals (Aldridge et al. 1997; Weibull et al., 1990; Fiehn, 2003). Many bacterial species take advantage of the phloem loading mechanism to acquire their preferred carbon source by producing effectors that target the efflux transporters which are mediated by genes in the SWEET family (Chen, 2014). Pathogen effectors bind directly to the SWEET gene promoter and induce expression, leading to increased sugar efflux from the cytoplasm into the apoplast for bacteria to utilize. By contrast, the xylem tissues carry the lowest amount of available carbon sources of all plant nutrient niches (Press and Whittaker, 1993; Zuluaga et al., 2013). Because the xylem is composed of dead and lignified cells it carries some cell wall degradation products which may be harmful to bacteria (Pieretti, 2012). This niche is a source of low amounts of amino acids, sugars and organic acids, but is an important source for necessary inorganic ions, especially potassium (Canny, 1995; Conti and Geiger, 1982).

Different plant niches are comprised of different levels of nutrients and metabolites, which are of variable availability to invading plant pathogens. In the context of this work, *Erwinia amylovora* has been illustrated to occupy nearly all of the above mentioned nutrient niches (all but for the phloem). As the pathogen progresses through its life cycle and moves through these niches, different metabolites become available or unavailable for use in satisfying metabolic requirements.

### 1.2.4 Iron Acquisition

The unique importance of iron in nearly all biological systems, macro and micro, means that the element has great influence on the relative success of an organismal population. In plant pathogens, iron acquisition basically controls pathogen activity, as the low bioavailability *in planta* dictates how, where, and when bacterial or fungal populations can grow (Expert, 1999). Iron plays an important role in multiple key biosynthetic pathways in all cells, and is a cofactor for numerous proteins and enzymes (Hantke, 2001). Its importance is due to the ability of iron to undergo reversible changes through several oxidation states which only differ by one electron (Culotta and Scott, 2016).

*In planta*, iron has a low bioavailability for pathogens because it is predominantly found as $Fe^{+3}$ ions, which have a very low solubility and are delivered to the various iron-binding molecules upon absorption (Crichton 1991; Lindsay, 1991; Smits and Duffy, 2011). Plants acquire iron through the roots via the following biochemical processes: release of reducing compounds, release of hydrogen ions (making iron more available by lowering soil pH), reduction of $Fe^{+3}$ to $Fe^{+2}$ in the root, and increased production of organic acids, especially citrate (Brown, 1978). Iron is then mobilized in the xylem by citrate or transported in the phloem by the non-proteogenic amino acid, nicotianamine (Brown, 1978; Pich et al., 1997). Once iron has been distributed throughout the plant it must be stored in cell organelles in order to ensure iron availability and prevent toxicity (Culotta and Scott, 2016). At the subcellular level, iron is mainly transported into the chloroplasts (80% of cellular iron), where it is utilized in photosynthesis and heme synthesis, the mitochondria, where it us used in respiration and heme synthesis, and the vacuole, where it is sequestered in order to maintain cytosolic iron levels (Culotta and Scott, 2016). The status of iron availability within the apoplast, where the majority of bacterial populations reside, is still unclear. Long distance iron transport from the root to the leaf cells is mediated by the citrate effluxer, FRD3 (ferric chelate reductase defective 3). Mutants defective in

*frd3* showed iron accumulation in the apoplast and vascular tissues, indicating that FRD3 plays a role in mediating citrate release into the apoplast, which allows iron to move between symplastically disconnected tissues (Roschzttardtz et al., 2011). $Fe^{+3}$ must be reduced in order to be transported into the cell, but a portion of these ions do remain insoluble in the apoplast of the mesophyll cells (Graziano et al. 2002).

Once bacteria have entered the host tissues, they must scavenge iron from the iron-transporting ligands, citrate and nicotianamine, by producing siderophores that will form soluble $Fe^{+3}$ complexes that will then be taken up by active transport mechanisms. Siderophores are low molecular weight compounds (in the range of 400–1,000 Da) with a high affinity for $Fe^{+3}$, which bind and transport iron ions which are then absorbed by the pathogen via highly selective membrane-associated ATP-dependent transport systems (Otto et al., 1992; Köster, 1995; Smits and Duffy, 2011). The manufacture of siderophores and the uptake proteins that recognize them is triggered by the derepression of specific genes as a response to low iron environments. In gram-negative bacteria, *E. amylovora* included, this process is usually a function of proteins in the ferric uptake regulator (Fur) family, encoded by *fur* genes (Hantke, 1981). The low iron concentration causes $Fe^{2+}$ ions to dissociate from repressors that are bound to the DNA upstream from siderophore production genes. The repressor then dissociates from the DNA, leading to gene transcription and subsequent siderophore production and secretion.

The fact that many bacterial plant pathogens are dependent on siderophore iron acquisition systems seems to indicate that iron is not readily available at the sites of infection, primarily the apoplast and xylem vessels, which may indicate that the plant host is utilizing a system of iron-withholding in order to combat pathogen colonization, further evidenced by the fact that iron is never in a readily available form outside of the host intracellular processes (Expert, 1999; Graziano et al. 2002).

The specific iron uptake system of *Erwinia amylovora* is dependent on the production of the major siderophore desferrioxamine-E (DFO-E), although $D_2$, $X_{1–7}$ and $G_1$ are also produced at significantly lower levels (Feistner et al., 1993). The genes that code for the production of DFO and its receptors are clustered together on the *E. amylovora* genome (Dellagi et al., 1998). Ferrioxamines are passed from the environment into the periplasm by the ferrioxamine receptor, FoxR (Kachadourian et al., 1996). Studies on the role of DFO and their receptors in the development of pathogenesis have found that *foxR*-deficient and *dfo*-deficient mutants are significantly less able to colonize floral tissues and cause floral necrosis, resulting in reduced virulence. This indicates that DFO-E, its receptors, and by extension iron itself, are necessary for colonization of flowers and the onset of infection, and that siderophores are important virulence factors (Dellagi et al., 1998). It also indicates that iron is not available for pathogen uptake without this system.

Infectious diseases occur as a result of a competitive interaction between the host and a pathogen. Investigation is underway into disease control methods via iron withholding by the host, utilizing current understanding of biological iron scarcity and high-affinity iron-binding molecules. These concepts may also be applied to nutrient withholding and acquisition in the future. The absorption of metabolites by bacterial pathogens is always parasitic by nature.

### 1.2.5 Tn*5* Mutagenesis

Transposable elements are short nucleic acid sequences that are capable of moving to new locations on a chromosome, without the need for close homology or the activity of the *rec* genes that are required for classical crossing over events (Berg, 1977; Berg and Berg, 1983). They were first described by the pioneering female scientist, Barbara McClintock, for which she won the Nobel Prize in 1983 (McClintock, 1950). They are found naturally occurring in both prokaryotes and eukaryotes (including complex organisms, like corn and humans), where they

cause mutations both at the site of insertion and alterations in the pattern of genes surrounding the site (McClintock, 1950; Mills et al., 2007). In bacteria, transposable elements can be found in the chromosome, plasmids and temperate phages. Bacterial transposable elements, or transposons, encode for a variety of functions, including antibiotic resistance, factors important in pathogenicity, as well as proteins necessary for transposition. Tn*5* is an artificial bacterial DNA transposon of approximately 5.7 kb that is frequently used in *in vitro* experimentation for random gene knockouts and genetic tagging because it is both highly transposable when introduced into a cell and fairly stable once integrated into the chromosome (Shaw and Berg, 1979). The insertion of the Tn*5* transposon is highly random and successful insertion confers resistance to the antibiotic kanamycin, making selection of mutants very simple using antibiotic selection plates. The transposon insertion results in the expression of the incorporated genes and the disruption of a random gene at the point of host chromosome insertion. Tn*5* transposition occurs through a "cut and paste" mechanism catalyzed by a transposase, in which the transposon is excised from the donor DNA segment and inserted into the target DNA at random, resulting in the duplication of 9 base pairs of the target sequence (Reznikoff, 2003). The Tn*5* transposase transposes the DNA segment contained between its 19 base pair mosaic end recognition sequences.

Tn*5* mutagenesis is a useful tool for analysis of metabolic processes because it can be utilized to quickly and easily generate large libraries of random mutants which can then be selected for desirable traits (Shaw and Berg, 1979). The transposon insertion site can be identified without difficulty by plasmid rescue and genetic sequencing because artificial transposons can be excised from the chromosome by cutting with a restriction enzyme that does not cut the mosaic ends (Bruijn, 1987). This allows for a large-scale forward genetics approach to the characterization of gene function across the genome.

### 1.2.6 Nutritional Auxotrophy

Insertional mutagenesis using Tn*5* transposition is a powerful experimental tool for molecular genetic analysis (Shaw and Berg, 1979). One such application is as a knockout mutagen, where the random insertion of the Tn*5* transposon is used to generate a large library of randomly mutated bacteria which can then be selected for the expression of desirable traits, such as auxotrophy (Meade et al., 1982; O'Hoy and Krishnapillai, 1985). Nutritionally auxotrophic strains of bacteria have been used to explore the intricacies of growth and disease requirements for many years, in both animal and plant systems (Bertels et al., 2012; Hoffman and Erbe, 1976; Ramos et al., 2015; Reznikoff, 2003). An auxotrophic strain can be described as a strain that is incapable of synthesizing one or more compounds or molecules that it requires for growth, as compared to a prototrophic strain which is fully capable of producing all of the compounds needed. Previous studies have utilized this method to generate non-pathogenic mutants of plant pathogenic bacteria, including *Agrobacterium tumefaciens, Xanthomonas oryzae* pv. *oryzae,* and *Erwinia amylovora*, however, these studies have been small-scale and did little to illustrate how auxotrophic and non-pathogenic mutants behave *in planta*, if mutant strains were tested in plants at all (Belleman and Geider, 1992; Collens et al., 2004; O'Hoy and Krishnapillai, 1985; Park et al. 2007; Ramos et al. 2014, 2015). Table 1 (below) summarizes some of the previous work on nutritional auxotrophy in plant-associated bacteria that have been tested *in planta*. Table 1 does not represent all of the work done in this regard, but it illustrates the approaches taken and the gaps in knowledge that remain. Much of this work has focused on the relationship of auxotrophy and nodulation in nitrogen-fixing soil bacteria (too many to be summarized in full here). By analyzing the genetic and metabolic causality of impaired nodulation, researchers can develop strains with greater nodulation and nitrogen-fixing capabilities for use in agriculture (diCenzo et al. 2015; de las Nieves Peltzer et al., 2008; Kummer and Kuykendall, 1989). Attempts have also been

made to develop antibiotic resistant strains of rhizobia that are still capable of nodulation at equal or greater levels as the wild type (Pain, 1978).

Only screens of auxotrophic mutants that are very large in scale and include *in planta* inoculations will characterize the trends in the metabolic habits of a pathogen in the context of the host cell. This is a novel approach for the analysis of *Erwinia amylovora* and its relationship to the host. Much of the initial work with nutritional auxotrophy was also performed before the technological capability to determine the exact gene in the biosynthetic pathway that each mutant is auxotrophic for (Belleman and Geider, 1992; Lippencott and Lippencott, 1966). This capability allows for greater understanding of the host-pathogen exchange at the biochemical level and reveals more detail regarding which specific genetic products are not being synthesized.

