MOLECULAR GENETIC STUDY OF ERWINIA AMYLOVOR A: rpoN, argD AND pyrC GENES

A Dissertation in
Plant Pathology
by
Laura Ramos Sepulveda

Submitted in Partial Fulfillment
of the Requirement
for a Degree of

Doctor in Philosophy
December 2014
The dissertation of Laura Ramos Sepulveda was reviewed and approved* by the following:

Timothy W. McNellis  
Associate Professor of Plant Pathology and Environmental Microbiology  
Dissertation Adviser  
Chair of Committee

Maria del Mar Jimenez-Gasco  
Assistant Professor of Plant Pathology and Environmental Microbiology  
Coordinator and Advisor of the Minor in Plant Pathology

Beth K. Gugino  
Associate Professor of Plant Pathology and Environmental Microbiology

Yinong Yang  
Associate Professor of Plant Pathology and Environmental Microbiology

Edward G. Dudley  
Associate Professor of Food Sciences

David M. Geiser  
Professor and Interim Department Head

*Signatures are on file in the Graduate School
ABSTRACT

Fire blight is the most destructive bacterial disease of apples and pears worldwide. Apples are the second most consumed fresh fruit in the United States. The causal agent of fire blight is *Erwinia amylovora*. Except for streptomycin, there is no registered product that can effectively prevent *E. amylovora* from infecting apple and pear orchards. Unfortunately, in the United States, streptomycin-resistant strains of *E. amylovora* have been found. A better understanding in general of the molecular genetics of the bacteria could lead not only to a better comprehension of the disease process but also the development of additional reliable management tools. In this research, three *E. amylovora* genes were studied: a regulatory gene called *rpoN*; and two metabolic genes called *argD* and *pyrC*. I found that the *rpoN* gene was essential for pathogenicity, for the hypersensitive response in non-host tobacco, and for the expression of the HrpN protein. The *rpoN* mutant was prototroph, indicating that the *rpoN* mutant non-pathogenic phenotype was not due to a defect in basic metabolism or growth. The *argD* gene is essential for the biosynthesis of the amino acid arginine in bacteria, including *E. amylovora*. *E. amylovora* *argD* mutant was an arginine auxotroph and did not cause disease or grow in host tissue. This study suggests that *E. amylovora* cannot obtain enough arginine from the host during infection. The *pyrC* gene is indispensable for the biosynthesis of pyrimidines. Surprisingly, even though the *E. amylovora* *pyrC* mutant was a uracil auxotroph, it was able to obtain uracil from the host, grow and cause disease. This study suggests that *E. amylovora* can obtain and utilize sufficient uracil from the host. The study of the last two genes gave us an insight about the nutrition requirements that the bacteria have during infection and after infecting the host. This knowledge could help develop potential fire blight controls.
TABLE OF CONTENTS

List of figures ........................................................................................................................................ vi
List of tables ........................................................................................................................................... viii

Acknowledgements ................................................................................................................................... ix

Chapter 1: Introduction to the molecular genetics of *Erwinia amylovora* ........................................ 1
  Background Information ......................................................................................................................... 2
    *Erwinia amylovora* ............................................................................................................................... 2
    Host ...................................................................................................................................................... 3
    Environment .......................................................................................................................................... 3
  Fire Blight Control Methods ................................................................................................................. 4
  The Problem ......................................................................................................................................... 6
  Molecular Genetics of *Erwinia amylovora* .......................................................................................... 8
    First Category: Pathogenicity Genes ..................................................................................................... 8
    Second Category: Virulence Genes ......................................................................................................... 9
    Third Category: Regulatory Genes ......................................................................................................... 11
  Contribution of this research to the study of fire blight ......................................................................... 13
  Objectives ............................................................................................................................................. 14
  References ............................................................................................................................................. 15

Chapter 2: The fire blight pathogen *Erwinia amylovora* requires the *rpoN* gene for pathogenicity in apple ................................................................................................................................. 22
  Abstract ............................................................................................................................................... 25
  Introduction ......................................................................................................................................... 26
  Material, Methods, Results and Discussion .......................................................................................... 28
  Acknowledgements ............................................................................................................................... 37
  References ............................................................................................................................................ 38

Chapter 3: Mutation of the *Erwinia amylovora argD* gene causes arginine auxotrophy, non-pathogenicity in apple and reduced virulence in pear .............................................................................. 42
  Abstract ............................................................................................................................................... 45
  Introduction ......................................................................................................................................... 46
  Material and Methods .......................................................................................................................... 48
  Results ................................................................................................................................................ 54
Chapter 4: Mutation of the *Erwinia amylovora* pyrC gene causes pyrimidine auxotrophy and attenuated virulence in apples trees and immature apple and pear fruits

Abstract ................................................................................................................. 80
Introduction ............................................................................................................. 81
Material and Methods.......................................................................................... 83
Results and Discussion........................................................................................ 85
Acknowledgement............................................................................................... 93
References ............................................................................................................. 94

Chapter 5: Conclusion........................................................................................... 98
Conclusion.............................................................................................................. 99
Future Work ......................................................................................................... 101
References ........................................................................................................... 103

Appendix A: Clean deletion of the *argD* gene in *Erwinia amylovora*................. 104

Appendix B: *yrfF* gene .......................................................................................... 112
References ........................................................................................................... 119
LIST OF FIGURES

Figure 1-1: Diagram of the genes in *E. amylovora* discussed in chapter 1 ........................................ 13  
Figure 2-1: Representation of the *rpoN* genomic neighborhood ...................................................... 28  
Figure 2-2: Pathogenicity assay in mature apple trees ................................................................. 31  
Figure 2-3: Pathogenicity assay in detached immature apple fruit ............................................... 32  
Figure 2-4: Hypersensitive response assay ............................................................. 33  
Figure 2-5: Western Blot assay ................................................................................................. 35  
Figure 2-6: Growth in minimal media ..................................................................................... 36  
Figure 3-1: Representation of the *argD* genomic neighborhood ........................................... 55  
Figure 3-2: Auxotrophy assay ............................................................................................... 56  
Figure 3-3: Map of the pCR2.1-arg*D* plasmid ................................................................. 57  
Figure 3-4: Growth in M9TN media supplemented with arginine ........................................... 58  
Figure 3-5: Pathogenicity assay in mature apple trees ......................................................... 59  
Figure 3-6: Pathogenicity assay in detached immature apple and pear fruits ..................... 61  
Figure 3-7: Pathogenicity and growth analysis of HKN06P1 and HKN06P1^InvR^ .................... 62  
Figure 3-8: Co-inoculations assay ......................................................................................... 63  
Figure 3-9: Plasmid stability assay ....................................................................................... 66  
Figure 3-10: Plasmid stability assay – Pathogenicity assay in apple trees ......................... 67  
Figure 4-1: Representation of the *pyrC* genomic neighborhood ........................................ 86  
Figure 4-2: Growth in M9TN media supplemented with uracil ............................................. 87  
Figure 4-3: Pathogenicity assay in detached immature apple and pear fruits ..................... 89  
Figure 4-4: Pathogenicity assay in mature apple trees assay and HR assay ..................... 91  
Figure 5-1: Impact of my research to the genetic knowledge of *E. amylovora* ................... 100  
Figure 5-2: Diagram of my research on the genetic knowledge of *E. amylovora* disease processes ........................................................................................................................................... 102

Figure Appendix A 1-1 ........................................................................................................... 106  
Figure Appendix A 1-2 ........................................................................................................... 107  
Figure Appendix A 1-3 ........................................................................................................... 109  
Figure Appendix A 1-4 ........................................................................................................... 110  
Figure Appendix A 1-5 ........................................................................................................... 110  
Figure Appendix A 1-6 ........................................................................................................... 111  
Figure Appendix B 1-1 .......................................................................................................... 114  
Figure Appendix B 1-2 .......................................................................................................... 114
Figure Appendix B 1-3 .................................................................115
Figure Appendix B 1-4 .................................................................116
Figure Appendix B 1-5 .................................................................117
Figure Appendix B 1-6 .................................................................117
Figure Appendix B 1-7 .................................................................118
LIST OF TABLES

Table 2-1: Plasmids and bacterial strains used in rpoN work ........................................41
Table 3-1: Plasmids, bacterial strains, and oligonucleotides used in argD work ..............77
Table 4-1: Plasmids, bacterial strains, and oligonucleotides used in pyrC work. ............97
ACKNOWLEDGEMENTS

I would like to thank Judy Sinn, Dr. Philip Jensen, Dr. Dharmendra Singh, and Dr. Steven Lee. They were really nice to me and also very patient with my English and my inexperience with molecular work when I arrived at Timothy McNellis’ laboratory in 2010. Especially, I would like to thank Steven Lee, whose laboratory work I continued; I really appreciate all the work he accomplished. I would also like to thank Sara Klee and Xin Lin for making the laboratory experience an even more enjoyable one.

I want to thank my Ph.D. committee members Drs. Maria Jimenez Gasco, Beth Gugino, Yinong Yang and Edward Dudley for being nice, for their time, knowledge, patience and input during our meetings once a semester. I want to thank Dr. Kari Peter and Brian Lehman at the Fruit Research Extension Center for their help with the apple trees and with the immature apple fruits we collected every summer. I want to thank everybody in the Department of Plant Pathology and Environmental Microbiology, specially Lori Long, Debra Clemmer, Dr. Jung-Eun Kim, Dr. Emily Pifiefer, Dr. Jill Demers, Freddy Magdama, Ilse Huerta, Dr. Maria Burgos, Yueying Chen and Dr. Seogchan Kang. Also, a special thanks to Dr. Chun Chen, who helped me with the clean deletion technique.

Lastly but not least, I would like to thank to my adviser, Timothy McNellis. He was very supportive, respectful, patient and nice to me. I want to thank him for sharing his knowledge and for pushing me to do better every day.

I appreciate receiving funding from the following sources: a Bunton Waller Graduate Fellowship, an Alfred P. Sloan Foundation Graduate Scholarship, The Pennsylvania State University College of Agricultural Sciences Graduate Student Competitive Grant Program, Grant #2010-65110-20488 from the Agriculture and Food Research Initiative Competitive
Grants Program of the United States Department of Agriculture National Institute of Food and Agriculture (Dr. Kang), The Department of Plant Pathology and Environmental Microbiology at The Pennsylvania State University, and the Sarah Chinn Kalser Faculty Research Assistance Endowment of the Pennsylvania State University (Dr. McNellis).
CHAPTER ONE

INTRODUCTION TO THE MOLECULAR GENETICS OF *ERWINIA AMYLOVORA*
BACKGROUND INFORMATION

*Erwinia amylovora*

Fire blight is the most economically important and destructive bacterial disease in apples and pears all around the world (van der Zwet *et al.*, 2012). It was the first plant disease that was found to be caused by bacteria (Arthur, 1885; Burrill, 1880; van der Zwet *et al.*, 2012) and the causal agent is *Erwinia amylovora* (Burrill, 1880). The disease is called fire blight because the infected branches of the tree look as if they were burned (Coxe, 1817; van der Zwet *et al.*, 2012). This branch dieback is the most characteristic symptom of fire blight disease and is often accompanied by bacterial ooze, the sign of the pathogen (van der Zwet *et al.*, 2012).

The fire blight disease cycle starts with the bacteria overwintering in the bark at the edge of a structure called the canker (Sackett, 1911; Miller 1929). In early spring, the bacteria inside the bark will start coming out as ooze, which is the source of the first inoculum and is transferred by rain, insects, and/or wind to healthy flowers (Miller, 1929; Rosen, 1929; Rosen, 1936; Gossard and Walton, 1917). The involvement of insects in the dispersion of the first inoculum is still controversial, but what is clear is that insects, specifically bees, are involved in the movement of the second inoculum, which occurs when the first inoculum bacteria are transferred to new healthy flowers (Waite, 1891).

After a short epiphytic stage on the flowers, the *E. amylovora* pass between the nectary guard cells, infecting the tree (Rosen 1936; Hildebrand, 1937). The bacteria move intercellularly between parenchyma cells until they reach the xylem (Hildebrand, 1937; van der Zwet *et al.*, 2012). Once the bacteria are in the xylem of the tree, they can move systemically through the plant (Goodman *et al.*, 1974; Suhayda and Goodman, 1981). It has been shown that the bacteria
can then move back and forth between the xylem and the intercellular spaces of the parenchyma tissue (Bogs et al., 1998; Goodman and White, 1981).

**Host**

In addition to apples (*Malus x domestica*) and pears (*Pyrus communis*), 129 species in 37 different genera of the family Rosaceae have been reported to be susceptible to fire blight (van der Zwet et al., 2012). Even though pears are more susceptible than apples to fire blight (van der Zwet and Keil, 1979), in our laboratory I used apples for the majority of our disease assays for two main reasons: first, we have found that pears rot faster than apples in storage and do not keep as well long-term, and secondly, apples are more economically important than pears in the United States as well as in the rest of the world. Apples are the second most consumed fresh fruit crop in the United States and account for about 50% of deciduous fruit tree production in the world (University of Illinois - Extension)

**Environment**

The disease triangle is the central concept of plant pathology (Agrios, 2005). It is based on the principal that disease is the result of an interaction between a host, a pathogen and the environment (Agrios, 2005). If any of the factors are not present, then the disease will not occur (Agrios, 2005). In order to have disease, the environment should be favorable for the pathogen and non-favorable for the host (Agrios, 2005).

In early spring, when temperatures are greater than 18.5°C, *E. amylovora* starts to multiply in the plant in order to come out as ooze from the infected cankers (van der Zwet and Keil, 1979). The ideal temperature for *E. amylovora*’s growth is 28°C (Brooks, 1926). The
highest temperature at which the bacteria can still cause disease is 32-35°C (van der Zwet and Keil, 1979). Soil temperature and intercellular humidity (IH) are also important for susceptibility. It has been shown that soil temperatures between 12°C and 32°C are favorable for the bacteria’s growth (Shaw, 1934). Furthermore, when the IH was 99.5%, the plants were yet more susceptible to fire blight (Shaw, 1935). The severity of the disease is regulated by many factors, including temperature, rainfall, relative humidity, and prevalence of insects. Warm (above 65°F), rainy days followed by periods of high humidity induce more disease development (Brooks, 1926; Miller, 1929; Tullis, 1929)

**FIRE BLIGHT CONTROL METHODS**

Apple growers try to prevent fire blight infection by selecting and planting resistant rootstocks and varieties (Mowry, 1969; Boyce, 1970; Thompson, 1971; Keil and van der Zwet, 1975; van der Zwet and Keil 1979; Vanneste, 2000). However growers find themselves in a dilemma when choosing between resistant rootstocks or susceptible dwarf rootstocks and between resistant cultivars (scions) or consumer-preferred susceptible cultivars. Unfortunately, most of the dwarf rootstocks which reduce the tree size and therefore labor costs are often more susceptible than the standard resistant rootstocks to fire blight, including the very important rootstock M.9 (van der Zwet et al., 2012; Vanneste, 2000). Furthermore, consumers prefer susceptible cultivars such as ‘Gala’, which is one of the most consumed apples in the United States (van der Zwet and Keil, 1979; van der Zwet et al., 2012).

Since there is no cure for fire blight, the only option that growers in Pennsylvania, USA have is to try to prevent the disease from entering their orchard. In order to do so, growers can spray the antibiotic streptomycin when necessary (Dunegan et al., 1954; Goodman, 1954;
Morgan and Goodman, 1955; Shaffer and Goodman, 1969) using a forecasting model like Maryblyt™ (Peter, 2014). The use of disease forecasting models is very important and influential when it comes to applying streptomycin, basically for two main reasons: cost and resistance management (Peter, 2014). When growers apply streptomycin, it costs them time, fuel for the tractor and sprayer, and money for the streptomycin (Peter, 2014). If growers spray streptomycin when environmental conditions are not favorable for infection, it is not cost effective and the chances of creating streptomycin-resistant *E. amylovora* strains are higher (Peter, 2014). Fortunately, streptomycin-resistance strains of *E. amylovora* have not yet been found in Pennsylvania (Peter, 2014).