Table 1. Nutritional Auxotrophy in Plant-Associated Bacteria

| Pathogen | Host | Affected Gene(s) | Gene Product(s) | Phenotype | Literature Source |
|---|---|---|---|---|---|
| *Pseudomonas fluorescens* strain 267 | Red Clover | Not identified | Thiamine; Niacin | Reduced root colonization; lack of plant growth promotion | Marek-Kozaczuk and Anna Skorupska, 2001 |
| *Acidovorax citrulli* | Cucurbits | *hisC* | His | Reduced and delayed disease; No HR in tobacco | Wang et al., 2011 |
| *Xanthomonas oryzae* pv. *oryzae* | Rice | *purD* | Purine | Reduced virulence | Park et al., 2007 |
| *Sinorhizobium meliloti* | Legumes | *proC, smb20003*[1]*; ilvI, ilvC, ilvD2, leuA1, leuC, leuD, leuB*[2] | Pro[1]; Ile, Val, Leu[2] | Impaired symbiosis[1]; Reduced nodulation and infection[2] | diCenzo et al. 2015[1]; de las Nieves Peltzer et al., 2008[2] |
| *Rhizobium leguminosarum* bv. *trifolii* | *Trifolium* spp. | Not identified | Riboflavin | Ineffective symbiosis | Schwinghamer, 1970 |
| *Agrobacterium tumefaciens* | Tobacco[1]; Bean[2] | Not identified[1,2] | Ade, Leu, Cys[1]; Ade, Met, Asp[2] | Growth limitation in culture[1]; Reduced infectivity | Collens et al., 2004[1]; Lippencott and Lippencott, 1966[2] |
| *Erwinia amylovora* | Apple[1,2]; Pear[3] | *argD*[1]*; pyrC*[2]; Not identified[3] | Arg[1]; Pyrimidine[2]; Leu[3] | Nonpathogenicity[1]; No effect on disease[2]; Nonpathogenicity[3] | Ramos et al., 2014[1], 2015[2]; Belleman and Geider, 1992[3] |
| *Bradyrhizobium japonicum* | Soybean | Not identified | His, Leu, Pro, Trp, Ile | Leu and Ile: normal nodulation; His, Pro, Trp: abortive nodules | Kummer and Kuykendall, 1989 |

### 1.2.7 *Erwinia amylovora*-Host Interactions

Much of the *Erwinia amylovora*-apple interaction is dependent upon and influenced by the ability of the pathogen to induce fire blight disease. It is now understood that this ability is predicated on the expression of the *hrp* type III secretion system, secretion of the type III effector DspA/E, and the production of the exopolysaccharide amylovoran (Malnoy et al., 2012).

The application of transposon-mediated mutagenesis for the deduction of genes required for *Erwinia amylovora* revealed the existence of several classes of genes involved in pathogenesis. The largest of these classes was comprised of genes that are required for both pathogenesis and the induction of the hypersensitive reaction in non-host plants (Willis et al., 1991). These genes were named *hrp* genes, for HR and pathogenicity. The second group was found to be required for pathogenicity but not for HR and were designated *dsp* because of their 'disease-specific' function (Barny et al. 1990). The final group of genes was named *ams* as they are required for the synthesis of amylovoran, which is a virulence factor in *E. amylovora* (Ayers et al. 1979).

The *E. amylovora hrp* and *dsp* genes are essential for the induction of disease. These two sets of genes are clustered in the genome within the Hrp pathogenicity island (PAI) (Oh et al., 2005). Like many other gram-negative bacteria, *E. amylovora* utilizes a type III secretion system (TTSS) to release effector proteins into host cells in order to manipulate the cellular machinery and induce disease. The main role of *hrp* genes is to encode the proteins that comprise and regulate the TTSS. The Hrp PAI contains three type III secretion operons (*hrpA, hrpC* and *hrpJ)* and three regulatory operons (*hrpXY, hrpS* and *hrpL)*(Oh et al., 2005). The expression of the genes in these operons is activated by the perception of plant apoplast conditions (or simulacra of said conditions), including pH, temperature and carbon and nitrogen levels (Wei et al. 1992). The induced signal cascade generates the Hrp type III secretion system which forms the basis for all *Erwinia* species pathogenesis (Frederick et al., 1993; Nizan et al., 1997). The TTSS is constructed

around the major protein HrpA, which forms the structure of the external pilus. From the pilus harpins, such as HrpW and HrpN, and effectors, such as DspA/E, are injected into the host (Wei et al., 1992). These proteins and their complex interactions are essential to *E. amylovora* pathogenicity by disrupting plant metabolism, components of the cell, or signaling pathways (Acsoy et al., 2017; Boureau et al., 2006; Bocsanczy et al., 2008). To date, 12 proteins have been described that are excreted by the TTSS (Nissinen et al., 2007).

*Erwinia amylovora* has two *dsp* genes (*dspA/E* and *dspB/F*) which are clustered neighboring the *hrp* cluster within a two gene operon (Bogdanove et al., 1998). The secretion of the DspA/E effector protein via the TTSS is dependent on DspB/F, which acts as a highly specific chaperone (Gaudriault et al., 2002). DspA/E function is essential for pathogenesis partially because it blocks callose deposition, which strengthens cell walls at the site of infection as a form of basal defense, which is mediated by salicylic acid (DebRoy et al., 2004). Four serine/threonine protein receptor kinases from the apple host have been identified as DspA/E-interacting proteins (Meng et al., 2006). These kinases interact directly with DspA/E and are highly conserved across apple cultivars, indicating targeting potential for new genetic control approaches. The role of DspA/E was also investigated via the expression of the β-glucuronidase (GUS) reporter system in transgenic *Arabidopsis thaliana* seedlings (Aksoy et al., 2016). Comparison of a mutant *ΔdspA/E* strain of *E. amylovora* to the wild type by monitoring of GUS activity indicated that expression of *dspA/E* is required to induce HR in non-host plants like *A. thaliana*.

The harpins, HrpN and HrpW, are glycine-rich proteins lacking in cysteine, that are involved in the induction of HR in non-hosts (Wei et al., 1992). HrpN is required for full virulence in the plant. More specifically, it has been shown that the C-terminal half of HrpN is essential for its secretion by *E. amylovora*, for its virulence activity on apple and pear (Sinn et al., 2008). Conversely, HrpW has not been shown to have a virulence function, in fact *hrpW* mutants

were equally as pathogenic as the wild type and acted as a negative HR effector in the non-host (Barny et al., 1999).

The bacterial ooze synthesized by *E. amylovora* is primarily comprised of the polysaccharides, amylovoran, a pathogenicity factor, and levan, a virulence factor (Ayers et al., 1979). Exopolysaccharides protect bacterial populations from desiccation, but are also thought to play a role in facilitate multiplication and movement through the host, and bypass the host plant defense systems (Belleman and Geider, 1992; Bogs et al., 1998; Ordax et al., 2010). Amylovoran mutants are fully non-pathogenic and are unable to multiply or move in the host, which emphasizes the essential role amylovoran plays in *E. amylovora* virulence. The 12 *ams* genes that code for amylovoran production are arranged on a single operon, controlled by the regulatory proteins RcsA and RcsB (Bugert and Geider, 1995). Higher amylovoran production is also positively correlated with greater virulence (Lee et al., 2010).

Amylovoran is the main factor in biofilm formation by *E. amylovora* (Koczan et al., 2009). The formation of biofilms is advantageous for bacteria because it allows communication easily via quorum sensing, enables greater nutrient acquisition, and creates a degree of protection from environmental factors that is not possible for a planktonic bacterium (Ramey et al., 2004). Biofilm formation is also positively correlated with bacterial virulence in planta (Lee et al., 2010). Biofilms have been demonstrated to form in planta and may play an active role in both pathogenesis and xylem colonization (Koczan et al., 2009; 2011).

A genome-wide examination of gene expression patterns during in vivo infection of pear fruits to uncover pathogenesis strategies of the organism, described 394 genes that were active during the process of infection (Zhao et al., 2005). These genes were identified based on sequence homology and divided based on putative function. Most notable in the context of this work is that 20.3% of the genes were involved in metabolism and 15.5% were involved in nutrient

acquisition. This clearly illustrates that our understanding of *Erwinia amylovora*, its relationship to the host, and its ability to derive resources from the host tissues, is far from complete.

### 1.2.8 Auxotrophy of *Erwinia amylovora* Illustrates Details of the Host Interactions

This work is directly built upon the work of a previous graduate student in the McNellis laboratory, Laura Ramos. Her work focused on the characterization of a pathogenic and a non-pathogenic auxotrophic *E. amylovora* mutant and the implications of those phenotypes in the context of the host tissue.

An arginine auxotroph with the Tn*5* insertion in the *argD* gene exhibited complete non-pathogenicity in apple and reduced pathogenicity in pear (Ramos et al., 2014). This indicates that *E. amylovora* is unable to obtain enough arginine from immature apple and pear tissues in order to induce disease development. The characterization of the *argD* mutant strain also facilitated the development of a novel plasmid that can be stably maintained in pathogen cells populating host tree tissues over extended periods of time. Long-term stable plasmids in host tissues had previously only been demonstrated in animal pathogenic systems.

The other mutant strain was a pyrimidine auxotroph with the Tn*5* insertion in the *pyrC* gene. Interestingly, this mutation had almost no effect on *E. amylovora* pathogenicity, indicating that there are sufficient supplies of pyrimidine in apple and pear tissues for the pathogen to function normally and induce full symptoms (Ramos et al., 2015).

The juxtaposition of these two mutant strains demonstrates the variability of the effects of auxotrophy on bacterial fitness. Furthermore, it sheds some light on the parasitic interaction of *E. amylovora* with the host. Some amino acids, molecules, and compounds can be acquired from the host, while others must be manufactured by *E. amylovora de novo*. Discovering which molecules are or are not available from the host will be an essential part of understanding the host-pathogen relationship as it occurs *in situ*. There remains a gap in the scientific knowledge regarding plant

pathogenic bacteria and their metabolic relationships with the host, relationships based on complex parasitic approaches and adaptions. *Erwinia amylovora* is an attractive model system for the study of host-pathogen interactions. It has the fascinating ability to occupy and migrate freely throughout the majority of the host plant nutrient niches. It is an easy pathogen to culture in the laboratory setting and its genome is easy to manipulate and well understood. As a cousin to well-studied animal pathogens like *Escherichia*, *Salmonella*, and *Shigella*, there are many resources for the comparison of genomic or metabolic traits. This work is a genome-wide, forward genetics approach to understanding the parasitic nature of the interactions between *Erwinia amylovora*, the fire blight pathogen, and its apple host.

# Chapter 2

# Methodology

## 2.1 Research Design

The goal of this research was to utilize a forward genetics approach to determine the essential biological molecules (metabolites) that *Erwinia amylovora* can and cannot obtain from the apple host tissues by analysis of how mutants with different biosynthetic pathways affected by the Tn*5* insertion express varying pathogenic phenotypes. In order to answer the objective questions (see Introduction pg. 2) a large pool of *E. amylovora* mutants were generated via Tn*5* mutagenesis, selected for auxotrophy with a selective media, confirmed as auxotrophic in a liquid selective media, and tested for virulence in immature apple fruits. Finally, the site of the Tn*5* insertion was by determined by isolating the genomic DNA from each mutant, cutting the genomic DNA with a restriction enzyme, ligating the DNA to itself, and inserting it as an artificial plasmid into electrocompetent *E. coli*. Circularized DNA containing the Tn*5* transposon behaves as a replicating plasmid conferring kanamycin resistance, and this process is called plasmid rescue. The rescued plasmid obtained from each auxotrophic mutant was then sent to the Genomics Core Facility at The Pennsylvania State University, University Park campus for Sanger sequencing of the *E. amylovora* DNA flanking the Tn*5* insertion (Fig. 1).

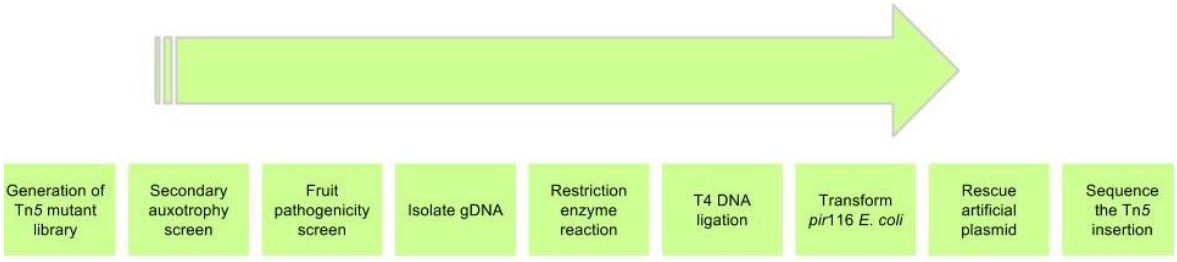


Figure 1. Work flow diagram.

**2.2 Data Collection Techniques**

The mutant library was generated via electroporation of an engineered Tn*5* transposon into a sample of electrocompetent *E. amylovora*, Pennsylvania strain HKN06P1, according to the manufacturer's instructions (EZ-Tn5<R6K γoriKan-2> transposome kit; Epicentre, Madison, WI). The mutant library was then stored in a suspension of 15% glycerol in a cryovial at -80°C from which subsamples were subsequently screened for auxotrophy on a selective media with kanamycin.

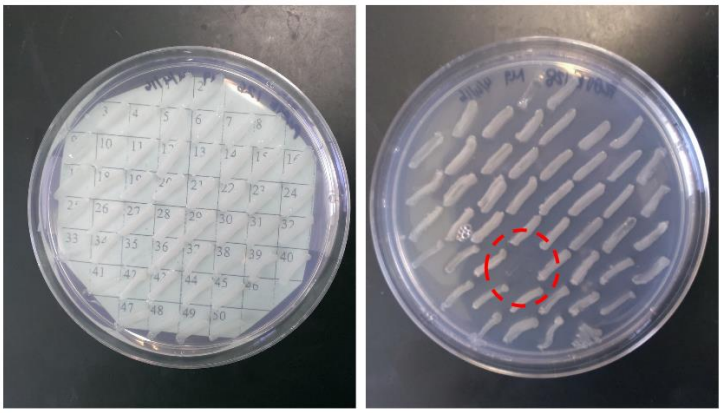

Figure 2. Fifty Tn*5* mutants screened on an LB Kan50 plate with grid (left); an auxotrophic mutant (red) did not grow on the M9 minimal media (right).

The preliminary auxotrophy screen was done by inoculating 100 µL of liquid Lysogeny broth (LB) with a loopful of bacteria from the frozen mutant library and spreading on an LB plate with 50 µL/mL kanamycin (Kan50) plate using the Copacabana method of spreading aqueous solutions on an agar plate surface with sterilized glass beads, incubated at 28°C for 2 days then stored at 4°C while in use. LB media consists of 10g NaCl, 20g tryptone, 15g agar, and 5g yeast extract per liter of water. Individual colonies of mutant bacteria were then transferred with a sterile toothpick to two comparative grid plates, one an LB Kan50 plate, the other an M9 minimal selection media plate and incubated overnight at 28°C (Fig. 2). Mutants with an auxotrophic phenotype (that did *not* grow on the M9 minimal media) were transferred from the LB Kan50

plate, suspended in sterile 15% glycerol and stored at -80°C. M9 media consists of M9 salts ($Na_2HPO_4$-$7H_2O$, $KH_2PO_4$, NaCl, and $NH_4Cl$), agar, $MgSO_4$, sorbitol, nicotinic acid, and $CaCl_2$.

Mutants identified as auxotrophic in the primary screen were then rescreened, this time in a liquid M9 minimal media supplemented with 50µg/ml kanamycin in order to confirm their auxotrophic status (Fig. 3). This was done both as a secondary confirmation and because the suspension of bacterial cells in liquid media assures that they are coming into contact with the media and antibiotic. Cells were transferred directly from the frozen glycerol suspension on a sterile toothpick in a roughly "colony-sized" amount (non-quantified) into 2 mL of liquid M9 plus

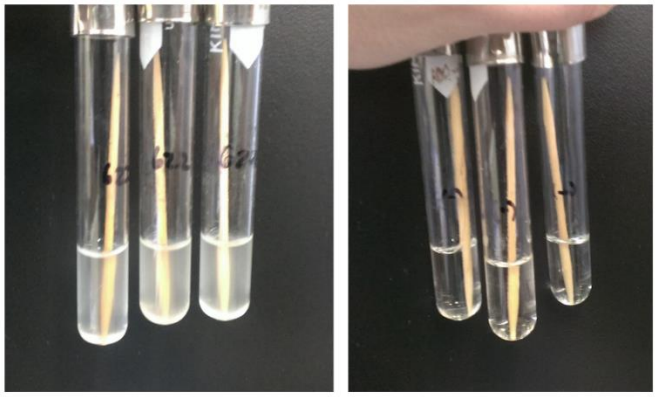

Figure 3. Experimental replications of a Prototrophic mutant (left) and an auxotrophic mutant (right) from secondary auxotrophic screen.

kanamycin (M9Kan50) broth and incubated in a shaker at approximately 250 rpm overnight at 28°C. Three replications were done for each mutant strain and strains that appeared to be partially auxotrophic, where only one or two of the tubes had bacterial growth, were subsequently re-tested.

The protocol for the secondary screen was later determined to be inefficient for the confirmation of mutants as auxotrophic as it allowed for many false positives (i.e. prototrophic mutants falsely described as auxotrophic). The transfer of bacteria directly from the frozen suspension yields a less consistent number of bacteria than transfer from bacteria grown on agar

plates so this assay was re-screened, resulting in a far smaller collection of auxotrophic mutants. For the rescreen, potentially auxotrophic mutants were streaked out on LB Kan50 agar plates with a sterile loop and incubated for 2 days at 28ºC. An approximated "colony-sized" amount of bacteria was then transferred into 2 mL of M9 minimal media plus kanamycin with a sterile toothpick and incubated in a shaker at approximately 250 rpm overnight at 28°C. Two replications were done per mutant along with a known prototroph as a positive control and a mock inoculation with a sterile toothpick as a negative control. Mutants rated as prototrophic were discarded, while those perceived as fully or partially auxotrophic were kept for the fruit pathogenicity screen.

Mutant strains that were confirmed as auxotrophic were then inoculated into immature 'Gala' apple fruits harvested from the Fruit Research and Extension Center in Biglerville, Pennsylvania, and incubated in a high humidity environment for 7 days at 22ºC (Fig. 4). It has been demonstrated that symptom development in immature apple fruits are a reliable indicator of *E. amylovora* virulence in apple trees (Lee et al. 2010). Daily qualitative symptoms were recorded based on the presence of water-soaked tissue, bacterial ooze from the lenticels or inoculation point, or necrotic tissue. The apples were prepared by surface disinfestation in 10% bleach for 10 minutes then rinsed 4-5 times with tap water. Then the blossom end was removed, the apple cut in half, and a small hole made on the upper surface with a sterile pipette tip. Two

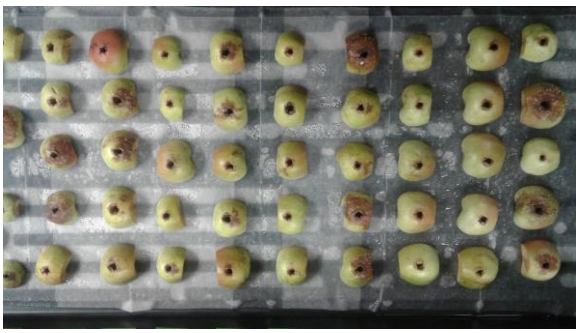

Figure 4. Pathogenicity assay in immature 'Gala' apple halves.

replications for each mutant strain were inoculated into the apple halves using sterile toothpicks from 2 day cultures on LB Kan50 plates at a non-quantitative rate of about “one colony” worth. The apples were arranged on vellum sheets sterilized with 70% EtOH over damp paper towels and stored in trays covered with humidity domes in a Conviron plant growth chamber in order to maintain high humidity for optimal bacterial growth. To maintain humidity distilled water was added daily to the damp paper towels and sprayed on the inside surface of the humidity domes.

Because the fruit pathogenicity screen results were qualitative rather than quantitative, it was decided that a re-screening of the mutants with a larger pool of experimental replicates would be more indicative of the effects of the Tn*5* insertion on symptom development. Furthermore, a new numerical rating system, rather than a descriptive one, would generate data that could be analyzed statistically. Five replicates per mutant were inoculated into immature ‘Gala’ apple fruits as per the previously described methods. The symptoms were then rated seven days post-inoculation on a 0 to 5 scale (Table 2). It was determined that the previous method of rating daily was unnecessary and that rating fruits at seven days post-inoculation was representative of full symptom development before secondary opportunistic infections began.

Table 2. Auxotrophic Tn*5* Mutant Symptom Rating Metric

| **Numerical Rating** | **Description of symptom development** |
|---|---|
| 0 | 0% coverage: No symptoms present |
| 1 | 10-15% coverage: Minor water-soaking and possible oozing from lenticels near the inoculation point |
| 2 | Up to 25% coverage: Water-soaking, if present, covers 25-30% of the apple surface; ooze from lenticels covers approximately 25% of the surface; possible minor necrosis around the inoculation point |
| 3 | Up to 50% coverage: 30-50% of the apple surface is producing ooze; major water-soaking covering approximately 50% of the surface; 25-50% of the tissue has necrotized |
| 4 | 75% coverage: Severe water-soaking covering upwards of 75% of the surface; 50-80% necrotic tissue and significant oozing |
| 5 | 90%+ coverage: Nearly the entire surface of the apple shows symptoms; may be fully water-soaked; major oozing and ooze may have filled the inoculation hole; greater than 75% necrotic tissue |

Tn*5* plasmid rescue was performed for all auxotrophic mutants, regardless of their pathogenicity, and sequencing was performed in order to determine the location of the Tn*5* transposon insertion. First, genomic DNA (gDNA) was isolated from each mutant strain (Wizard Genomic DNA purification kit, Promega, Madison, WI). The isolated gDNA concentration was measured using a Nanodrop 2000 spectrophotometer and samples with a high concentration of DNA (>120 μL/ mg) and low impurities (260/280 and 260/230 of ~2.00) were then cut using a restriction enzyme that does not cut the Tn*5* transposon insertion, such as *Eco*RI or *Eco*RV. The restriction enzyme product was then self-ligated using T4 DNA Ligase and transformed into *pir*116 electrocompetent *Escherichia coli* cells at 2,500 V and grown overnight at 37°C on an LB Kan50 plate. If any colonies grew, indicating a successful transformation, an individual colony was inoculated into 2.5 mL LBKan50 broth and incubated in a shaker at approximately 250 rpm overnight at 28°C. A miniprepartion was then performed to isolate the inserted artificial plasmid from this culture (E.Z.N.A.® Plasmid Mini Kit I, Omega Bio-Tek, Norcross, GA) and sent to the Penn State Genomics Core Facility at the Huck Institutes of the Life Sciences for Sanger sequencing.

# Chapter 3

## Results

10,750 total mutants from the generated Tn*5* mutant library were screened for auxotrophy over the course of this work, compared to the approximated 20,000 needed to represent genetic saturation, wherein every gene can be assumed to have been affected by the Tn*5* insertion. Genetic saturation was calculated to determine the amount of Tn*5* mutants to screen in order to have over 99.5% likelihood that the transposon was inserted into every gene. This calculation was based on the equation demonstrated in Figure 5. The initial auxotrophy confirmation screen, which had allowed for a high number of false negatives, resulted in 213 auxotrophic mutants (a rate of approximately 1.9%). The rescreening of these mutants with the refined protocol narrowed this pool to 47 confirmed auxotrophic mutants, for a final rate of 0.4%.

$$P = 1 - (1 - [X/G])N$$

P = Probability of finding an insertion in a given gene
X = Average gene size in E. amylovora (in base pairs)
G = Genome size of E. amylovora (in base pairs)
N = Number of insertion events in the screened population

Calculated from the literature (Krysan et al., 1999; Bocsanczy et al., 2008; Smits et al., 2010):

$$P = 1 - (1 - [939/3{,}805{,}874])20{,}000, \text{ or } P = 0.997$$

Figure 5. Equation for the determination of theoretical genetic saturation.