This summer (2014), growers suffered from hail damage (Peter, 2014). In this case, farmers should have sprayed streptomycin within 24 hours after the storm to prevent damaged apple trees from being infected with *E. amylovora*. Lastly, it is indispensable that growers monitor their orchards a couple of times a week for fire blight incidence (Beer, 1976; Peter, 2014).

If an apple or pear orchard gets infected with *E. amylovora*, the main thing the farmer should do is to cut the infected branches, 12 to 18 inches below the lowest symptomatic tissue (Alwood, 1902; Anderson, 1920; Chisholm, 1905; Isaac, 1902; Jackson, 1910; Lawrence, 1907; McLarty, 1923-1927; O’Gara, 1910, 1912, 1913; Tehon, 1914; Weldon, 1918). The pruned branches should be burned immediately; otherwise the bacteria could overwinter on the branches left on the ground (Fulton 1911; Peter, 2014). Unfortunately, if you prune during a fire blight epidemic you could cause more harm than good. When growers prune, it stimulates new growth, which is a more susceptible tissue to fire blight (Blake, 1934; Stewart, 1913; van der Zwet and Keil, 1970). Secondly, when growers prune during a fire blight epidemic they have to
be very careful while cleaning the pruning tools; otherwise they could infect healthy tissue later. Third, wounds are open entrances for the bacteria to infect. Growers should choose a dry day and spray the wounds with streptomycin no more than 24 hours after the pruning (Peter, 2014).

Another alternative is to use copper, which works because it kills the bacteria on contact (Clark, 1938; Keitt et al., 1932a; McCown, 1928, 1929, 1933; Musser, 1933; Pinckard et al., 1936; Reimer, 1926; Rosen, 1932, 1933, 1934; Sherbakoff, 1931-1932; Sherbakoff and Aindes, 1939; Peter, 2014). Unfortunately, copper will not kill the bacteria in the xylem or other internal tissues, and furthermore the copper causes fruit russetting (Keitt et al., 1932b; Reil et al., 1973; Rosen, 1936; Peter, 2014). Fruit russeting gives an undesired appearance to the fruits. OxiDate (hydrogen dioxide) is another product that kills the bacteria, but just like copper, it does not kill the bacteria inside the tree and causes fruit russetting (Peter, 2014). Other options exist, but they are in an experimental stage (Peter, 2014). Some experimental fire blight control products include Regalia (plant-based, Reynourtria sachalinensis), Serenade Optimum (bacterial-based, Bacillus subtilis), and Double Nickel (bacterial-based, Bacillus amyloliquefaciens). They are all bio-pesticides that are right now being evaluated as fire blight controls (Peter, 2014).

THE PROBLEM

Fire blight has been documented as an important apple and pear disease for more than two hundred years (Denning, 1794). Unfortunately, fire blight continues to cause major sporadic losses, for example, $68 million were lost in the northwestern part of the United States in 1998 (Bonn, 1999). In Michigan alone, growers lost $42 million to fire blight, including crop and trees losses in 2000, reduced crop for the next three to five years, and some young orchards that had to be removed and replaced (Norelli et al., 2003). Fire blight originated in the United States
and threatens to be even more economically significant in the future, as the disease continues spreading worldwide (Vanneste, 2000). Interestingly, China which is the major apple producer in the world (University of Illinois - Extension) has not seen fire blight symptoms yet (van der Zwet et al., 2012).

In the United States, with the exception of streptomycin, there are no registered products that can effectively manage fire blight (Dunegan et al., 1954; Goodman, 1954; Morgan and Goodman, 1955; Shaffer and Goodman, 1969). In fact, 90% of the agricultural use of streptomycin in the United States is for controlling fire blight disease (McManus et al., 2002). Streptomycin has been used in the United States since the 1950’s, but unfortunately it took only 10 years for streptomycin-resistant *E. amylovora* to be detected in the field. The first streptomycin-resistant *E. amylovora* was observed in 1971 in a Sacramento Valley pear orchard and still to this day researchers continue to find streptomycin-resistant strains in other locations in addition to Sacramento (Moller et al., 1981; Sholberg et al., 2001; Ponce de León et al., 2013).

A better understanding in general of the molecular genetics of *E. amylovora* could lead not only to a better comprehension of the process of disease but also to the development of additional management tools. Therefore, since the 1980’s, scientists have been working to identify *E. amylovora* genetic factors that contribute to the capacity of the bacteria to cause fire blight in apple and pear. *E. amylovora* genes contributing to virulence and pathogenicity continue to be discovered to this day.
MOLECULAR GENETICS OF *E. AMYLOVORA*

Most of the genetic factors that have been studied in *E. amylovora* can be assigned to three general categories. The first category is pathogenicity genes, which are genes that encode factors that are indispensable for disease development and that interact directly with the host to cause the disease. The second category is virulence genes, which are genes that are required for full disease development. The third category is regulatory genes, which are genes that regulate the expression of pathogenicity or virulence genes and do not encode any factor that directly interacts with the host.

**First Category: Pathogenicity Genes**

The amylovoran synthesis (*ams*), hypersensitive response and pathogenicity (*hrp*), and disease specific (*dsp*) genes are some of the most studied and most important *E. amylovora* pathogenicity genes discovered to date. Even though the *hrpL* gene is part of the *hrp* operon, which is a pathogenicity operon, it will be discussed in detail in the regulatory genes category because it encodes a transcription factor. This category will include some of the pathogenicity genes, with the exception of regulatory genes, which are going to be discuss in the regulatory category.

The *ams* operon is required for the biosynthesis of the exopolysaccharide amylovoran (Bugert & Geider, 1995). Amylovoran is indispensable for the protection of the bacteria against plant defenses. Amylovoran also creates a more suitable environment for the bacteria to grow and move in the xylem. The *hrp* operon is essential to elicit the hypersensitive response (HR) defense reaction in non-host plants and is indispensable for pathogenicity in host plants. In *E. amylovora*, the *hrcC* and *hrcA* genes encode structural proteins of the type III protein secretion
system (T3SS) (Kim et al., 1997). The \textit{hrpN} and \textit{hrpW} genes encode proteins that are secreted via the T3SS, with the HrpN protein being essential for virulence and the HR in tobacco (Wei \textit{et al.}, 1992), while the HrpW protein is not required for virulence, but does contribute to the HR in tobacco (Gaudriault \textit{et al.}, 1998; Kim & Beer, 1998). The \textit{dspA/E} genes are necessary for the induction of plant cell death (Gaudriault \textit{et al.}, 1997; Bogdanove \textit{et al.}, 1998) when the protein is translocated into the host by the T3SS (Bogdanove \textit{et al.}, 1998). HrpN also contributes to the translocation of the DspA/E protein into the plant cell (Bogdanove \textit{et al.}, 2008).

\textbf{Second Category: Virulence Genes}

This category will include genes that, even though they are not essential for disease, they contribute to disease development. In this category, I will be talking about genes that are involved in iron transport and sorbitol metabolism and transport; a gene that is indispensable for the production of levan exopolysaccharide; and one gene that is associated with bacterial protection against the plant defense responses.

For nutritional iron requirements, two essential genes for \textit{E. amylovora’s} virulence have been studied by Dellagi \textit{et al.}, 1998. In this study, they created two mutants: one was \textit{dfoA::Mu}dIlpR13; which had a mutation in the desferrioxamine biosynthesis pathway; and 
\textit{foxR::Mu}dIlpR13; which was a mutation of the ferrioxamine receptor FoxR. The desferrioxamines are siderophores that \textit{E. amylovora} produces to obtain iron from the environment and the ferrioxamine receptor FoxR is a membrane protein which is used by the ferrioxamines to re-enter the bacterial cell. Neither of the mutants \textit{dfoA} or \textit{foxR} caused fire blight symptoms when inoculated onto apple flowers, but both caused disease in apple seedlings. The \textit{dfoA} mutant caused the same disease incidence as the wild-type in the apple seedlings,
while the foxR mutant had reduced disease incidence. These results suggest that there is more iron available inside the host tissue compared to the apple flowers and that E. amylovora needs to be able to get sufficient iron during its short epiphytic stage on flowers before infecting the host.

For nutritional sorbitol requirements, Aldridge et al., 1997, studied two genes of E. amylovora in the sorbitol (srl) operon. The srl operon includes the genes: srlA, srlE, srlB, srlD, srlM and srlR. They created two different mutants: one was an srlE mutant and the other was an srlD mutant. Neither of the mutants was able to use sorbitol because of the mutations. The mutants were less virulent when inoculated in apple seedlings compared to the wild-type. Sorbitol is the main form of transported carbon in apples trees; consequently, the srl mutants could not use the sorbitol available in the plant and therefore they could not cause disease. In the presence of sorbitol the srl operon was expressed at high levels and it was repressed in the presence of glucose.

Geier and Geider, 1993 studied the levansucrose gene called lsc, which is indispensable for the production of levan. During the levan production assay, the wild-type produced more levan than the levansucrase mutants, in fact sometimes the levansucrase mutants produced no levan at all. Two levansucrase mutants were chosen to continue the study, Ea7/74-LS6 and Ea7/74-LS7. These two mutants were less virulent than the wild-type at early time points but at 12 days post inoculation (dpi) the mutants caused the same fire blight symptoms as the wild-type did. The mutants had delayed disease symptoms. This suggests that the levansucrase could be more important during early infection than later when disease has fully developed.

About the gene associated with the bacterial defense reaction against the plant, Berry et al., 2009 showed that the waaL gene was a virulence gene. The waaL gene is indispensable for
lipopolysaccharide biosynthesis, which is a component of the outer membrane of the bacteria. The *waaL* mutant had a 20% reduction in survival compared to the *E. amylovora* wild-type after 5, 10, 15 and 20 minutes growing in hydrogen peroxide. The survival of the bacteria in hydrogen peroxide mimics the reaction in the apple and pear host. After the plant recognizes the HrpN and DspA/E proteins, it will induce an oxidative burst in order to kill the invader. The wild-type *E. amylovora* should be capable of surviving this oxidative stress in order to cause fire blight. The *waaL* mutant had reduced twitching motility compared to the wild-type and the complemented strain. Furthermore, the *waaL* mutant had less necrotic area compared to the wild-type and complemented strain at 5, 7 and 9 dpi when inoculated in immature pears.

**Third Category: Regulatory Genes**

It was not until almost the twenty first century when researches started studying the regulatory genes in *E amylovora*. To this day, there is one operon (*rcs*) and two genes (*hns* and *hrpL*) that have been studied.

Bereswill and Geider, 1997 studied the regulators of capsular synthesis *rcsA* and *rcsB* in *E. amylovora*. The *rcsA* and *rcsB* genes belong to the *rcs* cluster, which is a very well conserved cluster in different bacteria like *E.coli, E. stewartii, Klebsiella pneumonia* and *E. amylovora*. The *rcs* genes have been very well studied in *E.coli*, where they have been shown to control colonic acid production. Bereswill and Geider showed that *rcsA* and *rcsB* mutants produced less amylovoran than the wild-type. But the *rcsB* mutant produced even more levan than the wild-type. The overexpression of *rcsA* and *rcsB* makes the bacteria overproduce amylovoran but reduces levan production to almost zero. The *rcsB* mutant did not cause fire blight in apple seedlings but it caused disease in immature pear fruit.
Wang et al., 2009 studied the \textit{rcsB}, \textit{rcsC}, and \textit{rcsD} genes. The \textit{rcsC} promoter is in an opposite direction to the promoter of the other two genes, suggesting that it is expressed separately. None of the mutants were pathogenic in pears and they grew six-fold less than the wild-type in the pear tissue. The \textit{rcsB}, \textit{rcsD} and \textit{rcsBD} mutants did not produce amylovoran, but the \textit{rcsC} mutant produced four times more amylovoran than the wild-type. Furthermore, the \textit{rcs} mutants showed slightly reduced motility on swarming plates compared to the wild-type.

Hildebrand \textit{et al.}, 2006 showed that \textit{hns}, which encodes a histone-like protein H-NS, was a virulence gene. Disruption of the chromosomal \textit{hns} gene caused a delay in fire blight symptom development in pear fruit slices compared to the wild-type. The \textit{hns} mutant produced two-fold more levan and more amylovoran than the wild-type. When a plasmid with an \textit{hns} gene copy was introduced into wild-type \textit{E. amylovora}, the bacteria exhibited reduced amylovoran and levan production. They showed that H-NS protein is a gene expression regulator that binds to the \textit{lsc}-promoter, which is suggested to control the expression of the \textit{rlsb} gene, which is an activator of levansucrase expression. Furthermore, it was suggested that H-NS negatively regulates the expression of the \textit{ams} operon by negatively controlling \textit{rcsA}.

Wei & Beer, 1995 showed that the \textit{hrpL} gene, which is a single-gene operon, positively regulates the expression of most \textit{hrp} genes. The HrpL protein is required for the HR and for causing disease in the host.

Figure 1-1 summarizes all \textit{E. amylovora} genes mentioned herein. The pathogenicity genes are shown in blue (Type III SS, DspA/E and amylovoran), the virulence genes are shown in green (levansucrase, iron and sorbitol acquisition and the survival of oxidative stress) and the regulatory genes are shown in black (\textit{rcs} operon, \textit{hns} and \textit{hrpL} genes).
Fig. 1-1 Diagram of the genes in *E. amylovora* discussed in this chapter. The type III protein secretion system (Type III SS) is necessary for the bacteria to inject the HrpN and DspA/E proteins inside the host cell. HrpN helps to translocate the DspA/E protein inside the plant cell, where both are indispensable for the induction of host cell death. Amylovoran is required for the bacteria to protect themselves against plant defenses. Levan is another polysaccharide like amylovoran that helps the bacteria to protect itself against the host defense. *E. amylovora* nutrition requirements include iron and sorbitol. When the plant recognizes the proteins HrpN and DspA/E, it induces an oxidative burst that the *E. amylovora* needs to overcome in order to be able to cause disease and reach the xylem. The regulatory gene *hns* regulates the expression of the *rcs* genes and levan production negatively. The *rcs* operon regulates the expression of amylovoran and levan positively and negatively respectively. The *hrpL* gene regulates the expression of the *hrp* operon, including the expression of the *hrpN* and *dspA/E* genes.

**CONTRIBUTION OF THIS RESEARCH TO THE STUDY OF FIRE BLIGHT**

In our molecular study of *E. amylovora* genes, we focused our attention on one regulatory gene, *rpoN* and two basic metabolic genes, *argD* and *pyrC*. In order to find these three genes, an *E. amylovora* strain called HKN06P1 (6p1; Lee *et al.*, 2010), was randomly transformed with an engineered transposon Tn5. I screened around 2,000 Tn5 mutants using detached immature apple fruit for loss of pathogenicity or reduced virulence. The *rpoN* and the *argD* mutants did not cause disease in immature apples while the *pyrC* mutant had reduced virulence.
OBJECTIVES

- Acquire a better understanding of the virulence regulatory system of *E. amylovora*
- Acquire a better understanding of the nutrition requirements of *E. amylovora*
REFERENCES


Anderson HW. (1920) Disease of Illinois fruit; fire blight of apple; pear blight or fire blight. Ill. Agric. Exp. Stn. Circ. 241:41-45,74-78.


Fulton HR. (1911) The persistence of *Bacillus amylovorus* in pruned apples twigs. Phytopathol. 1:68.


Peter K. (May 8, 2014) Penn State University-Extension. Disease update: be on alert for fire blight conditions later this week. The Pennsylvania State University.