### 3.1 Sequence Data

The results of the plasmid rescue and sequencing indicated that the auxotrophic mutants each bore the transposon insertion at some point along one of seven different metabolic or functional pathways: amino acid biosynthesis, nucleotide biosynthesis, nitrogen metabolism, sulfur metabolism, exopolysaccharide biosynthesis, survival protein biosynthesis, or encoding a

hypothetical or as yet undescribed protein (Fig. 6). All of the sequences received from the Genomics Core Facility were analyzed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) for nucleotide sequence comparison to known genomes, against the *Erwinia amylovora* CFBP1430 complete genome sequence, which has historically been the standard strain in many genetic and biochemical studies (Gaudriault et al. 1997; Dellagi et al. 1998).

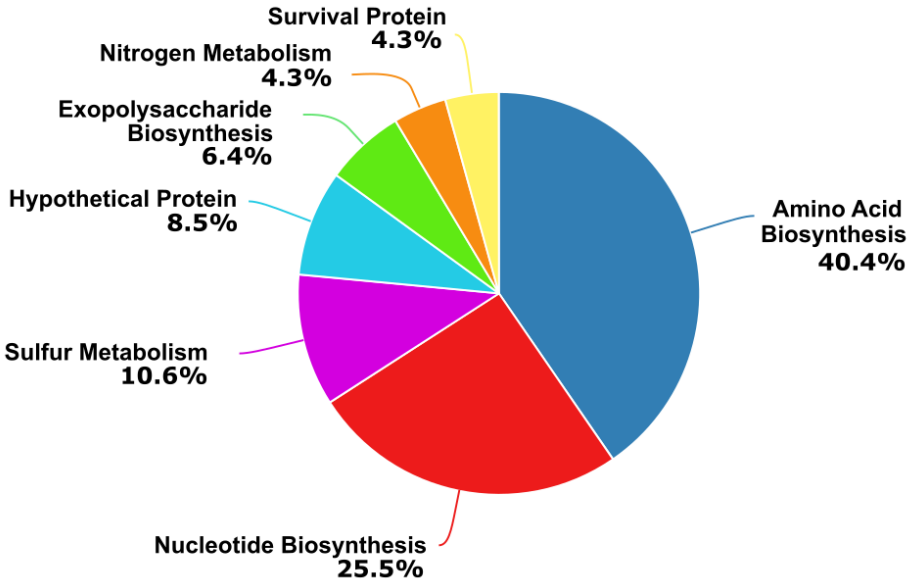


Figure 6. Distribution of pathway types affected by the insertion of the Tn*5* transposon.

### 3.2 Pathogenicity Data

Over the course of the winter of 2016, about 50 of the original confirmed auxotrophic mutants were rated for pathogenicity in immature 'Gala' apples based on the initial descriptive daily rating system, however these results were not reliable due to the effects of long-term cold storage on the immature apples, in addition to the decision to create a more refined rating system. After so many months in the cold room physiological changes in the apples coupled with a high rate of secondary fungal infection skewed the rate of pathogenicity in the assay. All of the pathogenicity assays were redone in the spring of 2017 using the scaled rating metric (Table 2)

and five replications, rather than two per mutant. The entirety of the pathway and phenotype data can be found in the Appendix.

A very high majority of the auxotrophic mutants had reduced pathogenicity (61.7%) or were completely asymptomatic (31.9%) in the pathogenicity fruit screens, as could be expected after disabling major biosynthetic pathways. This means that the pathogenicity was affected in approximately 93.6% of the auxotrophic mutants screened.

Table 3. Distribution of Pathogenicity Phenotypes by Affected Pathway

| **Affected Pathway or Gene Type** | **Pathogenicity Phenotype** | | | **Total** |
|---|---|---|---|---|
| | **Asymptomatic** | **Reduced Pathogenicity** | **Fully Pathogenic** | |
| Amino Acid Biosynthesis | 7 | 12 | 0 | 19 |
| Nucleotide Biosynthesis | 5 | 7 | 0 | 12 |
| Exopolysaccharide Biosynthesis | 1 | 2 | 0 | 3 |
| Sulfur Metabolism | 0 | 2 | 3 | 5 |
| Nitrogen Metabolism | 0 | 2 | 0 | 2 |
| Survival Protein | 1 | 1 | 0 | 2 |
| Hypothetical Protein | 1 | 3 | 0 | 4 |
| Total | 15 | 29 | 3 | 47 |

Most of the transposon insertion sites were in the biosynthesis pathways for either amino acids or nucleotides. Approximately 40% of the generated mutants were auxotrophic for an amino acid, which makes sense in the context of plant cellular metabolism. Only seven of the 20 proteinogenic amino acids were represented: cysteine, methionine, tryptophan, arginine, leucine, threonine, and histidine, as well as the pathways encoding precursors for the branched-chain amino acids (leucine, isoleucine, and valine) (Table 3). All five of the insertions into the arginine biosynthesis pathway were asymptomatic.

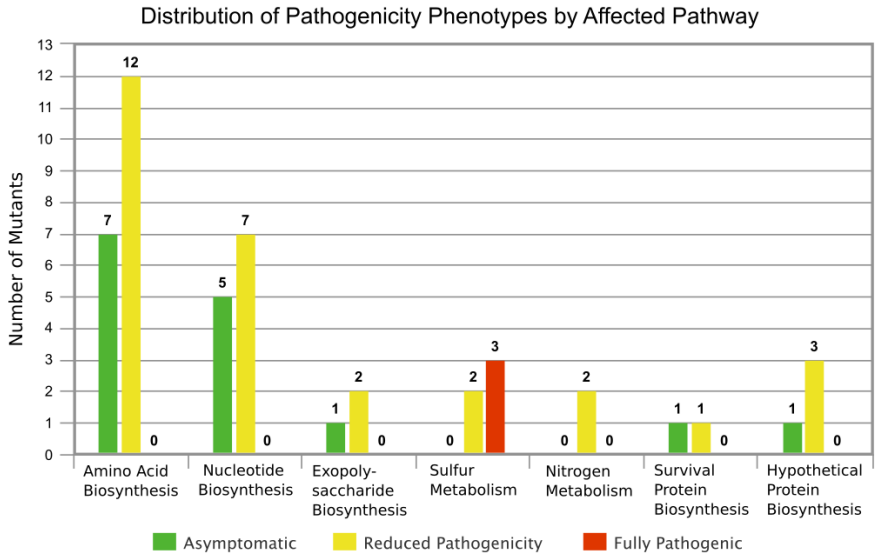


Figure 7. Variations in pathogenicity phenotype are divided by the biosynthesis pathway type.

Table 4. Mutants Auxotrophic for Amino Acid Biosynthesis

| **Mutant #** | **Affected Gene** | **Gene Product(s)** | **Biosynthesis Pathway** | **Disease Phenotype** |
|---|---|---|---|---|
| M161.21 | *argD* | Acetylornithine/ succinyldiaminopimelate aminotransferase | Arginine | Asymptomatic |
| M154.46 | *argE* | Acetylornithine deacetylase | Arginine | Asymptomatic |
| S1.36 | *argG* | Argininosuccinate synthase | Arginine | Asymptomatic |
| M131.23 | *argH* | Argininosuccinate lyase | Arginine | Asymptomatic |
| S7.13 | *argH* | Argininosuccinate lyase | Arginine | Asymptomatic |
| M163.4 | *cysQ* | Inositol-1-monophosphatase | Cysteine | Reduced |
| M129.18 | *hisD* | Histidinol dehydrogenase | Histidine | Reduced |
| M105.46 | *ilvC* | Ketol-acid reductoisomerase | Branched-chain amino acids (leucine, isoleucine, valine) | Reduced |
| M123.10 | *ilvC* | Ketol-acid reductoisomerase | Branched-chain amino acids | Reduced |
| M4.47 | *ilvE1* | Branched chain amino acid aminotransferase | Branched-chain amino acids | Reduced |
| M140.10 | *leuB* | 3-isopropylmalate dehydrogenase | Leucine | Reduced |
| M160.2 | *leuB* | 3-isopropylmalate dehydrogenase | Leucine | Asymptomatic |
| M134.35 | *leuC* | 3-isopropylmalate dehydratase large subunit | Leucine | Asymptomatic |
| M106.50 | *mdeA1* | Methionine gamma-lyase | Cysteine and methionine | Reduced |
| M129.46 | *metA* | homoserine transsuccinylase | Methionine | Reduced |
| M157.1 | *metB* | Cystathionine gamma-synthase | Methionine | Reduced |
| M157.42 | *thrB* | Homoserine kinase | Threonine | Reduced |
| M126.35 | *trpB* | Tryptophan synthase beta chain | Tryptophan | Reduced |
| M198.17 | *trpE* | Anthranilate synthase component I | Tryptophan | Reduced |

Twelve of the mutants were auxotrophic for a nucleotide, for a rate of 25.5% (Table 5). No bias toward purines versus pyrimidines was identified. Seven were in the pyrimidine biosynthesis pathway, and five were in the purine biosynthesis pathway. The transposon insertion into the *pyrD* gene occurred four times. Of these, three had reduced pathogenicity, and one was asymptomatic.

The remaining insertion pathway types followed the same trend of reduced or asymptomatic pathogenicity as in the amino acid and nucleotide biosynthesis mutants (Tables 5-7), with the exception of mutants auxotrophic for the sulfur metabolism pathway. Though only five mutants were in this group, they represent an interesting variation from the trend (Table 6). Three were fully pathogenic, while two had reduced pathogenicity. Mutants occurred in several unexpected pathways, as well (Table 7). These mutants were either for hypothetical or uncharacterized protein synthesis or were in pathway types that were not expected to result in an auxotrophic phenotype.

Table 5. Mutants Auxotrophic for Nucleotide Biosynthesis

| **Mutant #** | **Affected Gene** | **Gene Product(s)** | **Biosynthesis Pathway** | **Disease Phenotype** |
|---|---|---|---|---|
| M5.4 | *carA* | Carbamoyl-phosphate synthase small chain | Pyrimidine and Arginine | Asymptomatic |
| M.6.45 | *purA* | Adenylosuccinate synthetase | Purine | Asymptomatic |
| M110.31 | *purA* | Adenylosuccinate synthetase | Purine | Reduced |
| M128.36 | *purK* | Phosphoribosylaminoimidazole carboxylase ATPase subunit | Purine | Asymptomatic |
| M36.1 | *purU* | Formyltetrahydrofolate deformylase | Purine | Reduced |
| M158.18 | *purU* | Formyltetrahydrofolate deformylase | Purine | Reduced |
| M191.44 | *pyrB* | Aspartate carbamoyltransferase catalytic subunit | Pyrimidine | Asymptomatic |
| M46.31 | *pyrD* | Dihydro-orotate dehydrogenase | Pyrimidine | Asymptomatic |
| M129.28 | *pyrD* | Dihydro-orotate dehydrogenase | Pyrimidine | Reduced |
| M142.31 | *pyrD* | Dihydro-orotate dehydrogenase | Pyrimidine | Reduced |
| M156.9 | *pyrD* | Dihydro-orotate dehydrogenase | Pyrimidine | Reduced |
| S5.45 | *pyrD* | Dihydro-orotate dehydrogenase | Pyrimidine | Reduced |

Table 6. Mutants Auxotrophic for Nitrogen and Sulfur Metabolism

| Mutant # | Affected Gene | Gene Product(s) | Biosynthesis Pathway | Disease Phenotype |
|---|---|---|---|---|
| M155.32 | *gltB* | Glutamate synthase (NADPH) | Nitrogen metabolism | Reduced |
| M158.40 | *gltB* | Glutamate synthase (NADPH) | Nitrogen metabolism | Reduced |
| M5.1 | *cysN* | ATP sulfurylase | Sulfur metabolism | Pathogenic |
| M45.2 | *cysI3* | Sulfite reductase (NADPH) flavoprotein beta-component | Sulfur metabolism | Reduced |
| M131.39 | *cysI1* | Sulfite reductase (NADPH) flavoprotein alpha-component | Sulfur metabolism | Pathogenic |
| M148.15 | *cysI1* | Sulfite reductase (NADPH) flavoprotein alpha-component | Sulfur metabolism | Pathogenic |
| M151.13 | *nuoG* | NADH dehydrogenase I chain G | Sulfur metabolism | Reduced |

Table 7. Unexpected Mutants: Mutants Auxotrophic for Exopolysaccharide Biosynthesis, Survival Proteins, and Uncharacterized Proteins

| Mutant # | Affected Gene | Gene Product(s) | Biosynthesis Pathway | Disease Phenotype |
|---|---|---|---|---|
| M150.35 | *amsB* | Glycosyltransferase amsB | Exopolysaccharide biosynthesis | Reduced |
| M16.4 | *amsC* | Exopolysaccharide biosynthesis protein | Exopolysaccharide biosynthesis | Reduced |
| M10.29 | *amsE* | Putative glycosyltransferase | Exopolysaccharide biosynthesis | Asymptomatic |
| M11.31 | *EAMY_ 3259* | Hypothetical protein | Hypothetical | Reduced |
| M36.10 | *EAMY_ 3259* | Hypothetical protein | Hypothetical | Asymptomatic |
| M92.41 | *EAMY_ 3259* | Hypothetical protein | Hypothetical | Reduced |
| M8.17 | *surA* | Survival protein SurA precursor | Survival | Asymptomatic |
| M105.9 | *surA* | Survival protein SurA precursor | Survival | Reduced |
| M206.13 | *yibP* | Uncharacterized protein | Uncharacterized | Reduced |

# Chapter 4

## Discussion

This work reveals several common recurrences in affected biosynthetic pathways, leading to greater understanding of how nutrient acquisition plays a role in disease development in the context of the host tissues. These findings can provide a more comprehensive view of the host metabolites that are accessible to *E. amylovora*, which is poorly understood at this time. Although the ability to derive metabolites directly from the host tissues when a biosynthetic pathway is disabled does not necessarily indicate that the pathogen will parasitize the host tissues in this way under normal disease conditions, this study reveals greater details about the complex host-pathogen interactions that potentially occur. Biosynthetic pathways are highly regulated, such that metabolites may only be generated when concentrations are low. This study may indicate new details of the profile of available metabolites in the host tissues and how that influences bacterial biosynthetic processes as disease develops. As the bacteria colonize host tissues they may forgo certain biosynthetic pathways in favor of the absorption of intermediates from the host tissues. The potential metabolic parasitism occurring between the pathogen and host is a fascinating aspect of their relationship and illustrates the acclimative ability of the pathogen. Plant pathogenic bacteria are chemoheterotrophs, meaning that they must derive energy by ingesting intermediates (e.g. from the metabolism of amino acids, carbohydrates, etc.) (Goto, 1992). This characterization indicates that the host-pathogen interaction will always be dictated by the parasitic absorption of various molecules by the pathogen; a process which is influences by the availability of metabolites from the host tissues and by the ability of the pathogen to successfully parasitize them.

The various groups of mutants and affected biosynthesis pathways described and discovered in this work will serve an excellent indicator of the direction that further study in this

Table 8. Summary of Auxotrophic Mutants from Previous Tn*5* Virulence Screens (*=not screened in fruit)

| Recurrence in Virulence Screen | Affected Gene | Gene Products | Biosynthesis Pathway | Present in this screen? | Disease Phenotype |
|---|---|---|---|---|---|
| 1 | *amsA* | Putative tyrosine-protein kinase | EPS | No | Asymptomatic |
| 1 | *amsF-J precursor* | | EPS | No | * |
| 2 | *amsH* | phosphoribosylformylglycineamide synthetase | EPS | No | * |
| 2 | *argD* | bifunctional acetylornithine delta-aminotransferase / N-succinyldiaminopimelate aminotransferase | Arginine | Yes | * |
| 3 | *argG* | Argininosuccinate synthase | Arginine | Yes | Asymptomatic |
| 4 | *argH* | Argininosuccinate lyase | Arginine | Yes | Asymptomatic |
| 2 | *carB* | Carbamoyl-phosphate synthase large chain | Arginine | No | * |
| 2 | *cyaA* | Adenylate cyclase | cAMP | No | * |
| 1 | *EAMY_3259* | Hypothetical protein | Hypothetical | Yes | * |
| 1 | *glnA* | glutamine synthetase | Nitrogen metabolism | No | * |
| 1 | *guaB* | Inosine-5'-monophosphate dehydrogenase | Purine | No | * |
| 1 | *ilvD* | Dihydroxy-acid dehydratase | Branched-chain amino acids | No | * |
| 3 | *leuB* | 3-isopropylmalate dehydrogenase | Branched-chain amino acids | Yes | Asymptomatic |
| 1 | *mdeA1* | methionine gamma-lyase | Cysteine and methionine | Yes | * |
| 1 | *metB* | Cystathionine gamma-synthase | Methionine | Yes | * |
| 1 | *N/A* | Ornithine biosynthesis | Ornithine | No | * |
| 2 | *N/A* | Phosphoenolpyruvate Hpr | | No | * |
| 1 | *porF* | | | No | * |
| 2 | *proA* | Gamma-glutamyl phosphate reductase | Proline | No | * |
| 1 | *pstI* | | | No | * |
| 2 | *purL* | Phosphoribosylformyl-glycinamidine synthase | Purine | No | * |
| 1 | *purM* | Phosphoribosylamino-imidazole synthetase | Purine | No | * |
| 1 | *pyrC* | Dihydroorotase | Pyrimidine | No | * |
| 3 | *pyrD* | Dihydroorotate dehydrogenase | Pyrimidine | Yes | * |
| 1 | *thrA* | Homoserine dehydrogenase I | Threonine | No | * |
| 2 | *thrB* | Homoserine kinase | Threonine | Yes | * |
| 2 | *thrC* | Threonine synthase | Threonine | No | Reduced |
| 1 | *thyA* | Thymidylate synthase | Pyrimidine | No | * |
| 1 | *trpE* | Anthranilate synthase | Tryptophan | Yes | * |

vein should head. Thirty of the mutants identified as auxotrophic in this work were not previously described as such in virulence screens of approximately 12,000 Tn*5* mutants performed in the McNellis laboratory (Table 8) or in any *Erwinia amylovora* literature. These represent both the disruption of new genes in pathways that have had other gene disruptions previously described as leading to an auxotrophic phenotype, and genes in pathways that have never been characterized as resulting in auxotrophy. Combined these auxotrophic mutants represent a very large-scale, representative study of auxotrophy, metabolism, and pathogenicity in *Erwinia amylovora*.

The experimental approach taken in this work made it possible to examine mutants with intermediate phenotypes by avoiding the bias toward strong phenotypic expression that may have occurred had a reverse genetics or other approach been taken. The intermediate phenotypes, those of reduced pathogenicity, are of interest because eliminating bias toward clearer impacts caused by the transposon insertion gives a more accurate picture of the overall trends in metabolic activities that are occurring during the *E. amylovora*-apple interaction. These intermediate phenotypes tell the story of the rich complexities of biochemical interactions that are interwoven in parasitic relationships.

The very low incidence of auxotrophy may indicate that most genes that received the Tn*5* insertion were not involved in metabolic processes, or when they are, they are redundant. Alternatively, the metabolites required may potentially be obtained through the rearrangement of metabolites from other, related pathways, or the pathogen may be able to otherwise adjust to survival without the gene product(s). Previous studies concerning auxotrophic mutants of *Erwinia amylovora* found similarly low rates of auxotrophy, as follows: 1.6% (Steinberger and Beer, 1988) and 2.7% (Vanneste, et al, 1989). This low rate is also present in further unrelated organisms, as evidenced by the rate of auxotrophy of 0.5% in the filamentous cyanobacterium, *Anabaena variabilis* (Currier, et al, 1976). These auxotrophic mutant populations were generated

with a variety of approaches other than Tn*5* mutagenesis, indicating that the low rate of auxotrophy is not a result of only the mutagenic approach.

Other than the works of Ramos, very little work has been done to investigate auxotrophy and nutrient acquisition specifically in *Erwinia amylovora* (Ramos 2014, 2015). Even less genomic and metabolic information is available for other *Erwinia* species. Consequently, some genes and metabolic pathways described in this work were inferred by comparison to other bacteria, primarily *Escherichia coli*. Fortunately, bacteria in the family Enterobacteriaceae are quite similar to one another. *E. amylovora* is only distinguished from others in this family in terms of physiology and serology by its ability to grow weakly under anaerobic conditions, its requirement for nicotinic acid, and its inability to reduce nitrate to nitrite (Vanneste, 2000). This similarity is also reflected in the sequence analysis of the 16S rRNA genes, indicating a close relationship to enterobacteria including *Salmonella enterica* and *Escherichia coli* (Zhao, 2014).

The data indicate that the disruption of amino acid biosynthesis, nucleotide biosynthesis, survival protein biosynthesis, nitrogen metabolism, and exopolysaccharide biosynthesis pathways result in reduced or asymptomatic disease expression. This indicates that mutants with these disrupted pathways are *unable* to obtain sufficient amounts of the required metabolic products from the host tissue in order to complete the biosynthetic pathway and grow normally. Conversely, mutants with disrupted sulfur metabolism remained pathogenic, indicating that these mutants were *able* to obtain sufficient amounts of sulfur and sulfur metabolites from the host tissue in order to complete the biosynthetic pathway and grow normally.

### 4.1 Amino Acid Biosynthesis

The high rate of 40% is very illustrative of the importance of amino acids and possibly the metabolism of nitrogen. Amino acids are the initial products of nitrogen assimilation in plants, which are then transported to the various resource sink tissues, including fruits (Ortiz-Lopez et al,

2000). As evidenced by the reduction or complete lack of pathogenicity in these mutants, it is apparent that these amino acids must be synthesized *de novo*. Either *E. amylovora* lacks the ability to absorb extracellular amino acids or the metabolites necessary for their synthesis in the host plant environment (because they can on LB media), or the apple tissue does not have sufficient amounts of that particular amino acid in order to provide for the metabolic requirement. Full pathogenicity can only occur when the pathways for the synthesis of these amino acids (Table 4) are intact. In this regard, *E. amylovora* closely parallels the needs of other phytobacteria. Amino acid biosynthesis was found to be required for colonization by *Pseudomonas tolaasii* (Chung et. al, 2014), and *X. campestris pv. campestris* (Qian et. al, 2005) and by the rhizosphere colonizer *Pseudomonas fluorescens* (Simons et. al, 1997). In *Erwinia* species the major amino acids are alanine, glutamate, aspartate, and glycine, none of which appeared in this screen, with all other amino acids present at far lower levels (Goto, 1992). It is unusual that auxotrophic mutants for the amino acids most prevalent in *E. amylovora* cells did not occur in this screen, although this may coincidental, in that genetic saturation was not reached.

Interestingly, the most frequently disabled pathway, arginine biosynthesis, resulted in an asymptomatic phenotype in all five of the replications of the five mutants that occurred. This repetition affirms that the disabling of this pathway at any point leads to a truly auxotrophic mutant and affirms the complete lack of symptom development caused by this disruption, as previously reported (Ramos, 2014).

The occurrence of auxotrophic mutants in this group with a reduced pathogenicity may be a result of only partially reduced pathogenicity caused by the transposon insertion, or may be select cases of cross-contamination from other strains in the tray. While every effort was taken to prevent cross-contamination, it is still possible that the pathogen, which is highly motile, could have moved through water on the surface of the vellum sheets and spread to unintended apples. Further quantitative inoculations in the fruit would clarify these phenotypes. The reduced

pathogenicity phenotype may also have been a result of some level of alleviation of auxotrophy by metabolites involved in the biosynthesis of other amino acids or even by only partial gene disruption by the transposon. This ambiguity could be removed by making full deletions of the genes in question.