Rosen HR. (1933) The control of fire blight by the use of sprays. Am. Bee J. 73:165,169


CHAPTER TWO

THE FIRE BLIGHT PATHOGEN ERWINIA AMYLOVORA REQUIRES THE rpoN GENE FOR PATHOGENICITY IN APPLE

Chapter 2 was published in the journal of Molecular Plant Pathology (Ramos et al., 2013)
*Regarding this work accomplished, Judy Sinn helped me with the western blot assay and Emily Pfiefer helped me with the statistic analysis. Without their help it would not have been possible to submit the \textit{rpoN} manuscript within our deadline. The \textit{rpoN} manuscript was accepted with minor revisions. It was required for publication a pathogenicity assay in apple trees. Thanks to Brian Lehman and Noemy Halbrendt we were able to complete twice the assays and get our work published.
ABSTRACT

RpoN is a $\sigma^{54}$ factor regulating essential virulence gene expression in several plant pathogenic bacteria, including *Pseudomonas syringae* and *Pectobacterium carotovorum*. In this study, we found that mutation of *rpoN* in the fire blight pathogen *Erwinia amylovora* caused a non-pathogenic phenotype. The *E. amylovora rpoN* Tn5 transposon mutant *rpoN*1250::Tn5 did not cause fire blight disease symptoms on shoots of mature apple trees. In detached, immature apple fruits, the *rpoN*1250::Tn5 mutant failed to cause fire blight disease symptoms and grew to population levels eight orders of magnitude less than the wild-type. In addition, the *rpoN*1250::Tn5 mutant failed to elicit a hypersensitive response when infiltrated into non-host tobacco plant leaves, and *rpoN*1250::Tn5 cells failed to express HrpN protein when grown in *hrp*-inducing liquid media. A plasmid-borne copy of the wild-type *rpoN* gene complemented all the *rpoN*1250::Tn5 mutant phenotypes tested. The *rpoN*1250::Tn5 mutant was prototrophic on minimal solid and liquid media, indicating that the *rpoN*1250::Tn5 non-pathogenic phenotype was not due to a defect in basic metabolism or growth. This study provides clear genetic evidence that *rpoN* is an essential pathogenicity gene of *E. amylovora*, indicating that *rpoN* has similar function in *E. amylovora* as in *P. syringae* and *P. carotovorum*. 
INTRODUCTION

Fire blight is a very well-known plant bacterial disease, not only because it is the most economically important and destructive bacterial disease in apples and pears around the world, but also because it was the first disease of plants that was determined to be caused by bacteria (Arthur, 1885; Burrill, 1880; van der Zwet et al., 2012). The causal agent of fire blight is Erwinia amylovora and the disease is called fire blight because the infected parts of the tree look as if they were burned (Coxe, 1817; van der Zwet et al., 2012). The most important and characteristic sign of fire blight disease is bacterial ooze (van der Zwet et al., 2012). Fire blight has been a recognized disease problem for over two hundred years (Denning, 1794), and the disease continues to cause major sporadic losses, such as a $68 million loss in the northwestern United States in 1998 (Bonn, 1999) and a $42 million loss in the state of Michigan, USA, in 2000 (Busdieker, 2011). Unfortunately, fire blight threatens to be more economically important in the future, since the disease is still spreading around the world (Vanneste, 2000).

In recent decades, the development of molecular genetics techniques facilitated the identification of genetic factors that contribute to the ability of E. amylovora to cause fire blight disease. Three main classes of pathogenicity genes were found: ams, hrp, and dsp. The ams genes are involved in the biosynthesis of the exopolysaccharide amylovoran (Bugert and Geider, 1995). The hrp genes are required to trigger the hypersensitive response (HR) defense reaction in non-host plants and are necessary for pathogenicity in susceptible hosts. In E. amylovora, hrpL is necessary for the regulation and expression of most hrp genes (Wei and Beer, 1995); hrcC and hrcA encode structural components of the type III protein secretion system (T3SS) (Kim et al., 1997); and hrpN and hrpW encode proteins secreted via the T3SS that contribute to
E. amylovora pathogenicity and the HR in non-host leaves (Wei et al., 1992; Gaudriault et al., 1998; Kim and Beer, 1998). The dspA/E gene is required specifically for the fire blight disease process (Gaudriault et al., 1997; Bogdanove et al., 1998) and the DspA/E protein is translocated into host cells by the T3SS (Bocsanczy et al., 2008). HrpN assists in the translocation of DspA/E protein into the plant cell (Bocsanczy et al., 2008).

In an effort to identify additional genetic factors critical for E. amylovora pathogenicity, we mutated E. amylovora strain HKN06P1 (Table 1; Lee et al., 2010) using an engineered Tn5 transposon (EZ-Tn5<R6KγoriKan-2> Transposome Kit, Epicentre, Madison, WI, U.S.A.) and screened the mutants for loss of pathogenicity on detached immature apple fruit (Sinn et al., 2008; Lee et al., 2010). The ability of E. amylovora to grow and cause disease symptoms in immature apple fruits is a robust indicator of virulence in apple trees (Lee et al., 2010). One pathogenicity-defective E. amylovora mutant had a Tn5 insertion in the rpoN gene (Fig. 2-1). This mutant was named rpoN1250::Tn5 (Table 1) because the Tn5 transposon was inserted after rpoN coding region nucleotide number 1249. The location of the Tn5 insertion in rpoN1250::Tn5 was determined by Tn5 plasmid rescue and sequencing of the DNA flanking the Tn5 transposon. Briefly, rpoN1250::Tn5 genomic DNA was isolated (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, U.S.A.), digested with EcoRI (New England Biolabs, Ipswich, MA, U.S.A.), circularized with T4 DNA ligase (New England Biolabs), and introduced into Transformax EC100D pir-116 electrocompetent E. coli (Epicentre). Plasmid DNA was isolated from resulting kanamycin-resistant colonies (QIAquick Kit, Qiagen, Valencia, CA, U.S.A.) and sequenced at the Genomics Core Facility at The Pennsylvania State using sequencing primers provided with the EZ-Tn5<R6KγoriKan-2> Transposome Kit (Epicentre).
The position of the flanking DNA sequence in the *E. amylovora* strain CFBP 1430 genome (Smits *et al.*, 2010) was determined using NCBI's BLASTN program (Altschul *et al.*, 1990).

**Fig. 2-1** Representation of the *rpoN* genomic neighborhood, including the location of the Tn5 insertion in mutant *rpoN*1250::Tn5 and the genomic segment used for complementation.

**MATERIAL, METHODS, RESULTS AND DISCUSSION**

The *rpoN* gene is known to be essential for virulence in other important plant pathogenic bacteria (Hutcheson *et al.*, 2001; Chatterjee *et al.*, 2002). In *Pseudomonas syringae* pv. *syringae* and *Pectobacterium carotovorum* subsp. *carotovorum*, *rpoN* controls the expression of traits related to virulence, including the T3SS, flagella, alginate production (exopolysaccharide), and pili (Hutcheson *et al.*, 2001; Chatterjee *et al.*, 2002). Furthermore, in *P. syringae* pv. *glycinea* PG4180, *rpoN* controls the production of coronatine, a phytotoxin which functions as a virulence factor in bacterial blight of soybean (Alarcón-Chaidez *et al.*, 2003). It has also been demonstrated that *rpoN* controls flagellar protein expression, swimming and swarming, and
antifungal biocontrol activity of *Pseudomonas fluorescens* CHA0 (Péchy-Tarr et al., 2005). Expression of the *E. amylovora rpoN* gene is induced during infection of immature pear fruit tissue (Zhao et al., 2005), but the possible involvement of *rpoN* in *E. amylovora* pathogenicity has not been tested.

The *E. amylovora rpoN* gene encodes a predicted RNA polymerase $\sigma^{54}$ factor subunit. Homologous RpoN $\sigma^{54}$ factors are required for the activation and expression of virulence genes, including *hrp* genes, in plant pathogenic bacteria like *P. carotovorum* ssp. *carotovorum* (Chatterjee et al., 2002) and *P. syringae* pv. *syringae* (Xiao et al., 1994; Hutcheson et al., 2001). It has also been demonstrated that the RpoN $\sigma^{54}$ factor is part of a complex that activates the expression of *hrpL* in *P. syringae* pv. *syringae* (Xiao et al., 1994). Based on sequence similarity of *hrpL* and *rpoN* genes in *P. syringae* pv. *syringae* and *E. amylovora*, it has been hypothesized that RpoN is also part of a complex that activates the expression of *hrpL* in *E. amylovora* (Vanneste, 2000), which would probably make RpoN an essential pathogenicity and virulence regulator in *E. amylovora*. This is in contrast with the *E. amylovora* RpoS alternative sigma factor, which is required for environmental stress tolerance, but not for disease processes in apple (Anderson et al., 1998).

Indeed, the *rpoN*1250::Tn5 mutant did not cause fire blight symptoms in mature ‘Fuji’ trees inoculated by shoot tip wounding (Fig. 2-2). The *rpoN*1250::Tn5 mutant also did not cause fire blight disease symptoms in immature apple fruits at 7 days post inoculation (dpi) (Fig. 2-3A), and *rpoN*1250::Tn5 populations were 8 orders of magnitude lower than the wild-type in apple fruits at 7 dpi (Fig. 2-3B). The *rpoN* gene was amplified from wild-type *E. amylovora* HKN06P1 genomic DNA using forward primer 5´-CAGCGGCCTTGGCGTCTTGA-3´ and reverse primer 5´-GCCGCGTTTACATGCAGCG-3´ and 12 PCR cycles with a 60°C
annealing temperature. The resulting 1,792 bp product was cloned into pCR2.1 (TOPO TA Kit, Invitrogen, Grand Island, NY, U.S.A.), creating pCR2.1-rpoN (Table 1), and sequenced at the Pennsylvania State University Genomics Core Facility using T7 and M13R as forward and reverse primers respectively. This cloned segment of \textit{E. amylovora} HKN06P1 DNA was identical in sequence to that of the \textit{E. amylovora} CFBP 1430 reference genome, and included the entire \textit{rpoN} gene as well as 116 bp and 49 bp of flanking genes \textit{yhbH} and \textit{yhbG}, respectively (Fig. 2-1). Plasmids pCR2.1 (empty cloning vector; Table 1) and pCR2.1-rpoN were introduced into \textit{rpoN1250::Tn5} using electroporation, creating \textit{E. amylovora} strains \textit{rpoN1250::Tn5(pCR2.1)} and \textit{rpoN1250::Tn5(pCR2.1-rpoN)}, respectively (Table 1). Strain \textit{rpoN1250::Tn5(pCR2.1-rpoN)} caused fire blight symptoms when inoculated onto mature apple tree shoots and immature apple fruit, while strain \textit{rpoN1250::Tn5(pCR2.1)} did not (Fig. 2-2 and Fig. 2-3A). In addition, strain \textit{rpoN1250::Tn5(pCR2.1-rpoN)} population sizes in inoculated apple fruits were indistinguishable from wild-type population sizes at 7 dpi, while strain \textit{rpoN1250::Tn5(pCR2.1)} populations were similar to \textit{rpoN1250::Tn5} (Fig. 2-3B). Thus, pCR2.1-rpoN complemented the \textit{rpoN1250::Tn5} mutant pathogenicity defect on apple tree shoots and immature apple fruit. This indicates that the \textit{rpoN} gene is specifically required for \textit{E. amylovora} pathogenicity in apple and confirms that the \textit{rpoN1250::Tn5} pathogenicity defect is due to the Tn5 transposon insertion in the \textit{rpoN} gene.
Fig. 2-2 The *rpoN* gene was required for *E. amylovora* pathogenicity on apple tree shoots. Three-year-old, dormant, bare-rooted ‘Fuji’ apple trees were potted and grown in a greenhouse for 7 weeks and wound-inoculated on actively-growing shoot tips with the indicated *E. amylovora* strains at $1 \times 10^8$ cfu/ml in 10 mM MgCl$_2$. Twenty shoots total were inoculated with each strain; four trees were used for each strain and five shoots were inoculated on each tree. Each tree was inoculated with one strain only. Mock-inoculated shoots were wounded and inoculated with 10mM MgCl$_2$. The entire experiment was performed twice, with similar results each time. Inoculations were done as described previously (Jensen et al., 2003). The extent of shoot necrosis at 7, 14, and 21 days post inoculation (dpi) is shown. The extent of shoot necrosis was calculated by dividing the length of the necrotic portion of the shoot, measuring from the shoot tip, and dividing by total shoot length measured from the shoot tip to the shoot junction with the previous year’s wood, and expressed as a percent.
The *rpoN* gene was required for *E. amylovora* pathogenicity on immature apple fruits. (a) Immature ‘Gala’ apple fruit halves at 7 days post inoculation (dpi) with $2 \times 10^6$ CFU of the indicated bacterial strains into a wound on the surface of the fruit as described previously (Lee et al., 2010). Tissue necrosis and bacterial ooze are a symptom and a sign, respectively, of *E. amylovora* infection in immature apple fruits. (b) Bacterial populations in immature ‘Gala’ apple fruits halves at 7 dpi with $2 \times 10^6$ CFU of the indicated bacterial strains. Bars with the same letter are not significantly different according to a Tukey test ($\log_{10}$). The same apples shown in (a) were used for the assay shown in (b). The experiment was repeated three times with at least 5 fruit halves inoculated per strain. Every experiment repetition had similar results; results of representative experiments are shown.
E. amylovora elicits a hypersensitive response (HR) when infiltrated into the leaves of non-host tobacco plants (Giorgi et al., 2005). The HR is a mechanism used by plants that allows them to prevent the spread of pathogens inside their tissue and it is characterized by rapid, local cell death in the infected zone (Stakman, 1915; Morel et al., 1997). The degree of HR elicitation by E. amylovora in tobacco plants is correlated with virulence in apple fruits (Lee et al., 2010). To determine whether the rpoN1250::Tn5 mutant was defective in causing an HR on tobacco, the wild-type and the rpoN1250::Tn5 mutant strains were infiltrated into tobacco leaves. Wild-type E. amylovora caused an HR after 24 hours, while rpoN1250::Tn5 did not (Fig. 2-4). Furthermore, rpoN1250::Tn5(pCR2.1-rpoN) caused an HR, while rpoN1250::Tn5(pCR2.1) did not (Fig. 2-4), indicating that plasmid pCR2.1-rpoN complemented the rpoN1250::Tn5 mutant HR defect.

![Image](image_url)

**Fig. 2-4** The rpoN gene was required for the E. amylovora hypersensitive response (HR) in tobacco. Tobacco (Nicotiana tabacum L. cv. Glurk) leaf sections were infiltrated with suspensions of $1 \times 10^8$ CFU/mL in 10 mM MgCl$_2$ of the indicated strains using a syringe without a needle, as described previously (Sinn et al., 2008). The HR was indicated by collapse and death of the infiltrated region. The HR was scored at 24 hours post inoculation (hpi); photograph was taken at 72 hours hpi for better contrast. The tobacco plants were six weeks old. This experiment was repeated three times with at least eight tobacco plants used for each experiment. The experiment had identical results each time; a representative leaf is shown.
The T3SS-secreted HrpN protein is a critical component of *E. amylovora* virulence in hosts and is required for elicitation of the HR in tobacco (Wei *et al*., 1992). A defect in HrpN expression or secretion might contribute to the pathogenicity and HR defects of *rpoN1250::Tn5*. Therefore, we tested whether HrpN was expressed and secreted by *rpoN1250::Tn5* cells growing in *hrp*-inducing culture medium (Huynh *et al*., 1989; Sinn *et al*., 2008). Protein samples from *E. amylovora* cell pellet and cell-free culture supernatant fractions of were prepared as previously described (Lee *et al*., 2010; Sinn *et al*., 2008). The proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with a polyclonal anti-HrpN antibody (gift of S.V. Beer, Cornell University; Wei *et al*., 1992). As expected, HrpN protein was detected in wild-type cells and culture supernatant; in contrast, HrpN was not detected in cells or supernatant of *rpoN1250::Tn5* (Fig. 2-5). Furthermore, HrpN was detected in *rpoN1250::Tn5(pCR2.1-rpoN)* cells and supernatant, while HrpN was not detected in *rpoN1250::Tn5(pCR2.1)* cells and supernatant (Fig. 2-5). These results indicate that *rpoN1250::Tn5* mutant cells did not express HrpN protein in *hrp*-inducing media and that pCR2.1-*rpoN* restored HrpN expression and secretion by *rpoN1250::Tn5* cells. A *hrcC118::Tn5* T3SS mutant (Table 1) had detectable HrpN in the cells, but not the supernatant (Fig. 2-5) as expected (Wei *et al*., 2000). These results indicate that a lack of HrpN expression likely contributed to the pathogenicity and HR defects of the *rpoN1250::Tn5* mutant.
**Fig. 2-5** The *rpoN* gene was required for HrpN protein expression in cultured *E. amylovora* cells. Protein gel immunoblots detecting HrpN protein in cell pellets and cell-free culture supernatants are shown. The indicated strains were grown under *hrp*-inducing conditions and proteins from cell pellets and cell-free culture supernatant fractions were subjected to immunoblot analysis to detect the presence of HrpN protein as described previously (Sinn *et al*., 2008). The entire experiment was conducted twice with identical results.