Amino acids are often grouped by their properties, including charge, polarity, functional groups, size, and hydrophicity/-philicity. These characteristics are important in the formation of protein structures and in protein-protein interactions (Creighton, 1993). There are a few possible trends in property types from the amino acid mutants generated in this screen, although because genetic saturation was not reached it is not clear if amino acids in groups currently not represented would appear if the screen had been complete. All but one of the amino acid biosynthetic pathway types (those auxotrophic for arginine) are for hydrophobic amino acids. All three of the branched-chain amino acids, which comprise the aliphatic group, are represented. Five of the nine amino acids represented have nonpolar side chains. Further investigation would be needed into whether or not hydrophobicity, side chain type, or aliphatic composition are important factors in whether or not a mutant expresses an auxotrophic phenotype. Perhaps every amino acid would have at least one auxotrophic mutant upon reaching genetic saturation.

**4.2 Nucleotide Biosynthesis**

25% of the total mutants generated were auxotrophic for nucleotides. Purines and pyrimidines are the components of nucleic acid polymers, essential components of signal transduction pathways, and ATP (adenosine triphosphate), a nucleotide, is the universal currency of energy (Samant et al, 2008). The disruption of the biosynthetic pathways for their creation prevents normal cellular function, reproduction and survival.

A previous study found that a pyrimidine auxotrophic mutant with the transposon insertion in the *pyrC* gene retained full pathogenicity in trees and partial pathogenicity in fruits

(Ramos et al., 2015). During the course of infection it would seem that free nucleotides would become available to the pathogen as cellular degradation occurred. The study also indicated that some *de novo* pyrimidine biosynthesis was required in order for full pathogenicity in fruits. By contrast, the pyrimidine mutants from this work all expressed a reduced or asymptomatic disease phenotype, and as such were unable to obtain the nucleotides or precursors from the host necessary for full pathogenicity. Unfortunately, there were no *pyrC* mutants in order to make a direct comparison; however, this may be an instance of variation in the necessity for specific gene function in a pathway. Perhaps certain genes must function, while others are less necessary for disease development, due to redundancy or auxotrophy alleviation via specific metabolites. This could also be attributed to variation in inoculation concentration caused by lack of quantitative screening in this work. The declining state of the apples in cold storage is also an influence on fruit susceptibility, which could cause marked variation in pathogenicity. Immature fruits have also been demonstrated to have a higher susceptibility to storage diseases than fully mature fruits (Powell and Fulton, 1903).

The recurrence of five total mutants with the transposon insertion in the *pyrD* gene in this pathway, all with reduced pathogenicity, strongly supports the conclusion that the pyrimidine synthesis pathway is required for full virulence in fruits. In light of this work representing a survey of an estimated halfway point toward genetic saturation, perhaps this repeated incidence of insertion in the same gene may not be coincidence. This may be a case of transposon insertion bias based on the sequence. A small degree of Tn*5* transposon insertion bias toward G+C rich sequences has been described previously, however this bias has been suggested to have more relevance in organisms with a particularly A+T rich genome, such as *Saccharomyces cerevisiae* or humans (Green et al., 2012; Herold et al., 2008). Further investigation into the auxotrophy of pyrimidines in *E. amylovora* may be needed to clarify the level of pyrimidine accessibility to the pathogen.

**4.3 Sulfur Uptake and Metabolism**

The auxotrophic mutants in the sulfur uptake and metabolism pathways have transposon insertions in the *cysI1*, *cysI3*, *cysN* and *nuoG* genes. *CysI* genes encode components of the sulfite reductase complex, which synthesizes hydrogen sulfide from sulfite (Barrett and Chang, 1979). *CysN* encodes the production of sulfate adenylyltransferase subunit 1, which is involved in the synthesis of sulfite from sulfate. Both of these are steps in the hydrogen sulfide biosynthesis pathway, which metabolizes inorganic sulfur. The product of the *nuoG* gene is NADH-quinone oxidoreductase subunit G, which shuttles electrons from NADH (nicotinamide adenine dinucleotide, a coenzyme) via metal-binding to iron-sulfur centers, to quinones in the respiratory chain. This is a part of cellular redox reactions (Weidner et al., 1993).

Unlike several of the other affected pathway types, sulfur metabolic pathways always depend on at least some absorption of resources from the host tissues, as sulfur cannot by synthesized *de novo*. These mutants are likely defective in the assimilation of extracellular inorganic sulfur, which would explain why these mutants were unable to grow on the minimal media, which provides sulfur in the form of sulfate ($SO_4$). The presence of reduced or full pathogenicity despite the lack of inorganic sulfur uptake and metabolic processes means either that sulfur metabolism is not completely necessary in order for disease to occur or that some compounds or activities within the apple tissues were able to supplement the lack of gene function related to sulfur metabolism in these mutants. Sulfur is one of the six macronutrients required by plants and, though it is available at a rate of only 3-5% of that of nitrogen, it will still be present in plant host cells and intercellular spaces (Leustek, 1999). The low concentration of sulfur *in planta* is almost always in the form of the sulfur containing amino acids, cysteine and methionine, or their oxidation products or metabolites (Kocsis et al, 1998). It is likely that these sulfur mutants are incorrectly perceived as auxotrophs in minimal media because they are unable

to absorb or assimilate the inorganic sulfur provided, while in the plant tissue there is enough organic sulfur available to satisfy metabolic requirements.

Related to sulfur metabolism, four of the reduced pathogenicity mutants from the amino acid auxotrophic group were auxotrophic for methionine and cysteine. During the course of sulfur metabolism extracellular sulfate first reacts with ATP to form adenylyl sulfate which is then converted to 3'-phosphoadenylyl sulfate (PAPS). Finally, PAPS is reduced to sulfite, and then further reduced to sulfide. Sulfide molecules are incorporated into the structure of the sulfur-containing amino acids (Kanehisa, 2017). The necessity of *Erwinia amylovora* to absorb and metabolize extracellular sulfur sources may have contributed to the reduced pathogenicity phenotype for these mutants, rather than producing an asymptomatic mutant. Access to essential metabolites for these specific amino acids might have allowed partial or full completion of their biosynthesis pathways, leading to the limited ability to induce symptoms. Some plant-pathogenic bacteria require extracellular absorption of cysteine and methionine, such as several pathovars of *Xanthomonas campestris*, because of an inability to synthesize them from inorganic sulfur sources (Goto, 1992).

**4.4 Nitrogen Metabolism**

Two mutants auxotrophic for genes involved in nitrogen assimilation and metabolism were generated. The transposon insertion site for both mutants was *gltB,* which produces glutamate synthase, the enzyme that catalyzes the reaction which produces L-glutamate and $NADP^+$. The glutamate produced is utilized in the glutamine sythetase reaction which forms the amino acid glutamine, an essential component for nitrogen assimilation and metabolism (Temple et al, 1998). Both had a reduced pathogenicity in the fruit screen. Inorganic nitrogen is provided in the minimal media, in the form of ammonium ($NH_4^+$) so it is not unexpected that few auxotrophic mutants would appear in this screen. Only select points in the synthesis pathway

would result in a complete inability to absorb and process extracellular nitrogen. Others would disable only the synthesis into more specific products, such as amino acids or nucleotides.

The pathogen was able to derive at least some of the requisite nitrogen sources from the host tissues in order to induce a reduced pathogenicity phenotype, as evidenced by the partial virulence of these mutants. In the context of the nutrient sources provided, the auxotrophic mutants may have struggled to assimilate the inorganic nitrogen provided by the minimal media, because of the disabling of the *gltB* gene which is part of the biosynthesis pathway for the assimilation of inorganic nitrogen. Yet in the plant, as evidenced by the reduced pathogenicity genotype, the *gltB*$^{-}$ mutants were able to derive some level of organic nitrogen via amino acids in the tissues, or were able to derive other intermediates in order to complete the inorganic nitrogen assimilation pathway. The assimilation of organic nitrogen seems to make more sense in that these mutants were able to grow on the rich media which provided amino acids in the form of tryptone and yeast extract. *Erwinia amylovora* is unable to reduce nitrate to nitrite, therefore all assimilation of nitrogen by the pathogen will be via the ammonia pathway or via the absorption of amino acids (Vanneste, 2000).

The disabling of the inorganic nitrogen assimilation pathway has been described in other microorganisms, as well. Mutants of *Escherichia coli* and *Klebsiella aerogenes* that are deficient in glutamate synthase activity grow very poorly on nitrogen sources other than ammonia specifically due to glutamate starvation, supporting the findings in this work (Goss et al., 2001). Glutamate plays an essential role in all bacterial cellular metabolism and is consequently the most abundant metabolite (Commichau et al., 2006). It serves as an amino donor for nearly all nitrogen-containing metabolites and is highly regulated. Further investigation into the importance of glutamate in the metabolism, parasitism, and pathogenicity of *Erwinia amylovora* would be of great interest.

**4.5 Hypothetical Proteins**

Four mutants generated in this work were auxotrophic and encoded hypothetical or uncharacterized proteins. The three hypothetical proteins are predicted to exist but lack experimental evidence to identify their function. Similarly, the uncharacterized protein lacks experimental evidence, but it has a name, *yibP*, a putative function, and a predicted subcellular localization. This gene is a putative membrane protein. Membrane proteins are a structurally and functionally diverse group. The reduced pathogenicity of the *yibP* mutant would indicate that at least partial functionality of this protein is required in order for full pathogenicity to occur. The pathogen is unable to satisfy the need for whatever metabolites are required in full functionality of *yibP*. Further study of this this gene and others in the *yib* gene family may be of interest.

Initial investigation into the role of the *EAMY_3259* gene indicates some similarity to the translation factor *sua5*, which is essential in *Saccharomyces* species, based on comparison of the amino acid sequence. A database of orthologous proteins indicates that this is the uncharacterized protein sua5/yciO/yrdC (OmaBrowser). The yrdC/sua5 is a universal protein family which is essential for the production of threonylcarbamoyladenosine, which is a modification of the tRNA that aids in binding of the anticodon loop to ribosomes. Expression of *yrdC* is essential in *E. coli* (El Yacoubi et al., 2009).

The hypothetical protein, designated *EAMY_3259*, was also identified as auxotrophic in the previous Tn*5* mutant virulence screen in the McNellis lab, which further validates this designation. In the present study, two mutants in this gene had a reduced pathogenicity phenotype and one was asymptomatic. Despite the lack of information regarding these genes and their products, their identification in this work is of interest and may be of value as further research into uncharacterized genes in the *E. amylovora* genome continues.

**4.6 Exopolysaccharide Biosynthesis**

The mutant screen identified mutations in the *amsB*, *amsC*, and *amsE* genes. The *ams* operon is known to be required for the synthesis of the exopolysaccharide amylovoran by *E. amylovora* (Bugert and Geider, 1995*)*. Does the disabling of the EPS biosynthesis pathway create a pathogen that is unable to derive these resources from the host tissue or a mutant that is unable to utilize cellular metabolites to construct and excrete the polysaccharides? Why does the lack of an EPS coat prevent full pathogenicity from developing? These are questions that are not yet fully answered. Some exopolysaccharides are synthesized *de novo*, but are constructed from extracellular resources, especially carbon (Bennett and Billing, 1977). Because EPS is a component of the regulatory network via which *E. amylovora* would communicate through quorum sensing, perhaps an EPS$^-$ mutant is unable to signal amongst the population (Piqué et al, 2015). This could prevent the regulation of population dynamics required for colonization (Molina et al., 2005). Alternatively, the disruption of steps in the amylovoran pathway could have led to the accumulation of products which eventually interfere with other metabolic processes, as was described in mutations of the *cps* genes in *Erwinia stewartii* (Dolph et al., 1988).