In order to determine whether basic metabolic processes were intact in *rpoN1250::Tn5*, the ability of the *rpoN1250::Tn5* mutant to grow on minimal media was compared to that of the wild-type. The *rpoN1250::Tn5* mutant grew as much as the wild-type in liquid MM with 0.4% sorbitol (Fig. 2-6). These results indicate that *rpoN1250::Tn5* is a prototroph, and that the *rpoN1250::Tn5* defect in pathogenicity was not due to a disruption of a basic metabolic process that might affect its ability to grow in the host.
The *rpoN*1250::Tn5 mutant was not auxotrophic. (a) Growth of indicated strains streaked onto M9 minimal media (MM) agar with 0.4% sorbitol after 48 hours of growth at 28°C. (b) Growth of indicated strains in liquid MM with 0.4% sorbitol after 48 hours of shaking at 150 rpm at 28°C. The auxotrophic strain had a Tn5 insertion in the *porF* gene, which is predicted to encode an amidophosphoribosyltransferase. There was no significant difference between wild-type and *rpoN*1250::Tn5 at zero or 48 hours, based on a Tukey test (log₁₀). Experiments were repeated three times with similar results each time; results of representative experiments are shown.

Our results indicate that *rpoN* is essential for *E. amylovora* to cause fire blight symptoms in apple tree shoots and to grow and cause fire blight symptoms in detached, immature apple fruits. Our results also indicate that *rpoN* is essential for the *E. amylovora*-induced HR in non-host tobacco plant leaves. The results also show that the *E. amylovora rpoN* gene is required for production of HrpN protein in *hrp*-inducing media. The lack of HrpN protein production by *rpoN*184::Tn5 likely contributes to the lack of pathogenicity of *rpoN*184::Tn5 on apple fruits and shoots and the inability of *rpoN*184::Tn5 to trigger an HR in tobacco. Complementation of the *rpoN*1250::Tn5 mutant phenotype with pCR2.1-*rpoN* indicates that pCR2.1-*rpoN* contained a functional copy of the *rpoN* gene. Since the *rpoN*1250::Tn5 mutant was not an auxotroph, its
pathogenicity and non-pathogenicity defects are probably not due to a basic metabolic or growth defect. Instead, our results indicate that \textit{rpoN} plays a specific role in \textit{E. amylovora} virulence. The pathogenicity defect of the \textit{rpoN184::Tn5} mutant and its lack of HrpN expression are consistent with RpoN being a key virulence system expression regulator in \textit{E. amylovora}, just as it is in \textit{P. syringae} and \textit{P. carotovorum}.

\section*{ACKNOWLEDGEMENTS}

We thank Steven A. Lee for creating the \textit{E. amylovora} Tn5 mutant library that we screened, and we thank Steven V. Beer for providing the anti-HrpN polyclonal antibody. L.S.R. was supported in part by a Bunton-Waller graduate fellowship and an Alfred P. Sloan Foundation Graduate Scholarship; this work was supported in part by the Plant Pathology & Environmental Microbiology Department at The Pennsylvania State University.
REFERENCES


**Table 2-1** Plasmids and bacterial strains used in this work.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source, reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1</td>
<td>PCR cloning vector without an insert</td>
<td>TOPO TA Kit, Invitrogen</td>
</tr>
<tr>
<td>EZ-Tn5-rpoN</td>
<td>Plasmid rescued from ( rpoN_{1250}::\text{Tn5} )</td>
<td>This work</td>
</tr>
<tr>
<td>pCR2.1-rpoN</td>
<td>( rpoN ) cloned in pCR2.1</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Bacterial Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( E. amylovora ) strain HKN06P1</td>
<td>Wild-type strain</td>
<td>Lee <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>( hrcC_{118}::\text{Tn5} )</td>
<td>( E. amylovora ) HKN06P1 carrying a Tn5 transposon insertion in the ( hrcC ) gene</td>
<td>This work</td>
</tr>
<tr>
<td>( \text{porF auxotroph} )</td>
<td>( E. amylovora ) HKN06P1 carrying a Tn5 transposon insertion in the ( \text{porF} ) gene</td>
<td>This work</td>
</tr>
<tr>
<td>( rpoN_{1250}::\text{Tn5} )</td>
<td>( E. amylovora ) HKN06P1 carrying a Tn5 transposon insertion in the ( rpoN ) gene</td>
<td>This work</td>
</tr>
<tr>
<td>( rpoN_{1250}::\text{Tn5(pCR2.1)} )</td>
<td>( rpoN_{1250}::\text{Tn5} ) carrying pCR2.1</td>
<td>This work</td>
</tr>
<tr>
<td>( rpoN_{1250}::\text{Tn5(pCR2.1-rpoN)} )</td>
<td>( rpoN_{1250}::\text{Tn5} ) carrying pCR2.1-rpoN</td>
<td>This work</td>
</tr>
</tbody>
</table>
CHAPTER 3

MUTATION OF THE \textit{ERWINIA AMYLOVORA} \textit{argD} GENE CAUSES ARGinine
AUXOTROPHY, NON-PATHOGENICITY IN APPLE AND REDUCED VIRULENCE IN
PEAR

Chapter 3 will be published in Applied and Environmental Microbiology (Ramos \textit{et al}., in press)
*Regarding this work accomplished, Brian Lehman and Kari Peters helped us with the pathogenicity assay in apples trees. Their work was indispensable for the publication of the argD manuscript.
ABSTRACT

Fire blight is caused by *Erwinia amylovora* and is the most destructive bacterial disease of apples and pears worldwide. In this study, we found that the *E. amylovora argD* Tn5 transposon mutant *argD*1000::Tn5 was an arginine auxotroph that did not cause fire blight in apple and had reduced virulence in immature pear fruits. The *E. amylovora argD* gene encodes a predicted N-acetylcornithine aminotransferase enzyme, which is involved in the production of the amino acid arginine. A plasmid-borne copy of the wild-type *argD* gene complemented both the non-pathogenic and the arginine auxotrophic phenotypes of *argD*1000::Tn5. However, even when mixed with pathogenic or wild-type *E. amylovora* and inoculated onto immature apple fruit, the *argD*1000::Tn5 mutant still failed to grow, while the pathogenic strain grew and caused disease. Furthermore, the pCR2.1-*argD* complementation plasmid was stably maintained in the *argD*1000::Tn5 mutant growing in host tissues without any antibiotic selection. Therefore, the pCR2.1-*argD* complementation plasmid could be useful for expression of genes, markers, and reporters in *E. amylovora* growing in planta, without concern about losing the plasmid over time. The ArgD protein cannot be considered an *E. amylovora* virulence factor because the *argD*1000::Tn5 mutant was auxotrophic and had a primary metabolism defect. Nevertheless, these results are informative about the parasitic nature of the fire blight disease interaction, since they indicate that *E. amylovora* cannot obtain sufficient arginine from apple and pear fruit tissues, or from apple vegetative tissues, neither at the beginning of the infection process, nor after the infection has progressed to an advanced state.
INTRODUCTION

Fire blight is the most economically important and destructive bacterial disease in apples and pears that exists today. It is also a very well-known bacterial disease, in part because it was the first bacterial plant disease that was discovered (Burrill, 1880, Arthur, 1885). The causal agent of fire blight disease is the bacterium *Erwinia amylovora*, a nonobligate pathogen (Santander *et al*., 2014). When *E. amylovora* infects the different parts of an apple tree, they look as if they were burned; therefore the disease was named fire blight (Coxe, 1817). The bacterial ooze exuding from infected apple tree bark and/or infected apple tree fruit is the most important and characteristic sign of fire blight disease (Burrill, 1880, Arthur, 1885). *E. amylovora* also infects other members of the Rosaceae family, including quince, loquat, hawthorn, and other ornamentals (Momol and Yegen, 1993; Zilberstaine *et al*., 1996; van der Zwet and Keil, 1979).

Fire blight has been documented as an important plant disease problem for more than two hundred years (Denning, 1794). Unfortunately, fire blight disease continues to be the reason for major sporadic losses, for example a $68 million loss in the northwestern part of The United States in 1998 (Bonn, 1999) and a $42 million loss in the state of Michigan, USA, in 2000 (Norelli *et al*., 2003). Fire blight is also a perennial problem for apple and pear growers in regions where the disease is endemic (Vanneste, 2000). Furthermore, fire blight now threatens to be even more economically significant, as the disease continues to spread all around the world (Vanneste, 2000).

Since the 1980’s, scientists have been working to identify *E. amylovora* genetic factors that contribute to the capacity of the bacteria to cause fire blight in apple and pear. The *amylovoran synthesis (ams), hypersensitive response and pathogenicity (hrp), and disease*
specific \((\text{dsp})\) genes are some of the most studied and most important \textit{E. amylovora} virulence genes discovered to date (Bugert and Geider 1995; Wei and Beer 1995; Kim \textit{et al.}, 1997; Wei \textit{et al.}, 1992; Gaudriault \textit{et al.}, 1998; Kim and Beer, 1998; Gaudriault \textit{et al.}, 1997; Bogdanove \textit{et al.}, 1998; Bocsanczy \textit{et al.}, 200812).

Novel \textit{E. amylovora} genes contributing to virulence and pathogenicity continue to be discovered to this day, including recent examples such as \textit{waaL}, \textit{rpoN}, \textit{rcs}, \textit{luxS} and \textit{hns} (Rezzonico and Duffy, 2007; Wang \textit{et al.}, 2009; Ramos \textit{et al.}, 2013; Ancona \textit{et al.}, 2014; Berry \textit{et al.}, 2009; Hildebrand \textit{et al.}, 2006). These genetic studies provide a better understanding of the pathogen and have the potential to provide new methods to manage the disease. For example, salicylidene acylhydrazides can suppress the expression of type III secretion and amylovoran biosynthesis genes in \textit{Erwinia amylovora}, indicating that small molecules have the potential to be developed as fire blight controls (Yang \textit{et al.}, 2014). This is of practical importance, because with the exception of the antibiotic streptomycin, there are no registered products in the United States that can effectively control fire blight (Dunegan \textit{et al.}, 1954; Goodman, 1954; Morgan and Goodman, 1955; Shaffer and Goodman, 1969). In fact 90\% of the streptomycin used in the United States in agriculture is used to control fire blight (McManus \textit{et al.}, 2002). Unfortunately, streptomycin resistance has been detected in \textit{E. amylovora} field populations in different parts of the world including the United States, Mexico and Canada (Moller \textit{et al.}, 1981; Sholberg \textit{et al.}, 2001; Ponce de León Door \textit{et al.}, 2013).

In order to discover additional genetic aspects critical for \textit{E. amylovora} pathogenicity, we mutagenized \textit{E. amylovora} strain HKN06P1 (Table 1; 36) with an engineered Tn5 transposon. The \textit{E. amylovora} mutants were screened for loss of virulence on immature apple fruits. One of the \textit{E. amylovora} mutants that did not cause disease in immature apple fruits had a Tn5
transposon insertion in the \textit{argD} gene. The \textit{E. amylovora} \textit{argD} gene is predicted to encode an enzyme called N-acetylornithine aminotransferase, which is involved in step number four of the arginine biosynthesis pathway (Sakanyan \textit{et al}., 1992; Riley and Glansdorff, 1983; Ledwidge and Blanchard, 1999; Qu \textit{et al}., 2007). N-acetylornithine aminotransferase transfers an amino group from L-glutamate to the aldehydic carbon of N-acetyl-L-glutamate semialdehyde to produce N-acetyl-L-ornithine (Ledwidge and Blanchard, 1999). Like most aminotransferases, N-acetylornithine aminotransferase is dependent on pyridoxal 5'-phosphate as catalytic cofactor (Hartmann \textit{et al}., 2003.). In \textit{E. amylovora}, the \textit{argD} gene does not appear to be part of an operon and is separated from the rest of the \textit{arg} genes, just as in \textit{Escherichia coli} and \textit{Xanthomonas campestris} (Riley and Glansdorff, 1983; Ledwidge and Blanchard, 1999; Qu \textit{et al}., 2007). However, in some other bacteria, such as \textit{Streptomyces clavuligerus}, all the \textit{arg} genes, including \textit{argD}, are clustered in an operon (Rodríguez-García \textit{et al}., 2000.). The \textit{E. coli} ArgD protein is involved lysine biosynthesis as well (Ledwidge and Blanchard, 1999); however, this dual enzymatic capacity of ArgD has not been demonstrated in \textit{E. amylovora}. In the present study, we show that mutation of the \textit{E. amylovora argD} gene causes arginine auxotrophy, non-pathogenicity in apple and reduced virulence in immature pear fruits.

**MATERIALS AND METHODS**

**Screening process for loss of pathogenicity.**

\textit{E. amylovora} strain HKN06P1 was mutagenized with an engineered Tn5 transposon according to the manufacturer’s instructions (EZ-Tn5<R6KγoriKan-2> Transposome Kit, Epicentre, Madison, WI, U.S.A.). Around 2,000 \textit{E. amylovora} mutants were screened for loss of pathogenicity in detached immature ‘Gala’ apple fruits. During the initial round of screening,
one immature apple fruit was inoculated per mutant. Mutants that did not cause disease by 10 days post inoculation (dpi) were then inoculated onto 5 immature apples during the second screening round. Mutants that did not cause disease during the second screening were inoculated onto 10 apples in the third screening. During the third screening, the 10 apples were inoculated with 20 µl of mutant bacterial suspension in 10 mM MgCl₂ with an absorbance of 0.1 (λ = 600), as measured using a Spectronic 20+ spectrophotometer (Spectronic Instruments Inc., Irvine, CA, USA). Screening conditions were 28°C and relative humidity (RH) of 100%. The \textit{argD}1000::Tn5 (Table 1) mutant did not cause fire blight disease in detached immature apple fruits and was selected for further study.

**Molecular characterization of the Tn5 transposon insertion in the argD1000::Tn5 mutant**

Genomic DNA (gDNA) was isolated from the \textit{argD}1000::Tn5 mutant (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, U.S.A.). 5 µg of \textit{argD}1000::Tn5 gDNA were digested in a total reaction volume of 100 µl, with 10µl of 10X EcoRI enzyme buffer and 2µl of EcoRI enzyme (New England Biolabs, Ipswich, MA, U.S.A.). The reaction was run overnight at 37°C with a 50 µl of mineral oil overlay. The digestion reaction was cleaned (Qiagen QIAquick Gel Extraction Kit, Valencia, CA, U.S.A.) and eluted in 35 µl of EB buffer. \textit{argD}1000::Tn5 gDNA EcoRI fragments were circularized with 10µl of 10X buffer T4, 2µl of T4 DNA ligase (New England Biolabs) in a 100 µl reaction volume at room temperature overnight. Then, the ligation reaction was cleaned (QIAquick Gel Extraction Kit) and eluted in 20 µl of EB. The circularized \textit{argD}1000::Tn5 gDNA fragments were introduced into Transformax EC100D pir-116 electrocompetent \textit{E. coli} (Epicentre) using an Electroporator 2510 (Eppendorf AG, Hamburg, Germany) at 2,500 volts. EZ-Tn5-\textit{argD} plasmid DNA (Table 1) was isolated from
resulting kanamycin-resistant colonies (QIAquick Kit, Qiagen, Valencia, CA, U.S.A.) and used as template for DNA sequencing according to the EZ-Tn5<\textit{R6K} \gamma \textit{oriKan-2}> Transposome Kit manufacturer instructions (Epicentre) at the Genomics Core Facility at The Pennsylvania State University at University Park. The position of the flanking DNA sequence was then determined on the \textit{E. amylovora} strain CFBP 1430 genome sequence (Smits \textit{et al.}, 2010.) using NCBI's BLASTN program (Altschul \textit{et al.}, 1990.). This mutant was named \textit{argD1000::Tn5} because the Tn5 transposon was inserted after the \textit{argD} gene coding region nucleotide number 999.