Other affected pathway groups in this work have a mixed population of mostly absolute auxotrophic mutants and some partial auxotrophic mutants. The EPS$^-$ mutants, however, are all partials. Because these inoculations were non-quantitative and were categorized based a perceived rating of reduced opacity in the growth media compared to prototrophic mutants, their characterization is speculative. It is possible that these mutants have a reduced ability to grow in minimal media due to the transposon insertion, but it is also possible that these specific replications happened to have a great enough variation in inoculation amount in order to be falsely perceived as partially auxotrophic. Further quantitative screenings could determine whether or not EPS$^-$ mutant can be partial or fully auxotrophic. Previous Tn*5* virulence screens in the literature and generated in the McNellis laboratory have identified auxotrophic *ams* mutants,

so it is not unprecedented, however this ambiguity due to the non-quantitative inoculation approach does merit further investigation (Belleman and Geider, 1992).

Finally, there is also a possibility that the *ams* mutants were confirmed as auxotrophic in the preliminary screens because they might have been less able to survive on the media, prior to testing for auxotrophy. The lack of exopolysaccharide production could severely impact survival of these mutants when grown on agar media rather than broth, due to rapid desiccation (Roberson and Firestone, 1992).

**4.7 Survival Protein Biosynthesis**

In the closely related species, *E. coli*, *surA* was initially described as having a role in survival in the stationary phase, when bacteria are metabolically active, but non-dividing (Tormo et al, 1990). In normal disease conditions, most bacteria will spend most of their cycle in a non-dividing phase due to nutrient availability as a limiting factor. Upon onset of the stationary phase, the synthesis of many genetic products is increased. Tormo et al found that *surA* was only required during the stationary phase, with the implication that some metabolic processes only occur during this phase. However, more recent publications regarding *surA* have described the protein product as a primary chaperone for the transport of integral β-barrel proteins synthesized in the cytoplasm for incorporation into the outer membrane (Sklar, et al, 2007). A lack of *surA* production resulted in a reduced outer membrane density. It was hypothesized that the disabling of the *surA* gene resulted in a compromised outer membrane. SurA was demonstrated to have some flexibility in the transport of non-specific proteins to the outer membrane.

The role of survival genes has not been heavily investigated in *Erwinia amylovora*, but the similar roles they play in both *E. coli* and *Dickeya dadantii* indicate that *surA* should have the same or similar function (Rondelet and Condemine, 2012). Of the two *surA*¯ mutants in this work, one was a partial auxotroph with reduced pathogenicity and the other was an asymptomatic, full

auxotroph. The pathogen, then, was unable to obtain the required metabolites in order to complete its metabolic processes and pathways. Perhaps the compromised outer membrane reduced the bacteria's ability to survive in the low pH environment of the apple apoplast or affected the ability of the bacterium to uptake metabolites. This gene could also have a pleiotropic effect. Further exploration of how reduced outer membrane concentration affects pathogenicity could shed more light on this subject.

### 4.8 In the Context of the Plant Host Tissue

This work not only reveals insights into how bacterial metabolism occurs as disease develops, but also how the physiology and metabolism of the apple tissues is shaped by infection. This work must be examined in the context of the plant host tissues.

A preliminary assay of the amino acid profile of apple fruit tissues detected glycine, threonine, alanine, arginine, proline, tyrosine, the branched-chain amino acids, phenylalanine, lysine, and most likely, tryptophan, while a more contemporary examination suggested that nine free amino acids were available in measurable levels above ~50 nmoles/g fresh weight (Hulme, 1951; Fish, 2012). The amino acids detected in 'Golden Delicious' and/or 'Red Delicious' apple tissues were as follows: Ala, Asn, Asp, Glu, Gln, Pro, Ser, and Thr. The highest reported amino acid levels were for glutamine and threonine. Other studies have detected levels of at least 18 proteinogenic amino acids in apple fruit tissues, indicating that amino acid levels vary widely across cultivar, growing season, and fruit age (Sugimoto et al., 2011). Available nitrogen was also detected as ammonia ($NH_3$) at a rate of 2.5 mmoles/kg dry weight, a percentage of 20% of the total dry weight. The ability of an auxotrophic mutant to parasitize the tissue for a specific amino acid is dependent on the free availability of that amino acid. An analysis of protein changes in apple during ripening and senescence found that approximately 34% of the proteins in the tissue were involved in energy and metabolism (Shi, 2014). That is to say, the apple tissue is neither a

minimal nor a rich media for pathogen nutrition. It is complex and variable. Further exploration of this relationship, with special attention to the shifting availability of metabolites would be of value, both in this disease system and in others. Furthermore, detection of amino acids by tissue analysis might not reflect whether these are actually available for pathogen uptake.

The changes caused by ripening and senescence are of particular relevance to this work. As the apple fruit ripens levels of isoleucine increase in the tissue to levels 20-fold those initially measured, while other amino acids fluctuated wildly throughout the apple aging processes (Sugimoto et al., 2011). The influence of changing amino acid levels and availabilities, coupled with changes in phytohormone expression and increased respiration will have a great impact on the ability of invading pathogens to colonize tissues and absorb nutrients. Investigation into how *E. amylovora* colonization and parasitism is impacted by changing apple physiology may be informed by the findings in this work in the future. Perhaps nutrient availability is a contributing factor in the ability of *E. amylovora* to infect immature apples, but not mature apples. It has been demonstrated that the immature apple fruits have higher nutrient concentrations (Nachtigall and Dechen, 2006).

In the context of the apple tree as a whole, the fruit serves as a sink in resource allocation. How does this shape resource availability in the fruit tissues? Are resources in greater quantity or in forms that are more bioavailable for pathogen uptake due to the need of the plant to transport resources to the fruit efficiently? Analyses of nutrient availability as apple fruits develop on the tree from blossom to harvest indicate that nutrients are at their highest levels during initial fruit development, followed by an abrupt initial drop in nutrients in the first three weeks (Nachtigall and Dechen, 2006). Nutrients then continue to drop as the fruit grows until stabilizing at about 10 weeks post-bloom. This drop in nutrient concentration is caused by chemical dilution, where increases in fruit biomass lead to dilution of nutrient concentrations. Nutrients measured accumulated in the quantities as follows (from highest to lowest): K> N> P> Mg = Ca> Fe> Mn>

B> Cu> Zn. The immature apple fruits utilized in this work were harvested approximately six weeks after full bloom, meaning that the nutrient concentration was well into decline at the time of harvest.

Less information is available regarding sulfur concentrations and availability in the apple fruit tissue. It has been demonstrated that methionine, the sulfur-containing amino acid, is converted to ethylene, the phytohormone implicated in fruit ripening via the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (Knee, 1984). It is also evident that sulfur intermediates are available in sufficient quantities to be utilized by *E. amylovora* auxotrophs from this work.

The mutants with genes disabled in the hydrogen sulfide biosynthesis pathway (*cysN*, *cysI1*, and *cysI3*) were able to colonize the apple tissues at a rate comparable to that of the wild type. Why then does *E. amylovora* retain this metabolic pathway? There are many examples of naturally-occurring auxotrophic mutants that are at times able to even outcompete prototrophic strains in certain environments, such as in the work of Zamenhof and Eichhorn, in which *Bacillus subtilis* mutants auxotrophic for histidine and tryptophan were found to have a selective advantage when grown on media in which the amino acids required by the respective auxotrophs were provided in excess (Zamenhof and Eichhorn, 1967). They hypothesized that this advantage was due to saving energy because of fewer biosynthetic steps. This advantage is only conferred in highly specific environments, where the auxotroph is able to fully satisfy the requirement, which as demonstrated in this work, includes immature apple tissues in the context of sulfur metabolites. It can be inferred that *Erwinia amylovora* must stably maintain these genes because of its complex lifestyle, which throughout a growing season requires the ability to retain populations epiphytically on floral and leaf tissues, as well as within a variety of internal plant tissues, such as the xylem and fruit tissues (Rosen, 1929; Miller and Schroth, 1972). Each of these niches requires different metabolic functions adaptable to the profile of intermediates that the pathogen is able to derive from each. This variation in gene expression in different host niches is illustrated in the life

cycle of *Salmonella enterica*, a human enteric pathogen. *Salmonella* species are able to colonize both the human gut and some fruits and vegetables. It was found that the pathogen expresses a distinct set of plant-associated genes when inoculated in tomatoes, which only partially overlap with those expressed in the animal host (Moraes, et al, 2016). This variation may be present in *Erwinia amylovora* as well, where subsets of genes are differentially expressed in different environments, though perhaps not the degree required for the occupation of both plants and animals. The advantage conferred by auxotrophy is highly contextual.

**4.9 Experimental Pitfalls**

There are several pitfalls that prevent the formation of more definite conclusions from the data that come from both the experimental design and from characteristics inherent to the processes involved.

Mutagenesis via the insertion of the Tn*5* transposon is not a true deletion of the gene, but rather a disruption that can lead to greater variation in phenotype. The insertion of the transposon may not fully disable the gene, leading to a false phenotype classification caused by partial gene function. False phenotypes could also be generated if the disruption of a gene does not fully disable the biosynthetic pathway, allowing metabolites to interact or accumulate. Furthermore, the insertion of the Tn*5* transposon is not completely random. The transposon inserts with a bias toward sequences containing higher levels of the nucleotides, guanine and cytosine, which could clearly lead to bias toward select segments of the DNA sequence and prevent total genetic saturation at a large scale (Green, et al., 2012).

There is potential for the alleviation of auxotrophy by metabolites from similar pathways, particularly with auxotrophic mutants in the pathways for the biosynthesis of amino acids and nucleotides, as compounds for their pathways can come from a particularly broad range of carbon and nitrogen sources.

Although the mutant pool tested represents over halfway to the point of assumed genetic saturation, the small subset of auxotrophic mutants addressed in this work may not be large enough for the interpretations of pathways and phenotypes to be deemed statistically significant. Particularly, the variation in test group size and lack of replications per mutant in the fruit pathogenicity assay may not be statistically meaningful data. Ten replications per mutant may have given a better indication than five.

The varying ages and sizes of the apples could have had an effect on pathogen success. This variation, coupled with their long term cold storage, means that there was possibly significant variation in the availability of metabolites in the tissues. This is not necessarily an experimental pitfall and may be an interesting point for further study. The relationship of changing metabolic rates as the fruit ages may have interesting effects on the ability of a pathogen to colonize the tissues. This aspect would be of particular interest in the context of post-harvest pathology and storage.

Due to the large scale approach taken in this work, genetic complementation was not performed for each mutant. Rather, the larger numbers of mutants were interpreted as verification of each phenotype. When the transposon is inserted into the same gene several times, it can be inferred that the resulting phenotype is caused by the disruption of that gene with greater confidence. However, without genetic complementation, none of the phenotypes can be confidently verified. There is no proof that the phenotype is caused by the transposon insertion in the gene and that no other defects in the genetic code are occurring. Future work could verify the auxotrophy of the mutants generated in this work in order to indicate that defects outside of the intended transposon-induced disruptions are not affecting the mutant phenotype.

# Chapter 5

## Future Work and Conclusions

In a more general sense, this research may serve as a fantastic jumping off point for further examination of nutritional parasitism in *Erwinia amylovora* and other bacterial plant pathogens. Genetic solutions for the control of fire blight and other bacterial diseases of perennial crops have great potential for circumventing the troubles presented by traditional means of control, such as antibiotic applications. Further understanding of the nutritional needs and parasitic behaviors of *Erwinia amylovora* may eventually contribute to the development of successful resistant apple and pear cultivars, or non-pathogenic biocontrol strains which function by competitive inhibition.

As previously mentioned, this survey represents an approximation of the halfway point to genetic saturation. The previous identification of *pyrC* as an auxotrophic mutant in *E. amylovora*, which was not found in this screen, indicates that complete genetic saturation was not achieved. Further screening will be essential to generating a holistic understanding of the host-pathogen trophic interactions that occur in this system.