\textbf{Polymerase chain reaction protocols.}

The polymerase chain reaction (PCR) was used to amplify the \textit{argD} gene from wild-type \textit{E. amylovora} HKN06P1 gDNA using a thermal cycler (Eppendorf), oligonucleotide primers P1 and P2 (Table 1), and Advantage 2 polymerase reaction mix (Mountain View, CA, USA). The thermal cycler was programmed for 2 minutes at 95°C followed by 12 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 90 seconds at 72°C.

To verify the position of the Tn5 transposon in the chromosome of \textit{argD1000::Tn5}, PCR was performed using primers P1, P2, FP and RP (Table 1). The pre-heating, denaturation, annealing and extension time and temperature were the same as described above, except that a 4 minute extension time was used to amplify the \textit{argD1000::Tn5} mutant \textit{argD} gene including the Tn5 transposon insertion.

\textbf{Plasmid introductions and complementation of the \textit{argD1000::Tn5} mutant.}

The amplified wild-type \textit{E. amylovora} HKN06P1 \textit{argD} gene PCR product of 1,448 base pairs (bps) was cloned into pCR2.1 (TOPO TA Kit, Invitrogen, Grand Island, NY, USA)
according to the manufacturer’s instructions, creating pCR2.1-argD (Table 1). The pCR2.1-argD insert was sequenced at the Genomics Core Facility (The Pennsylvania State University, University Park) using T7 and M13R as reverse and forward primers, respectively. The cloned segment included 145 bps upstream of the argD gene start codon and 90 bps downstream of the stop codon. These upstream and downstream DNA segments included 77 bps and 58 bps of the argD flanking genes yhfK and pabA, respectively. The 1,448 bps cloned segment was identical in sequence to that of the E. amylovora CFBP 1430 reference genome. Plasmids were introduced into E. amylovora competent cells using Electroporator 2510 (Eppendorf).

Quantitative growth analysis in M9 minimal media.

The wild-type strain was grown overnight in LB broth; the argD1000::Tn5 mutant was grown overnight in LB broth supplemented with 50 µg/ml of kanamycin (Sigma-Aldrich, St. Louis, MO, U.S.A.); and strains argD1000::Tn5(pCR2.1) and argD1000::Tn5(pCR2.1-argD) (Table 1) were grown overnight in LB broth supplemented with 100 µg/ml of carbenicillin (RPI, Mount Prospect, IL, U.S.A.). All the strains were grown at 28°C with rotary shaking at 200 rpm. Cell were pelleted at 13,000 rpm for 1 minute and re-suspended in a 1.5 µl centrifuge tube and washed with 10 mM MgCl$_2$, twice. All strains were re-suspended in 10 mM MgCl$_2$ to a final absorbance of 0.1 ($\lambda = 600$). Then, 3.5 ml of this re-suspension was added to 33.5 ml of M9 minimal media broth supplemented 0.5 mM thiamine, and 1 mM nicotinic acid (M9TN media) with 2 mM sorbitol as the carbon source. Cultures were grown at 28°C with rotary shaking at 150 rpm. Each strain population was quantified at 0 and 48 hours after starting the culture by serial dilution plating (Sambrook et al., 1989; Sinn et al., 2008).
For the arginine auxotrophy tests, strains were grown overnight, pelleted, and re-suspended in 10 mM MgCl₂ to an absorbance of 0.1 (λ = 600) as described above. Then, 3.5 ml of this re-suspension was added to 33.5 ml of M9TN with 2 mM sorbitol and 0.1 mg/ml arginine (Sigma-Aldrich). Cultures were grown at 28°C with rotary shaking at 150 rpm. Culture populations were quantified at 0 and 48 hours after starting the culture by serial dilution plating.

**Pathogenicity assay in apple trees**

Two year-old, dormant, bare-rooted ‘Gala’ apple trees with EMLA 26 rootstocks (Adams County Nursery, Aspers, PA, USA) were potted and grown in a greenhouse for seven weeks. Then the trees were wound-inoculated on actively-growing shoot tips with the indicated *E. amylovora* strains at 1×10⁸ cfu/ml in 10 mM MgCl₂, as described previously (Jensen et al., 2003). Four trees were used for each strain and five shoots were inoculated on each tree. The average size of the branches used was 41.4 cm (st. dev: ± 6.62 cm). Each tree was inoculated with one strain only. Mock-inoculated shoots were wounded and treated with 10 mM MgCl₂. The entire experiment was performed twice. The extent of shoot necrosis was measured at 7, 14, and 21 days post inoculation (dpi) and calculated by dividing the length of the necrotic portion of the shoot, measuring from the shoot tip, and dividing by total shoot length measured from the shoot tip to the shoot junction with the previous year’s wood, and expressed as a percent.

**Quantitative growth analysis in immature apple and pear fruits.**

Immature apple and pear fruits were collected at The Pennsylvania State University Fruit Research and Extension Center orchards in Biglerville, PA. Bacteria were suspended in 10 mM MgCl₂ to an absorbance of 0.1 (λ = 600). Then, 20 µl of this cell suspension was introduced
onto wounds on the skin side of halved immature ‘Gala’ apple and ‘Bosc’ pear fruits (Sinn et al., 2008). Bacterial populations in the immature fruits were quantified at 0 and 7 dpi by macerating the apples in 10 mM MgCl₂, followed by serial dilution plating, and expressed as colony-forming units (CFU) per gram of plant tissue (Lee et al., 2010.).

The nalidixic acid-resistant derivative of the wild-type E. amylovora HKN06P1 strain (HKN06P1⁴nalR; Table 1) was created by plating E. amylovora HKN06P1 from the -80°C glycerol stock on LB plates without antibiotics and growing them for two days at 28°C. Then, all the bacteria that had grown were spread evenly across LB plates supplemented with 25 µg/ml of nalidixic acid (Sigma-Aldrich). Resulting nalidixic acid-resistant colonies were tested for pathogenicity in immature apple fruit.

For mixed inoculations, HKN06P1⁴nalR and argD1000::Tn5 were separately suspended in 10 mM MgCl₂ to an absorbance of 0.1 (λ = 600). Then, a 1:1 mixture was prepared and immature apple fruit halves were inoculated with 20 µl of the combination. The population densities were measured by serial dilution plating at 0 and 7 dpi. The serial dilutions were plated on LB supplemented with 25 µg/ml of nalidixic acid for selecting HKN06P1⁴nalR and on LB supplemented with 50 µg/ml of kanamycin for selecting argD1000::Tn5.

**Plasmid stability assay in apple trees and immature apple fruits**

Inoculated apple tree shoots were collected at 21 dpi, and the bases of the shoots (2-3 cm segments) were surface-disinfected with 50% bleach for 5 minutes, rinsed once with de-ionized water, and ground using a mortar and pestle. In the case of the apple fruit, at 7 dpi the whole apple was ground in 1 ml of 10 mM MgCl₂. Extracts from apple tree shoots and apple fruits inoculated with argD1000::Tn5(pCR2.1-argD) were plated on LB supplemented with 50 µg/ml
of kanamycin and on LB supplemented with 50 µg/ml of kanamycin + 100 µg/ml of carbenicillin. Extracts from apple trees and apple fruits inoculated with HKN06P1\textsuperscript{NaIR}(pCR2.1) and HKN06P1\textsuperscript{NaIR}(PCR2.1-argD) (Table 1) were plated on LB supplemented with 25 µg/ml with nalidixic acid and on LB supplemented with 25 µg/ml of nalidixic acid + 100 µg/ml of carbenicillin. The apple tree and apple fruit extracts were processed separately.

**Statistical Analysis**

Statistical analyses were performed using PROC GLM in SAS (version 9.2, SAS Institute, Cary, NC). The strains were analyzed as the independent variable, and the growth (log\textsubscript{10} cfu) as the dependent variable. *Post-hoc* comparisons were evaluated using the Tukey test, with $\alpha=0.05$. Error bars represent the standard error of the mean of three, four or five replicate samples per strain. The number of replicates varied depending on the assay.

**RESULTS**

**Auxotrophy assays.**

One of the non-pathogenic mutants that we identified had a Tn5 transposon insertion in the *argD* gene (*argD\textsubscript{1000}:Tn5; Fig. 3-1). The *argD\textsubscript{1000}:Tn5* mutant was expected to be auxotrophic due to a disruption of the arginine biosynthesis pathway. As expected, the *argD\textsubscript{1000}:Tn5* mutant did not grow in M9 minimal media supplemented with thiamine and nicotinic acid (M9TN) media with sorbitol as the carbon source, while the wild-type *E. amylovora* grew in the same media (Fig. 3-2). Supplementation of M9 minimal media with thiamine and nicotinic acid allows wild-type *E. amylovora* bacteria to grow by several orders of magnitude; in contrast, wild-type *E. amylovora* does not grow by even a single order of
magnitude in M9 minimal in the absence of thiamine and nicotinic acid (23). Therefore, the use of M9TN media allows for easier identification of auxotrophic *E. amylovora* mutants.

![Diagram of gene positions](image)

**Fig 3-1** Illustration of the *argD* gene and flanking genes *yhfK* and *pabA* showing the genome nucleotide position of the Tn5 insertion in the *argD*1000::Tn5 mutant and the genomic DNA segment used for complementation (A). Genome nucleotide positions are numbered according to their homologous positions in the *E. amylovora* CFBP 1430 complete genome sequence. PCR primer (Table 1) hybridization locations and directions are indicated. PCR analysis and agarose gel electrophoresis was used to verify Tn5 transposon insertion in the *argD* gene in the *argD*1000::Tn5 mutant (B). A 1,448 bp PCR product was amplified from wild-type genomic DNA (gDNA), but not from *argD*1000::Tn5 mutant (*argD*) gDNA, using primers P1 and P2, as expected. When PCR extension time was increased to 4 minutes, using primers P1 and P2, a 3,449 bp PCR product was amplified from *argD*1000::Tn5 mutant gDNA, representing the *argD*
gene interrupted by Tn5. As expected, primer combinations P1 and RP, and FP and P2 amplified 303 and 1,145 bp products, respectively, further confirming the presence of a Tn5 transposon insertion in the argD gene of the argD1000::Tn5 mutant. Control PCR reactions with no gDNA template produced no PCR products.

**Fig 3-2** Auxotrophy of the argD1000::Tn5 mutant. Bacterial population densities of the indicated strains growing in M9 minimal media supplemented with thiamine and nicotinic acid (M9TN media) and sorbitol as the carbon source are shown at 0 and 48 hours after starting the culture, as determined by serial dilution plating. Values are the mean of three replicates and error bars represent standard error of the mean, p=0.05. Bars with the same letter have no statistically significant difference based on a Tukey test (log_{10}). This experiment was performed three times with similar results each time. CFU, colony-forming units.

A genomic DNA (gDNA) segment that included the argD gene and parts of the flanking genes yhfK and pabA (Fig. 3-1A) was ligated into pCR2.1 to create plasmid pCR2.1-argD (Fig. 3-3). The pCR2.1 and pCR2.1-argD plasmids were introduced into the argD1000::Tn5 mutant to create strains argD1000::Tn5(pCR2.1) and argD1000::Tn5(pCR2.1-argD), respectively. The argD1000::Tn5(pCR2.1-argD) strain grew in M9TN with sorbitol as the carbon source, while
strain \textit{argD1000::Tn5}(pCR2.1) did not grow in the same media (Fig. 3-2). Addition of arginine into the M9TN media with sorbitol as the carbon source restored the growth of the \textit{argD1000::Tn5} mutant (Fig. 3-4).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{pCR2.1-argD_map.png}
\caption{Map of the pCR2.1-\textit{argD} plasmid used to complement the \textit{argD1000::Tn5} mutant. The wild-type \textit{argD} complementation fragment (Fig. 3-1) was inserted with direction of \textit{argD} transcription opposite to that of the \textit{Plac} promoter in pCR2.1. Unique restriction sites are indicated in bold.}
\end{figure}
Fig. 3-4 Restoration of growth of the argD1000::Tn5 mutant in minimal media by addition of arginine. Bacterial population densities of the indicated strains growing in M9 minimal media supplemented with thiamine and nicotinic acid (M9TN media) with sorbitol as the carbon source and supplemented with 0.1 mg/ml arginine are shown at 0 and 48 hours after starting the culture, as determined by serial dilution plating. Values are the mean of three replicates and error bars represent standard error of the mean, p=0.05. There was no statistically significant difference between the wild-type and the argD1000::Tn5 mutant at any time point according to a Tukey test (log_{10}). This experiment was performed three times, with similar results each time. CFU, colony-forming units.

**Pathogenicity assays**

The argD1000::Tn5 mutant did not cause fire blight disease when inoculated onto apple trees by shoot tip wounding at 7, 14 or 21 dpi, while the wild-type strain caused extensive and progressive shoot necrosis (Fig. 3-5). Although a small amount of host tissue necrosis occurred at the argD1000::Tn5 inoculation sites, systemic symptom progression beyond the point of inoculation did not occur. Strain argD1000::Tn5(pCR2.1-argD) caused progressive necrosis indistinguishable from the wild-type, while strain argD1000::Tn5(pCR2.1) did not cause any progressive fire blight symptoms at any of the time points measured (Fig. 3-5). There was no
statistically significance difference between the wild-type and \( \text{argD}1000::\text{Tn}5(\text{pCR}2.1-\text{arg}D) \), or between \( \text{argD}1000::\text{Tn}5 \) and \( \text{argD}1000::\text{Tn}5(\text{pCR}2.1) \), based on Tukey tests \( (\log_{10}) \).

**Fig. 3-5** The \( \text{argD}1000::\text{Tn}5 \) mutant was not pathogenic in apple trees. Actively-growing shoot tips were inoculated with the indicated \( E. \text{amylovora} \) strains at \( 10^8 \) CFU/ml by shoot tip wound inoculation. Values are the mean of three replicates, and error bars represent standard error of the mean, \( p=0.05 \). Bars with the same letter have no statistically significant difference based on a Tukey test \( (\log_{10}) \). The entire experiment was performed twice, with similar results each time.
Similarly, \textit{argD}1000::Tn5 did not cause fire blight disease signs or symptoms when inoculated onto immature apple fruits at 7 dpi (Fig. 3-6A) and only occasionally caused minor necrosis on pear fruits at 5 dpi (Fig. 6B), while apple and pear fruits inoculated with wild-type \textit{E. amylovora} exhibited extensive necrosis and frequently had bacterial ooze at 7 and 5 dpi, respectively (Fig. 3-6A and 3-6B). Furthermore, the \textit{argD}1000::Tn5 mutant failed to grow in immature apple fruits (Fig. 3-6C), while the wild-type \textit{E. amylovora} bacterial population densities increased by about eight orders of magnitude by 7 dpi. The \textit{argD}1000::Tn5 mutant population density grew by four orders of magnitude after 5 dpi in immature pear tissue, about two orders of magnitude lower than the wild-type (Fig. 3-6D).