Future work could include:

- Testing mutants auxotrophic for amino acids on minimal media supplemented with those amino acids as a way to confirm the nature of the auxotrophy.
- Quantitative inoculations in order to get a more accurate representation of the rate of pathogenicity, including revealing instances of reduced pathogenicity in some mutant strains, which may have been inaccurately reported here.
- Quantification of bacterial populations during of the fruit assay would also indicate if the asymptomatic mutants were able to survive in the fruit, despite their inability to cause disease. The various auxotrophic mutants may have retained a certain level of

infectivity (ability to invade and replicate in the host tissues) that is not indicated by the level of pathogenicity.

- Inoculating select mutants from each pathway type in immature pear fruits. Pears tend to have a higher susceptibility to *Erwinia amylovora* than apples, however some Tn*5* mutants have been found to cause disease differently (or not at all) in pear fruits (Ramos et al., 2014). This variability in pathogenicity may be of interest.
- Inoculating mutants in young (approx. 2 years old) apple trees would indicate mutant strains' ability to induce fire blight symptoms in woody tissues. Different tissues mean different forms and bioavailability of metabolites and other compounds. Will nutrient and metabolite availability in the vascular tissues differ significantly from availability in the fruit, a resource sink? And is that difference enough to cause variations in phenotype?
- Comparing auxotrophic mutant growth in immature apples to their ability to grow in isolated fruit extract from immature apples may shed more light on whether or not amino acids or other metabolites are in readily available forms for the pathogen to absorb. Analysis of tomato root exudate indicated that the amino acids necessary for colonization by *Pseudomonas fluorescens* were present, but the levels of bio-availability were too low for colonization to occur (Simons et. al, 1997). This may be the case for *E. amylovora* colonization as well.
- There is potential for harnessing nutritional immunity in the development of resistant apple cultivars that could be influenced by this study. Plant hosts attempt to sequester nutrients in order to restrict the growth of invading pathogens. There is precedence for this approach with iron uptake, however the same principles could be utilized in researching the mechanisms by which bacteria attain nutrients, amino acids, and

other metabolites from the host and how these processes can be mitigated (Terwilliger et al, 2015).

In conclusion, this work will have been the most comprehensive look into *Erwinia amylovora* nutrient acquisition potential *in planta* to date. The large scale and broad spectrum approach can help to initiate further study of the facets of metabolism and parasitism involved in this complex host-pathogen interaction.

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# APPENDIX

Appendix Table 1. Summary of all Tn*5* Auxotrophic Mutant Insertion Sites and Pathogenicity Phenotypes Determined in this Study

| Mutant | Sequence date | Qual20 | Insertion Site | Affects: | Pathway Disrupted | Pathway Type | Pathogenicity | Averaged Symptom Score | Auxotrophic in virulence screen? |
|---|---|---|---|---|---|---|---|---|---|
| M150.35 | 11/2/16 | 1043 | amsB | Glycosyltransferase amsB | Exopolysaccharide biosynthesis | EPS | Reduced | 0.2 | no |
| M16.4 | 11/2/16 | 1031 | amsC | Exopolysaccharide biosynthesis protein | Exopolysaccharide biosynthesis | EPS | Reduced | 2.4 | no |
| M10.29 | 11/2/16 | 1019 | amsE | Putative glycosyltransferase | Polysaccharide biosynthesis | EPS | Asymptomatic | 0 | no |
| M161.21 | 11/2/16 | 1033 | argD | Acetylornithine/ succinyldiaminopimelate aminotransferase | Arginine biosynthesis | Amino acid metabolism | Asymptomatic | 0 | yes |
| M154.46 | 11/2/16 | 1034 | argE | Acetylornithine deacetylase | Arginine biosynthesis | Amino acid metabolism | Asymptomatic | 0 | no |
| S1.36 | 3/29/17 | 1029 | argG | Argininosuccinate synthase | Arginine biosynthesis | Amino acid metabolism | Asymptomatic | 0 | yes |
| M131.23 | 11/2/16 | 1043 | argH | Argininosuccinate lyase | Arginine biosynthesis | Amino acid metabolism | Asymptomatic | 0 | yes |
| S7.13 | 3/29/17 | 972 | argH | Argininosuccinate lyase | Arginine biosynthesis | Amino acid metabolism | Asymptomatic | 0 | yes |
| M5.4 | 11/2/16 | 1035 | carA | Carbamoyl-phosphate synthase small chain | Pyrimidine biosynthesis; glutamine metabolic process | Nucleotide metabolism | Asymptomatic | 0 | no |
| M131.39 | 11/2/16 | 1043 | cysI1 | Sulfite reductase (NADPH) flavoprotein alpha-component | Hydrogen sulfide biosynthesis | Sulfur metabolism | Pathogenic | 3.2 | no |
| M148.15 | 11/2/16 | 1036 | cysI1 | Sulfite reductase (NADPH) flavoprotein alpha-component | Hydrogen sulfide biosynthesis | Sulfur metabolism | Pathogenic | 3 | no |

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| M45.2 | 3/29/17 | 1053 | cysI3 | Sulfite reductase (NADPH) flavoprotein beta-component | Hydrogen sulfide biosynthesis | Sulfur metabolism | Reduced | 0.8 | no |
| M5.1 | 10/2/16 | N/A | cysN | ATP sulfurylase | Sulfur metabolism | Sulfur metabolism | Pathogenic | 4.2 | no |
| M163.4 | 11/2/16 | 1007 | cysQ | Inositol-1-monophosphatase | Cysteine biosynthesis | Amino acid metabolism | Reduced | 1.4 | no |
| M11.31 | 11/2/16 | 1072 | EAMY_3259 | Hypothetical protein | Hypothetical | Hypothetical | Reduced | 0.6 | yes |
| M36.10 | 11/2/16 | 1029 | EAMY_3259 | Hypothetical protein | Hypothetical | Hypothetical | Asymptomatic | 0 | yes |
| M92.41 | 11/2/16 | 1014 | EAMY_3259 | Hypothetical protein | Hypothetical | Hypothetical | Reduced | 0.2 | yes |
| M155.32 | 3/29/17 | 1029 | gltB | Glutamate synthase (NADPH) | Nitrogen metabolism | Nitrogen metabolism | Reduced | 1.4 | no |
| M158.40 | 11/2/16 | 997 | gltB | Glutamate synthase (NADPH) | Nitrogen metabolism | Nitrogen metabolism | Reduced | 2 | no |
| M129.18 | 11/2/16 | 1017 | hisD | Histidinol dehydrogenase | Histidine biosynthesis | Amino acid metabolism | Reduced | 1.2 | no |
| M105.46 | 11/2/16 | 1022 | ilvC | Ketol-acid reductoisomerase | Branched-chain amino acid biosynthesis | Amino acid metabolism | Reduced | 0.6 | no |
| M123.10 | 11/2/16 | 1040 | ilvC | Ketol-acid reductoisomerase | Branched-chain amino acid biosynthesis | Amino acid metabolism | Reduced | 0.2 | no |
| M4.47 | 10/2/16 | N/A | ilvE1 | Branched chain amino acid aminotransferase | Branched-chain amino acid biosynthesis | Amino acid metabolism | Reduced | 0.8 | no |
| M140.10 | 11/2/16 | 1037 | leuB | 3-isopropylmalate dehydrogenase | Leucine biosynthesis | Amino acid metabolism | Reduced | 0.2 | yes |
| M160.2 | 11/2/16 | 1031 | leuB | 3-isopropylmalate dehydrogenase | Leucine biosynthesis | Amino acid metabolism | Asymptomatic | 0 | yes |
| M134.35 | 11/2/16 | 1019 | leuC | 3-isopropylmalate dehydratase large subunit | Leucine biosynthesis | Amino acid metabolism | Asymptomatic | 0 | no |
| M106.50 | 3/29/17 | 1041 | mdeA1 | Methionine gamma-lyase | Cysteine and methionine | Amino acid metabolism | Reduced | 2.2 | intermediate |

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|---|---|---|---|---|---|---|---|---|---|
| | | | | | biosynthesis | | | | |
| M129.46 | 11/2/16 | 1042 | metA | Homoserine transsuccinylase | Methionine biosynthesis | Amino acid metabolism | Reduced | 0.8 | no |
| M157.1 | 11/2/16 | 1006 | metB | Cystathionine gamma-synthase | Methionine biosynthesis | Amino acid metabolism | Reduced | 0.2 | yes |
| M151.13 | 11/2/16 | 1019 | nuoG | NADH dehydrogenase I chain G | metal binding; redox reactions | Sulfur metabolism | Reduced | 1.4 | no |
| M.6.45 | 11/2/16 | 1013 | purA | Adenylosuccinate synthetase | AMP biosynthesis | Nucleotide metabolism | Asymptomatic | 0 | no |
| M110.31 | 11/2/16 | 1023 | purA | Adenylosuccinate synthetase | AMP biosynthesis | Nucleotide metabolism | Reduced | 0.6 | no |
| M128.36 | 11/2/16 | 1023 | purK | Phosphoribosylaminoimidazole carboxylase ATPase subunit | Purine biosynthesis; in operon with purE | Nucleotide metabolism | Asymptomatic | 0 | no |
| M36.1 | 11/2/16 | 1033 | purU | Formyltetrahydrofolate deformylase | Purine biosynthesis | Nucleotide metabolism | Reduced | 2.8 | no |
| M158.18 | 11/2/16 | 1020 | purU | Formyltetrahydrofolate deformylase | Purine biosynthesis | Nucleotide metabolism | Reduced | 1.6 | no |
| M191.44 | 3/29/17 | 1035 | pyrB | Aspartate carbamoyltransferase catalytic subunit | Pyrimidine biosynthesis | Nucleotide metabolism | Asymptomatic | 0 | no |
| M46.31 | 3/29/17 | 1017 | pyrD | Dihydro-orotate dehydrogenase | UMP biosynthesis | Nucleotide metabolism | Asymptomatic | 0 | yes |
| M129.28 | 11/2/16 | 1045 | pyrD | Dihydro-orotate dehydrogenase | UMP biosynthesis | Nucleotide metabolism | Reduced | 0.6 | yes |
| M142.31 | 11/2/16 | 1040 | pyrD | Dihydro-orotate dehydrogenase | Pyrimidine biosynthesis | Nucleotide metabolism | Reduced | 0.4 | yes |
| M156.9 | 11/2/16 | 998 | pyrD | Dihydro-orotate dehydrogenase | Pyrimidine biosynthesis | Nucleotide metabolism | Reduced | 0.8 | yes |
| S5.45 | 3/29/17 | 1015 | pyrD | Dihydro-orotate dehydrogenase | Pyrimidine biosynthesis | Nucleotide metabolism | Reduced | 0.2 | yes |
| M8.17 | 11/2/16 | 1045 | surA | Survival protein SurA precursor | Survival in stationary phase (in *E. coli)* | Survival | Asymptomatic | 0 | no |
| M105.9 | 11/2/16 | 1031 | surA | Survival protein SurA precursor | Survival in stationary state (in *E.* | Survival | Reduced | 0.8 | no |

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|---|---|---|---|---|---|---|---|---|---|
| | | | | | *coli*) | | | | |
| M157.42 | 11/2/16 | 1009 | thrB | Homoserine kinase | Threonine biosynthesis | Amino acid metabolism | Reduced | 0.8 | yes |
| M126.35 | 11/2/16 | 1030 | trpB | Tryptophan synthase beta chain | Tryptophan biosynthesis | Amino acid metabolism | Reduced | 1.2 | no |
| M198.17 | 3/29/17 | 1017 | trpE | Anthranilate synthase component I | Tryptophan biosynthesis | Amino acid metabolism | Reduced | 0.2 | no |
| M206.13 | 3/29/17 | 1054 | yibP | Uncharacterized protein yibP | Putative membrane protein | Uncharacterized | Reduced | 2.2 | no |