Strain \textit{argD}1000::Tn5(pCR2.1-\textit{argD}) caused necrosis and bacterial ooze on immature apple fruits at 7 dpi (Fig. 3-6A) and immature pear fruits at 5 dpi (Fig. 3-6B), while strain \textit{argD}1000::Tn5(pCR2.1) did not cause any fire blight symptoms (Fig. 3-6A and 3-6B). Strain \textit{argD}1000::Tn5(pCR2.1-\textit{argD}) grew as well as the wild-type in immature apple and pear fruit tissue, while strain \textit{argD}1000::Tn5(pCR2.1) failed to grow in immature apple and pear fruit tissue (Fig. 3-6C and 3-6D). In immature apple fruits, there was no statistically significant difference between the wild type and \textit{argD}1000::Tn5(pCR2.1-\textit{argD}) population densities at 7 dpi, or between the \textit{argD}1000::Tn5 and \textit{argD}1000::Tn5(pCR2.1) population densities at 7 dpi, based on Tukey tests ($\log_{10}$) (Fig. 3-6C). In immature pear fruits, there was no statistically significant difference between the wild type and \textit{argD}1000::Tn5(pCR2.1-\textit{argD}) population densities at 5 dpi; however, there were statistically significant differences between the wild-type and \textit{argD}1000::Tn5 population densities, and between the \textit{argD}1000::Tn5 and \textit{argD}1000::Tn5(pCR2.1) population densities at 5 dpi, based on Tukey tests ($\log_{10}$) (Fig. 6D).
Fig. 3-6 Pathogenicity assay and quantitative growth in detached immature apple and pear fruit. (A) Immature ‘Gala’ apple fruit halves at 7 days post inoculation (dpi) with the indicated bacterial strains. Tissue necrosis and bacterial ooze are a symptom and a sign, respectively, of fire blight disease in immature apple fruit. (B) Immature ‘Bosc’ pear fruit halves at 5 dpi with the indicated bacterial strains. (C) Bacterial populations in immature ‘Gala’ apple fruit halves at 7 dpi with the indicated bacterial strains, as determined by serial dilution plating. The same apples shown in (A) were used for the quantitative growth assay shown in (C). (D) Bacterial populations in immature ‘Bosc’ pear fruit halves at 5 dpi with the indicated bacterial strains. In (C) and (D), values are the mean of three replicates, and error bars represent standard error of the mean, p=0.05. Bars with the same letter have no statistically significant difference based on the Tukey test (log$_{10}$). Experiments were performed at least three times with at least five immature apple and pear fruit halves inoculated per strain, with the same results each time.
A nalidixic acid-resistant derivative of the wild-type *E. amylovora* HKN06P1 strain was identified (HKN06P1<sup>NalR</sup>) that produced similar symptoms to HKN06P1 (Fig. 3-7A) and grew as well as HKN06P1 (Fig. 3-7B) in immature apple fruit. When the *argD1000::Tn5* mutant was inoculated onto immature apple fruit in a 1:1 mixture with HKN06P1<sup>NalR</sup>, the *argD1000::Tn5* mutant did not grow, while HKN06P1<sup>NalR</sup> grew by eight orders of magnitude after 7 dpi (Fig. 3-8).

**Fig. 3-7** Fire blight symptoms and quantitative growth analysis in immature apple fruit inoculated with wild-type *E. amylovora* strain HKN06P1 and a nalidixic acid-resistant derivative (HKN06P1<sup>NalR</sup>). (A) Strains HKN06P1 and HKN06P1<sup>NalR</sup> produced similar fire blight symptoms in immature apple fruits at 7 days post inoculation (dpi). (B) Strains HKN06P1 and HKN06P1<sup>NalR</sup> grew to similar population densities a 7 dpi as determined by serial dilution plating. Extracts from fruits inoculated with HKN06P1 were plated in LB without antibiotic selection, and extracts from fruits inoculated with HKN06P1<sup>NalR</sup> were plated on LB supplemented with 25 µg/ml nalidixic acid. The immature apple fruits shown in (A) were used for the quantitative growth analysis shown in (B). Bars with the same letter have no statistically significant difference based on a Tukey test (log<sub>10</sub>).
Fig. 3-8  Bacterial growth in immature apple fruits after inoculation with a 1:1 mixture of HKN06P1_NalR and the argD1000::Tn5 mutant. Bacterial population densities were determined by serial dilution plating; HKN06P1_NalR populations were determined by plating on LB supplemented with 25 µg/ml nalidixic acid, while argD1000::Tn5 populations were determined by plating on LB supplemented with 50 µg/ml kanamycin. Values are the mean of four replicates, and error bars represent standard error of the mean, p=0.05. Bars with the same letter have no statistically significant difference based on a Tukey test (log10). The experiment was performed twice with similar results each time.

Plasmid stability in planta.

The inability of the argD1000::Tn5 mutant to grow in apple fruit tissue or cause disease symptoms in apple trees implied that an intact arginine biosynthesis pathway was required for E. amylovora to grow and cause disease in apples. This requirement appeared to be cell-autonomous, since the argD1000::Tn5 mutant did not grow when co-inoculated with the pathogenic HKN06P1_NalR strain (Fig. 3-8). Therefore, we hypothesized that the pCR2.1-argD plasmid would be stably maintained in the argD1000::Tn5 mutant growing in planta.
The pCR2.1 and pCR2.1-argD plasmids were introduced into HKN06P1\textsuperscript{NalR} competent cells to produce strains HKN06P1\textsuperscript{NalR}(pCR2.1) and HKN06P1\textsuperscript{NalR}(pCR2.1-argD), respectively. Immature apples were inoculated with strains HKN06P1\textsuperscript{NalR}(pCR2.1), HKN06P1\textsuperscript{NalR}(pCR2.1-argD) and argD1000::Tn5(pCR2.1-argD). At 0 dpi, all three strains had similar population densities as determined by serial dilution plating of immature apple fruit extracts on LB media supplemented with the appropriate antibiotics (Fig. 3-9A). At 7 dpi, the nalidixic acid-resistant \textit{E. amylovora} populations in immature apple fruits inoculated with HKN06P1\textsuperscript{NalR}(pCR2.1) and HKN06P1\textsuperscript{NalR}(pCR2.1-argD) were the same as the kanamycin-resistant \textit{E. amylovora} populations growing in fruits inoculated with argD1000::Tn5(pCR2.1-argD), indicating that all three strains were growing to the same population level. In contrast, at 7 dpi, the carbenicillin-resistant \textit{E. amylovora} populations in immature apple fruits inoculated with HKN06P1\textsuperscript{NalR}(pCR2.1) and HKN06P1\textsuperscript{NalR}(pCR2.1-argD) were 3-4 orders of magnitude lower than the carbenicillin-resistant \textit{E. amylovora} population growing in fruits inoculated with argD1000::Tn5(pCR2.1-argD). Furthermore, the kanamycin-resistant and carbenicillin-resistant \textit{E. amylovora} populations in fruit inoculated with argD1000::Tn5(pCR2.1-argD) were equal (Fig. 9A). These results indicated that fewer than 0.1\% of \textit{E. amylovora} cells in fruits inoculated with HKN06P1\textsuperscript{NalR}(pCR2.1) and HKN06P1\textsuperscript{NalR}(pCR2.1-argD) were resistant to carbenicillin after 7 days of growth in plant tissue. In contrast, the results indicate that 100\% of \textit{E. amylovora} cells in fruits inoculated with argD1000::Tn5(pCR2.1-argD) were resistant to carbenicillin after 7 days of growth in plant tissue. Plasmid preparations from randomly-selected, carbenicillin-resistant \textit{E. amylovora} colonies obtained during this analysis indicated that pCR2.1 and pCR2.1-argD were maintained as plasmids, as analyzed by agarose gel electrophoresis (Fig. 3-9B).
Similar results were obtained from plasmid stability assays in apple trees. ‘Gala’ variety apple trees were inoculated by shoot tip wounding with strains HKN06P1\textsuperscript{NalR} (pCR2.1), HKN06P1\textsuperscript{NalR} (pCR2.1-argD) and \textit{argD1000::Tn5}\textsuperscript{argD}, and each strain produced similar progression of fire blight necrosis (Fig. 3-10). At 21 dpi, the nalidixic acid-resistant \textit{E. amylovora} populations in tissues from trees inoculated with HKN06P1\textsuperscript{NalR} (pCR2.1) and HKN06P1\textsuperscript{NalR} (pCR2.1-argD) were the same as the kanamycin-resistant \textit{E. amylovora} populations growing in trees inoculated with \textit{argD1000::Tn5}\textsuperscript{argD} (Fig. 3-9C), indicating that all three strains grew similarly in the trees. In contrast, at 21 dpi, the carbenicillin-resistant \textit{E. amylovora} populations in tissues from trees inoculated with HKN06P1\textsuperscript{NalR} (pCR2.1) and HKN06P1\textsuperscript{NalR} (pCR2.1-argD) were 3-5 orders of magnitude lower than the carbenicillin-resistant \textit{E. amylovora} population growing in trees inoculated with \textit{argD1000::Tn5}\textsuperscript{argD} (Fig. 3-9C). Moreover, the kanamycin-resistant and carbenicillin-resistant \textit{E. amylovora} populations in fruit inoculated with \textit{argD1000::Tn5}\textsuperscript{argD} were equal (Fig 3-9B). These results indicated that fewer than 0.1\% of \textit{E. amylovora} cells in tissues of trees inoculated with HKN06P1\textsuperscript{NalR} (pCR2.1) and HKN06P1\textsuperscript{NalR} (pCR2.1-argD) were resistant to carbenicillin at 21 dpi. In contrast, the results indicate that 100\% of \textit{E. amylovora} cells in tissues of trees inoculated with \textit{argD1000::Tn5}\textsuperscript{argD} were resistant to carbenicillin at 21 dpi. Plasmid preparations from randomly-selected, carbenicillin-resistant \textit{E. amylovora} colonies obtained during this analysis indicated that pCR2.1 and pCR2.1-argD were maintained as plasmids, as analyzed by agarose gel electrophoresis (Fig. 3-9D).
The pCR2.1-argD plasmid was stably maintained in the argD1000::Tn5 mutant growing in planta. (A) Plasmid stability assay in immature apple fruits. Immature apple halves were inoculated with the indicated E. amylovora strains and bacterial populations were determined at 0 and 7 days post inoculation (dpi) by serial dilution plating of the fruit extract on LB plates supplemented with either kanamycin, carbenicillin or nalidixic acid, as represented by the indicated bar fills. (B) Plasmid preparations from randomly-selected carbenicillin-resistant isolates from the experiment shown in (A), resolved by agarose gel electrophoresis. Plasmid preparations from wild-type(pCR2.1) and wild-type(pCR2.1-argD) strains which had not passed through plants serve as controls. A plasmid preparation from wild-type E. amylovora carrying no foreign plasmids is also included. (C) Plasmid stability in apple trees inoculated with the indicated E. amylovora strains. ‘Gala’ apple trees were inoculated by shoot tip wounding with the indicated E. amylovora strains, and bacterial populations were determined at 21 dpi by serial dilution plating shoot tissue extract on LB plates supplemented with either kanamycin, carbenicillin or nalidixic acid, as represented by the indicated bar fills. (D) Plasmid preparations from randomly-selected carbenicillin-resistant isolates from the experiment shown in (C), resolved by agarose gel electrophoresis. Plasmid preparations from wild-type(pCR2.1) and wild-type(pCR2.1-argD) strains which had not passed through plants serve as controls. In (A) and (C), values are the mean of three replicates and error bars represent standard error of the mean, p=0.05. Bars with the same letter have no statistically significant difference based on a Tukey test (log_{10}).
Fig. 3-10  HKN06P1\textsuperscript{NalR} (pCR2.1), HKN06P1\textsuperscript{NalR} (pCR2.1-argD), and \textit{argD}1000::Tn5 (pCR2.1-argD) caused similar fire blight disease progression in apple trees. Actively-growing shoot tips of ‘Gala’ apple trees were inoculated with the indicated \textit{E. amylovora} strain at 10\textsuperscript{8} CFU/ml by shoot tip wound inoculation. The extent of shoot necrosis at 7, 14, and 21 days post inoculation (dpi) are shown. Values are the mean of three replicates, and error bars represent standard error, \( p=0.05 \). Bars with the same letter have no statistically significant difference based on a Tukey test (log\textsubscript{10}). The entire experiment was performed twice, with similar results each time.

**DISCUSSION**

The \textit{E. amylovora} \textit{argD}1000::Tn5 mutant was expected to be auxotrophic for arginine due to disruption of the arginine biosynthesis pathway. For example, disruption of the \textit{argD} gene in the cyanobacteria \textit{Anabaena} sp. Strain PCC 7120 and the bacterium \textit{Sinorhizobium meliloti} Rmd201 caused an arginine auxotroph phenotype (Floriano \textit{et al.}, 1994; Kumar \textit{et al.},
The *argD*1000::Tn5 did not grow in M9TN media with sorbitol as the carbon source, which is consistent with the *argD*1000::Tn5 mutant being an auxotroph. Addition of arginine into the M9TN media with sorbitol as the carbon source restored *argD*1000::Tn5 mutant growth, showing that indeed *argD*1000::Tn5 was an arginine auxotroph. This result also suggests that the *argD*1000::Tn5 mutant can transport arginine from the extracellular environment into the cell and use the extracellular arginine, despite having a disruption in the arginine biosynthesis pathway. Plasmid pCR2.1-argD restored *argD*1000::Tn5 growth in M9TN media with sorbitol as the carbon source, indicating that pCR2.1-argD complemented the *argD*1000::Tn5 mutant auxotroph phenotype and confirming that the *argD*1000::Tn5 auxotrophic phenotype was due to the Tn5 transposon insertion in the *argD* gene.

The inability of the *argD*1000::Tn5 mutant to cause fire blight in apple trees and immature apple and pear fruits like the wild-type indicates that an intact arginine biosynthesis pathway is required for *E. amylovora* to survive and grow in the host and cause fire blight disease. The pCR2.1-argD plasmid complemented the *argD*1000::Tn5 mutant’s ability to cause fire blight in apple trees and immature apple fruits, and restored normal growth in immature pear fruits, confirming that the *argD*1000::Tn5 mutant pathogenicity and virulence defects were due to the Tn5 transposon insertion in the *argD* gene.

The *argD*1000::Tn5 mutant failed to grow at all in immature apple fruits, which is consistent with the lack of symptoms in apple fruits and shoots inoculated with *argD*1000::Tn5 mutant. It is interesting to note that the *argD*1000::Tn5 mutant grew by several orders of magnitude in immature pears, although this growth was two orders of magnitude lower that the growth of the wild-type. This could be partly explained by the greater susceptibility of pears overall to fire blight compared to apple (Thompson and Ockey, 2000) and possibly by the
presence of more arginine in pear fruit compared to apple. For example, a study of ‘Beurre’ pear fruit and ‘Jonathan’ apple fruit found that the pears contained approximately twice as much arginine as the apples (Pilipenko et al., 1999).

The fact that \textit{argD1000::Tn5} was able to transport and use extracellular arginine from the M9TN media supplemented with sorbitol and arginine, but was not able to cause fire blight symptoms in apple shoots or immature fruits, was not able to grow in immature apple fruit and had greatly reduced growth in pear fruit tissues, suggests that wild-type \textit{E. amylovora} cells do not obtain sufficient arginine from the host during infection and must synthesize their own arginine; hence, the dependency on \textit{argD}. This suggests either that host tissues tested did not contain sufficient arginine to support normal \textit{E. amylovora} growth, or that \textit{E. amylovora} cannot extract sufficient arginine from the host tissues tested to support normal growth.

The \textit{pCR2.1-argD} plasmid appears to be stably maintained in \textit{E. amylovora} strain \textit{argD1000::Tn5(pCR2.1-argD)} growing \textit{in planta}. The kanamycin-resistant and carbenicillin-resistant \textit{E. amylovora} populations growing in immature apple fruits and apple trees inoculated with \textit{argD1000::Tn5(pCR2.1-argD)} were the same, indicating that the \textit{argD1000::Tn5(pCR2.1-argD)} cells maintained the \textit{pCR2.1-argD} plasmid over 7 dpi in immature apple fruit and 21 dpi in apple trees. The maintenance of the \textit{pCR2.1-argD} plasmid in \textit{E. amylovora} strain \textit{argD1000::Tn5(pCR2.1-argD)} growing \textit{in planta} occurred without antibiotic selection, and reflects a requirement for the arginine biosynthesis pathway for \textit{E. amylovora} growth \textit{in planta}. Furthermore, these results imply that \textit{E. amylovora} requires an intact arginine biosynthesis pathway even at stages of infection when severe symptoms and necrosis are evident, when host cellular contents are probably readily available to the pathogen. This supports the hypothesis that \textit{E. amylovora} cannot acquire sufficient arginine for its needs from host tissue, and must
synthesize its own arginine in order to grow. This is further supported by the mixed-inoculum experiment, which indicates a cell-autonomous requirement for argD activity for *E. amylovora* growth in the host. This situation contrasts with *E. amylovora* iron acquisition requirements, for example. The siderophore-based, high-affinity iron acquisition of *E. amylovora* is required mainly for initial floral infection and epiphytic growth, but not for infection through shoot wound sites or for systemic spread and necrosis in pear seedlings, when *E. amylovora* appears to be able to acquire sufficient iron through low-affinity uptake systems (Dellagi *et al.*, 1998).

Furthermore, since the pCR2.1-argD plasmid was very stable in the *argD1000::Tn5*(pCR2.1-argD), the pCR2.1-argD plasmid could be a useful new tool for fire blight research because of its antibiotic-free selection in *E. amylovora* strain *argD1000::Tn5*(pCR2.1-argD) growing *in planta*. The pCR2.1-argD plasmid could be used to express genes of interest and study them *in planta* without losing the plasmid over time because of no antibiotic selection. Plasmid loss from plant pathogens growing *in planta* due to lack of antibiotic selection is commonly observed (Hasnain and Sherwani, 1994; Boe *et al.*, 1987; Smith and Bidochka, 1998). Lastly, there has been some interest in using ArgD as an antibiotic target in bacteria (Rajaram *et al.*, 2007). However, arginine biosynthesis is a highly conserved pathway (Sakanyan *et al.*, 1992; Riley and Glansdorff, 1983; Ledwidge and Blanchard, 1999; Qu *et al.*, 2007; Hartmann *et al.*, 2003; Rodríguez-García *et al.*, 2000; Slocum, 2005; Xu *et al.*, 2007), which limits its utility as an antibiotic target.

In summary, this study indicates that *E. amylovora* cannot obtain sufficient arginine from host tissues, neither at the beginning of infection, nor later when the disease is in an advanced state. This would explain why *E. amylovora* needs an intact arginine biosynthesis pathway to cause fire blight disease in apple trees and immature apple and pear fruits, and why the pCR2.1-
argD complementation plasmid was stably maintained in the argD1000::Tn5 mutant growing in apple fruits and apple trees in the absence of antibiotic selection. Although the ArgD protein is not specifically involved in *E. amylovora* virulence and therefore cannot be considered an *E. amylovora* virulence factor, the results presented here provide new insight into the parasitic interaction of *E. amylovora* with the host. Finally, the pCR2.1-argD plasmid could be a useful new tool for fire blight research for expressing genes in *E. amylovora* growing *in planta*.

ACKNOWLEDGEMENTS

We thank Steven A. Lee for creating the *E. amylovora* Tn5 mutant library that we screened. L.S.R. was supported in part by: a Bunton Waller Graduate Fellowship; an Alfred P. Sloan Foundation Graduate Scholarship; a grant from the Pennsylvania State University College of Agricultural Sciences Graduate Student Competitive Grant Program; grant # 2010-65110-20488 from the Agriculture and Food Research Initiative Competitive Grants Program of the United States Department of Agriculture National Institute of Food and Agriculture; and the Department of Plant Pathology and Environmental Microbiology at The Pennsylvania State University. This research was also supported in part by funding from the Sarah Chinn Kalser Faculty Research Assistance Endowment of the Pennsylvania State University (T.W.M).
REFERENCES


**TABLE 3-1** Plasmids, bacterial strains, and oligonucleotides used in this work.

<table>
<thead>
<tr>
<th><strong>Plasmids</strong></th>
<th><strong>Description</strong></th>
<th><strong>Source, reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1</td>
<td>PCR cloning vector without an insert (Kan&lt;sup&gt;R&lt;/sup&gt;/Carb&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>TOPO TA Kit, Invitrogen</td>
</tr>
<tr>
<td>EZ-Tn5-argD</td>
<td>Plasmid rescued from argD1000::Tn5 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This work</td>
</tr>
<tr>
<td>pCR2.1-argD</td>
<td>argD cloned in pCR2.1 (Kan&lt;sup&gt;R&lt;/sup&gt;/Carb&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This work</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Bacterial Strains</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. amylovora</em> strain HKN06P1</td>
</tr>
<tr>
<td>argD1000::Tn5</td>
</tr>
<tr>
<td>argD1000::Tn5(pCR2.1)</td>
</tr>
<tr>
<td>argD1000::Tn5(pCR2.1-argD)</td>
</tr>
<tr>
<td>HKN06P1&lt;sup&gt;NalR&lt;/sup&gt;</td>
</tr>
<tr>
<td>HKN06P1&lt;sup&gt;NalR&lt;/sup&gt;(pCR2.1)</td>
</tr>
<tr>
<td>HKN06P1&lt;sup&gt;NalR&lt;/sup&gt;(pCR2.1-argD)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Oligonucleotides</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
</tr>
<tr>
<td>P2</td>
</tr>
<tr>
<td>FP</td>
</tr>
<tr>
<td>RP</td>
</tr>
</tbody>
</table>
CHAPTER 4

MUTATION OF THE ERWINIA AMYLOVORA pyrC GENE CAUSES PYRIMIDINE AUXOTROPHY AND ATTENUATED VIRULENCE IN APPLE TREES AND IMMATURE APPLE AND PEAR FRUITS
*Regarding this work accomplished, Brian Lehman and Kari Peters helped us with the pathogenicity assay in apples trees.
ABSTRACT

*Erwinia amylovora* is the causal agent of fire blight disease, which affects apple and pear production worldwide. The *E. amylovora pyrC* gene encodes a predicted dihydroorotase enzyme involved in pyrimidine biosynthesis. Here, we discovered that the *E. amylovora* pyrC244::Tn5 mutant was a uracil auxotroph. Unexpectedly, the *E. amylovora pyrC244::Tn5* mutant grew as well as the wild-type in detached immature apple and pear fruits. However, fire blight symptoms caused by the *pyrC244::Tn5* mutant in immature apple and pear fruits were attenuated compared to those caused by the wild-type. The *pyrC244::Tn5* mutant was slightly less virulent than the wild-type in apple tree shoots, but this difference was statistically significant only at seven days post inoculation and not at later time points. A plasmid-borne copy of the wild-type *pyrC* gene complemented the pyrimidine auxotrophy and reduced symptom phenotypes of the *pyrC244::Tn5* mutant. These results suggest that *E. amylovora* can obtain sufficient pyrimidine from the host to support bacterial growth, although *de novo* pyrimidine synthesis by *E. amylovora* is required for full symptom development. This situation contrasts with mammalian pathogens such as *Bacillus anthracis*, *Escherichia coli*, and *Salmonella enterica*, for which *de novo* nucleotide biosynthesis is critical for growth in human blood.
INTRODUCTION

Fire blight is the most economically significant and destructive bacterial disease in the world affecting apples and pears (van der Zwet et al., 2012). The causal agent of fire blight is *Erwinia amylovora* (Burrill, 1880). The most characteristic symptom and sign of fire blight are host necrosis and bacterial ooze, respectively (Coxe, 1817; van der Zwet et al., 2012). Even though fire blight has been documented as an important apple and pear disease problem for more than two hundred years (Denning, 1794; Arthur, 1885; Burrill, 1880), the disease continues to be the reason for major sporadic losses. A $68 million loss in the northwestern part of the United States in 1998 (Bonn, 1999) and a $42 million loss in the state of Michigan, USA, in 2000 (Norelli et al., 2003) are two examples. Fire blight is also a perennial problem for apple and pear growers in regions where the disease is endemic (Vanneste, 2000). Furthermore, fire blight threatens to be even more economically important as it continues to spread all around the world (Vanneste, 2000).

A better comprehension of the molecular genetics of *E. amylovora* could lead not only to a better understanding of the process of disease but also to the development of reliable controls. For several decades, scientists have been working to identify *E. amylovora* pathogenicity and virulence genes that contribute to the capacity of the bacteria to cause fire blight disease in apple and pear, and novel *E. amylovora* genes contributing to virulence and pathogenicity continue to be discovered, such as *luxS*, *rcs*, *rpoN*, *waaL*, and *hns* (Rezzonico and Duffy, 2007; Wang et al., 2009; Ramos et al., 2013; Ancona et al., 2014; Berry et al., 2009; Hildebrand et al., 2006). This type of knowledge could provide the basis for the development of new methods to manage the disease. For example, salicylidene acylhydrazides have the potential to be developed as fire
bliight controls because they can suppress the expression of type III secretion and amylovoran biosynthesis genes in *E. amylovora* (Yang et al., 2014).

Knowledge of *E. amylovora* nutritional requirements during infection could also be useful for developing disease control methods. For example, it is known that high-affinity, siderophore-based iron uptake is important during initial floral infection, but is less important after infection is established (Dellagi et al., 1998). In addition, the ability to metabolize sorbitol contributes to virulence in apple shoots, but is not required for virulence in pear fruits (Aldridge et al., 1997). An *E. amylovora* arginine auxotroph was not pathogenic on apple and pear, indicating that an intact arginine biosynthesis pathway is required for *E. amylovora* to grow in host tissues (Ramos et al., in press). However, our understanding of *E. amylovora* nutritional requirements while growing in host tissues is still incomplete.

In the present study, we have examined the effect of mutation of the *E. amylovora pyrC* gene on bacterial growth and pathogenicity. The *E. amylovora pyrC* gene encodes a predicted dihydroorotase protein (Choi and Zalkin, 1990; Wilson et al., 1992) which is an enzyme involved in step number three of the bacterial pyrimidine biosynthesis pathway, catalyzing the cyclization of N-carbamyl-L-aspartate to L-dihydroorotate (McPhail and Shepherdsone, 2006; Schenk-Gröninger et al., 1995; Ogawa and Shimizu, 1995).
MATERIALS AND METHODS

Mutant screening and Tn5 transposon insertion analysis

The Tn5 mutants were created by the mutagenesis of *E. amylovora* strain HKN06P1 (Lee *et al.*, 2010) with an engineered Tn5 transposon, according to the manufacturer’s instructions (EZ-Tn5<R6KγoriKan-2> Transposome Kit, Epicentre, Madison, WI, U.S.A.), and mutant screening was performed as described elsewhere (Ramos *et al.*, in press). The location of the Tn5 insertion in *pyrC*244::Tn5 was determined by Tn5 plasmid rescue according to the kit manufacturer’s instructions, sequencing of the DNA flanking of the Tn5 transposon in plasmid EZ-Tn5-*pyrC* using primers FP and RP (Table 1; Fig. 4-1a) at the Penn State University Genomics Core Facility at University Park, and locating the DNA sequences on the *E. amylovora* strain CFBP 1430 reference genome (Smits *et al.*, 2010) using the NCBI’s BLASTN program (Altschul *et al.*, 1990).

Plasmids

A gDNA section that included the entire *pyrC* coding region plus 141 base pairs (bp) upstream of the *pyrC* gene start codon and 81 bp downstream of the *pyrC* stop codon was amplified with 15 cycles of the PCR using primers P1 and P2 (Table 1), Advantage II polymerase (Clontech, Mountain View, CA, U.S.A.) and an annealing temperature of 55°C, and then cloned into plasmid pCR2.1 (Invitrogen, Grand Island, NY, U.S.A.) according to the manufacturer’s instructions, creating plasmid pCR2.1-*pyrC* (Table 1). The segment of *E. amylovora* DNA cloned in pCR2.1-*pyrC* was sequenced using primers T7 and M13R, and the sequence was found to be identical to that of the published CFBP 1430 genome (Smits *et al.*, 2010) using NCBI BLAST (Altschul *et al.*, 1990). Plasmids pCR2.1-*pyrC* and pCR2.1 were
introduced into the *pyrC*244::Tn5 competent cells using Electroporator 2510 (Eppendorf AG, Hamburg, Germany) at 2,500 volts, in order to create the strains *pyrC*244::Tn5(pCR2.1-*pyrC*) and *pyrC*244::Tn5(pCR2.1), respectively (Table 1).

**Growth analysis in media**

Growth in M9TN media and uracil auxotrophy tests were performed as described elsewhere (Ramos *et al.*, in press).

**Apple tree inoculations**

Two-year-old, dormant, bare-rooted ‘Gala’ apple trees grafted onto ELMA 26 rootstocks (Adams County Nursery, Aspers, PA) were potted in general purpose potting mix (Pro Mix BX) and grown in a greenhouse for six weeks. Tree inoculations and fire blight disease measurements were performed as described elsewhere (Jensen *et al.*, 2003). Inoculated shoots were selected to be as similar to each other as possible, with an average length of 28.7 cm (st. dev: ± 1.2 cm).

**Growth analysis in fruits**

Bacterial populations in the immature fruits were quantified by macerating the fruits in 10 mM MgCl₂, followed by serial dilution plating, and expressed as colony-forming units (CFU) per gram of plant tissue, as previously described (Lee *et al.*, 2010).

**Hypersensitive response (HR)**

Six-week-old tobacco (*Nicotiana tabacum* L. cv. Glurk) plants grown under 300 μE·m⁻²·s⁻¹ light intensity at 24°C with 40% RH were used. Leaf sections were infiltrated with
bacterial suspensions with absorbance of 0.1 ($\lambda$ = 600 nm) in 10 mM MgCl$_2$ using a syringe without a needle, as described previously (Sinn et al., 2008).

Statistics

Statistical analyses were performed using PROC GLM in SAS (version 9.2, SAS Institute, Cary, NC). The strains were analyzed as the independent variable, and the growth ($\log_{10}$ cfu) as the dependent variable. *Post-hoc* comparisons were evaluated using the Tukey test, with $\alpha$=0.05. Error bars represent the standard error of the mean of three, four or five replicate samples per strain. The number of replicates varied depending on the assay.

RESULTS AND DISCUSSION

An *E. amylovora* pyrC mutant was identified during the screening of two thousand Tn5 mutants for reduced virulence on immature ‘Gala’ apple fruits. The *E. amylovora* pyrC mutant was named *pyrC*244::Tn5 (Table 1) because the Tn5 transposon was inserted after pyrC gene coding region nucleotide number 243 (Fig. 4-1a). The presence of a Tn5 transposon insertion in the pyrC gene of the *pyrC*244::Tn5 mutant was confirmed by polymerase chain reaction (PCR) analysis of *pyrC*244::Tn5 mutant genomic DNA (gDNA) (Fig. 4-1b).
The *E. amylovora* pyrC244::Tn5 mutant had a Tn5 transposon insertion into the *pyrC* gene. (a) Illustration of the *pyrC* gene, flanking genes *dinI* and *yceB*, and the location of the Tn5 insertion in the *pyrC* gene (triangle). The *E. amylovora* CFBP 1430 complete genome sequence was used to number the genome nucleotide positions. Locations and directions of oligonucleotide primers P1, P2, RP, and FP (Table 1) are indicated by half arrows; the segment of DNA used to complement the *pyrC*::244Tn5 mutant is indicated by the bracket. (b) Confirmation of Tn5 transposon insertion in the *pyrC* gene of the *pyrC*::244Tn5 mutant by polymerase chain reaction (PCR) amplification of genomic DNA (gDNA). A product of 1,083 base pairs (bp) was amplified from wild-type *E. amylovora* gDNA using primers P1 and P2, as expected, while no similar product was amplified from *pyrC*::244Tn5 mutant (pyrC) gDNA. PCR amplification of *pyrC*::244Tn5 mutant gDNA using primers pairs P1 + RP and FP + P2 amplified products of 889 bp and 383 bp length, respectively, indicating the presence of a Tn5 transposon insertion into the *pyrC* gene in the *pyrC*::244Tn5 mutant.
The *E. amylovora* pyrC244::Tn5 mutant was predicted to be auxotrophic due to a disruption of the pyrimidine biosynthesis pathway, a component of primary metabolism. As expected, the *pyrC*244::Tn5 mutant did not grow in M9 minimal media broth supplemented with 0.5 mM thiamine and 1 mM nicotinic acid (M9TN media) with 2 mM sorbitol as the carbon source, while the wild-type grew in the same media (Fig 2a).

**Fig. 4-2** Pyrimidine auxotrophy of the *pyrC*244::Tn5 mutant. (a) Growth of the indicated *E. amylovora* strains in M9 minimal media supplemented with thiamine and nicotinic acid (M9TN media) with sorbitol as the carbon source at 0 and 24 hours after starting the culture. (b) Growth of the *pyrC*244::Tn5 mutant was restored when M9TN minimal media with sorbitol was supplemented with uracil. Values are the mean of three or more replicates and error bars represent standard error, *p* = 0.05. Bars with same letter have no statistically significant difference based on a Tukey test (log_{10}). Each experiment was repeated three times with similar results each time; results of representative experiments are shown. CFU, colony-forming units.
Strain \textit{pyrC}244::Tn5(pCR2.1-\textit{pyrC}) grew in the M9TN media, while strain \textit{pyrC}244::Tn5(pCR2.1) did not (Fig 2a). There was no statistically significant difference between the growth of the wild-type and strain \textit{pyrC}244::Tn5(pCR2.1-\textit{pyrC}) in M9TN media (Fig. 4-2a), indicating that the pCR2.1-\textit{pyrC} plasmid fully complemented the \textit{pyrC}244::Tn5 auxotrophic phenotype. This indicates that the auxotrophic phenotype of the \textit{pyrC}244::Tn5 mutant was indeed due to the Tn5 disruption of the \textit{pyrC} gene, and that plasmid pCR2.1-\textit{pyrC} contained a functional copy of the \textit{pyrC} gene.

As expected, when the M9TN media was supplemented with 0.1 mg/ml uracil, the \textit{pyrC}244::Tn5 mutant grew as well as the wild-type (Fig 2b). This result was consistent with the \textit{E. amylovora} \textit{pyrC} gene product playing a role in pyrimidine biosynthesis. Furthermore, the results were also consistent with results for \textit{pyrC} disruption in other plant-associated bacteria such as \textit{Pseudomonas putida} and \textit{Sinorhizobium meliloti}, where a disruption in their \textit{pyrC} genes lead to a uracil auxotrophy phenotype (Lee and Cooksey, 2000; Nogales et al., 2006).

Unexpectedly, the \textit{pyrC}244::Tn5 mutant grew in immature ‘Gala’ apple fruits just as well as wild-type \textit{E. amylovora} (Fig 4-3a). There was no statistically significant difference between the wild-type and the \textit{pyrC}244::Tn5 population densities at 7 days post inoculation (dpi). Even though the \textit{pyrC}244::Tn5 mutant population was the same as the wild-type in the immature apple fruits at 7 dpi, apples inoculated with \textit{pyrC}244::Tn5 showed attenuated fire blight symptoms compared to apples inoculated with the wild-type (Fig 4-3b). Apples inoculated with strain \textit{pyrC}244::Tn5(pCR2.1-\textit{pyrC}) showed fire blight symptoms and signs similar to apples inoculated with the wild-type strain (Fig 4-3b). In contrast, apples inoculated with strain \textit{pyrC}244::Tn5(pCR2.1) showed attenuated fire blight symptoms and signs similar to apples
inoculated with the *pyrC*244::Tn5 mutant (Fig 4-3b). Strains *pyrC*244::Tn5(pCR2.1-*pyrC*) and *pyrC*244::Tn5(pCR2.1) grew in the immature apple fruits to population densities similar to the wild-type (Fig 4-3a).

**Fig. 4-3** Growth and virulence of wild-type, *pyrC*244::Tn5, *pyrC*244::Tn5(pCR2.1-*pyrC*) and *pyrC*244::Tn5(pCR2.1) *E. amylovora* strains in immature apple and pear fruits. (a) Growth of indicated strains in immature apple fruits at 0 and 7 days post inoculation (dpi) as determined by tissue maceration followed by serial dilution plating. (b) Symptoms on representative apple fruit halves at 7 dpi with the indicated *E. amylovora* strains; tissue necrosis and bacterial ooze are a symptom and a sign, respectively of fire blight. (c) Growth of indicated strains in immature pear fruits at 0 and 5 dpi. (d) Symptoms on representative apple fruit halves at 5 dpi with the indicated *E. amylovora* strains. Values are the mean of three or more replicates and error bars represent standard error, *p*=0.05. Bars with same letter have no statistically significant difference based a Tukey test (log10). Experiments were repeated three times with similar results each time; results of representative experiments are shown. CFU, colony-forming units.
Similarly, in immature ‘Bosc’ pear fruits, the wild-type, *pyrC244::Tn5*, *pyrC244::Tn5*(pCR2.1-*pyrC*) and *pyrC244::Tn5*(pCR2.1) strains grew to the same bacterial population levels at all time points tested (Fig.4-3c), with no statistically significant differences between any of the strains at any of the time points. Even though all four strains grew to the same population levels in the pears, *pyrC244::Tn5* and *pyrC244::Tn5*(pCR2.1) were less virulent than the wild-type and the *pyrC244::Tn5*(pCR2.1-*pyrC*) in terms of symptom development (Fig. 4-3d).

In ‘Gala’ apple trees inoculated by shoot tip wounding, the *pyrC244::Tn5* mutant was slightly less virulent than the wild-type at 7 dpi (Fig. 4-4a). However, differences between strains *pyrC244::Tn5*, *pyrC244::Tn5*(pCR2.1-*pyrC*) and *pyrC244::Tn5*(pCR2.1) were not statistically significant at 7 dpi. Furthermore, at 14 and 21 dpi, all four strains caused severe fire blight symptoms, and there were no statistically significant differences in virulence between any of the four strains tested. When infiltrated into the leaves of non-host *Nicotiana tabacum* cv. ‘Glurk’ plants, the wild-type and strains *pyrC244::Tn5*, *pyrC244::Tn5*(pCR2.1-*pyrC*) and *pyrC244::Tn5*(pCR2.1) caused a typical hypersensitive response (Morel and Dangl, 1997, Fig. 4-4b), indicating that the *pyrC244::Tn5* mutant triggered typical non-host defense responses in tobacco.
Virulence and avirulence of wild-type, pyrC244::Tn5, pyrC244::Tn5(pCR2.1-pyrC) and pyrC244::Tn5(pCR2.1) E. amylovora strains in ‘Gala’ apple tree shoots and tobacco leaves. (a) Shoot tips were wound-inoculated with a drop of bacterial suspension with an absorbance of 0.1 ($\lambda = 600$ nm) of the indicated E. amylovora strains. The extent of shoot necrosis at 7, 14, and 21 days post inoculation (dpi) is shown. The extent of shoot necrosis was calculated by dividing the length of the necrotic portion of the shoot (measuring from the inoculated shoot tip), by the total length of the shoot as measured from the shoot tip to the shoot junction with the previous year’s wood, and expressed as a percent. Values are the mean of three or more replicates and error bars represent standard error of the mean, $p = 0.05$. Bars sharing a letter have no statistically significant difference based a Tukey test ($\log_{10}$). The experiment was performed twice with similar results each time; results of a representative experiment are shown. CFU, colony-forming units. (b) Leaf segments of non-host tobacco (Nicotiana tabacum L. cv. ‘Glurk’) were infiltrated with bacterial suspension with an absorbance of 0.1 ($\lambda = 600$) of the indicated E. amylovora strains. The hypersensitive response (HR) was scored as tissue collapse at 24 hours post inoculation (hpi); photo was taken at 72 hpi. The experiment was repeated twice with similar results each time and using several replicate leaves for each experiment; a representative leaf is shown.
The clear and absolute auxotrophic phenotype of the *pyrC*244::Tn5 mutant growing in minimal M9TN media contrasts sharply with the ability of the *pyrC*244::Tn5 mutant to grow normally in immature apple and pear tissues and to cause fire blight disease symptoms, particularly in tree shoots. These results suggest that *E. amylovora* is able to obtain sufficient pyrimidines from host tissue, including shoot tissue, in order to support growth and disease development. During plant cell death in the course of the fire blight disease interaction, it is likely that plant RNA is rapidly degraded into free nucleotides, which are presumably accessible to *E. amylovora*. However, the attenuated symptoms caused by the *pyrC*244::Tn5 mutant indicate that some *de novo* pyrimidine biosynthesis by *E. amylovora* is required for full virulence. These results provide insight into the nutritional requirements of *E. amylovora* growing in host tissues.

The results that we obtained are different from human pathogen studies with the same pyrimidine pathway. It has been shown that human bacterial pathogens like *Staphylococcus aureus*, *Bacillus anthracis*, *Escherichia coli*, and *Salmonella enterica* with defects in pyrimidine synthesis are not able to grow in blood (Truong *et al.*, 2013; Samant *et al.*, 2008). Therefore, there has been some effort to specifically target the *pyrC* gene in *Staphylococcus aureus* as a therapeutic approach (Truong *et al.*, 2013). However, our study indicates that a similar approach would be unlikely to succeed in the case of fire blight disease, suggesting that not all primary metabolic pathways may be suitable antibiotic targets in pathogens.
ACKNOWLEDGEMENTS

We thank Steven A. Lee for creating the *E. amylovora* Tn5 mutant library that we screened. L.S.R. was supported in part by: a Bunton Waller Graduate Fellowship; an Alfred P. Sloan Foundation Graduate Scholarship; a grant from the Pennsylvania State University College of Agricultural Sciences Graduate Student Competitive Grant Program; grant # 2010-65110-20488 from the Agriculture and Food Research Initiative Competitive Grants Program of the United States Department of Agriculture National Institute of Food and Agriculture; and the Department of Plant Pathology and Environmental Microbiology at The Pennsylvania State University. This research was also supported in part by funding from the Sarah Chinn Kalser Faculty Research Assistance Endowment of the Pennsylvania State University (T.W.M). The authors declare no conflicts of interest.
REFERENCES


**Table 4-1** Plasmids, bacterial strains, and oligonucleotides used in this work.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source, reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1</td>
<td>PCR cloning vector without an insert (Kan(^R)/Carb(^R))</td>
<td>TOPO TA Kit, Invitrogen</td>
</tr>
<tr>
<td>EZ-Tn5-pyrC</td>
<td>Plasmid rescued from pyrC244::Tn5 (Kan(^R))</td>
<td>This work</td>
</tr>
<tr>
<td>pCR2.1-pyrC</td>
<td>pyrC cloned in pCR2.1 (Kan(^R)/Carb(^R))</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Bacterial Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. amylovora</em> strain</td>
<td>Wild-type strain</td>
<td>Lee <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>pyrC244::Tn5</td>
<td><em>E. amylovora</em> HKN06P1 carrying a Tn5 transposon insertion in the pyrC gene (Kan(^R))</td>
<td>This work</td>
</tr>
<tr>
<td>pyrC244::Tn5(pCR2.1)</td>
<td>pyrC244::Tn5 carrying pCR2.1 (Kan(^R)/Carb(^R))</td>
<td>This work</td>
</tr>
<tr>
<td>pyrC244::Tn5(pCR2.1-pyrC)</td>
<td>pyrC244::Tn5 carrying pCR2.1-pyrC (Kan(^R)/Carb(^R))</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5′-TGATCAGCATGACAGCCTCCGGTCTTA-3′</td>
<td>This work</td>
</tr>
<tr>
<td>P2</td>
<td>5′-CCGCTCTTACCAGTAACAATCGCCGG-3′</td>
<td>This work</td>
</tr>
<tr>
<td>FP</td>
<td>5′-GCCAACGACTACGACTACGAGC-3′</td>
<td>EZ-Tn5 Kit, Epicentre</td>
</tr>
<tr>
<td>RP</td>
<td>5′-GAGCCAATAGTGCGAAGAACACCCGAGAA-3′</td>
<td>EZ-Tn5 Kit, Epicentre</td>
</tr>
</tbody>
</table>
CONCLUSION

The three main genes that I studied in *Erwinia amylovora* were *rpoN*, *argD* and *pyrC* (See Chapters 2, 3 and 4, respectively). My research results provide new information about fire blight disease processes. The *rpoN* gene is a regulatory pathogenicity gene and *argD* and *pyrC* are involved in basic metabolic pathways, and they provide insight about the nutritional requirements that the bacterium has during infection and after infecting the host.

Our study (Ramos *et al.*, 2013), together with Ancona *et al.*, 2014, showed that *rpoN* is a regulatory gene that controls the expression of the *hrpL* gene, which regulates the expression of other pathogenicity and virulence genes including the *hprN* and *dspA/E* genes. Furthermore, *rpoN* has a similar function in *E. amylovora* as in *Pseudomonas syringae* and *Pectobacterium carotovorum*. Our study also provides information about nutritional requirements of *E. amylovora* growing in host tissues. The *E. amylovora argD* mutant was able to obtain and use extracellular arginine from M9 minimal media supplemented with thiamine, nicotinic acid, sorbitol and arginine, but it was not able to obtain sufficient arginine from the host tissue. This suggests that an intact arginine biosynthesis pathway is required by *E. amylovora* for normal growth and disease symptom development in the host. In contrast, although the *E. amylovora pyrC* mutant was strictly auxotrophic for pyrimidine, it grew nearly as well as the wild-type in fruits and caused fire blight in apple trees, although symptoms were somewhat attenuated in fruits and trees. This suggests that *E. amylovora* can obtain sufficient pyrimidines from host tissue to support growth and fire blight symptom development, although *de novo* pyrimidine synthesis is necessary for full virulence. These results are different from findings in some human bacterial pathogens, which require *de novo* pyrimidine synthesis for growth in host blood.
Figure 5-1 shows all the genes discussed in Chapter 1 with the addition of the genes that I studied and described in this dissertation.

**Fig. 5-1** Diagram of the impact of my research on the genetic knowledge of *E. amylovora* disease processes. This is the same figure as Figure 1-1, except that my findings have been added. The three genes studied during my graduate studies are in red, *rpoN*, *argD* and *pyrC*. The *rpoN* gene controls the expression of the *hrpL* gene, which in turn controls many virulence functions in *E. amylovora*. *E. amylovora* cannot get sufficient arginine (yellow circles) from the host in order to grow and cause disease; therefore, it needs an intact arginine biosynthesis pathway. In contrast, the bacteria can get sufficient uracil (light green circles) from the host but it needs the pyrimidine biosynthesis pathway for full virulence.
**Future work**

It would be interesting to investigate if the \textit{argD} and \textit{pyrC} mutants can grow in host flowers. Can the \textit{argD} and \textit{pyrC} mutants obtain and use extracellular arginine and pyrimidine, respectively, from the host flower tissue for growth or/and disease development? We tried to answer this question, but unfortunately the apple flowers were treated with streptomycin the day before we collected them, which interfered with the growth analysis.

Regarding the \textit{argD} mutant, it would be interesting to investigate why some apples tree branches of 15 cm or shorter showed fire blight symptoms when inoculated with the \textit{argD} mutant. During the pathogenicity assays in apple trees, the preferred size of the branches is 28-40 cm long. Occasionally, some small branches of 15 cm or less were also inoculated with the \textit{argD} mutant, and they showed fire blight symptoms at 28 days post inoculation (dpi) during the first repetition of the assay and at 14 and 21 dpi during the second repetition of the assay. Since such small branches are not typically used in this assay, the data results were not shown in the \textit{argD} manuscript or chapter 3. A detailed analysis of shoot size relationship with susceptibility to the \textit{argD} mutant was beyond the scope of the \textit{argD} study; however, it would be interesting to test this is in a future study. Interestingly, it has been shown that nitrogen supply could be in the form of free amino acids, mainly arginine, in succulent and young branches more often that in old branches (Tromp, 1983; Cheng \textit{et al.}, 2003).

It would be also interesting to study if nitrogen application times or/and different kinds of nitrogen fertilizer have an input in the ability of the \textit{argD} mutant to obtain and use extracellular arginine from the host. It has been shown previously that different kinds and different application time points of nitrogen fertilizer have different input in the quantity of free amino acids, specially arginine, inside the plants tissue (Edwards, 1986; Tromp and Ovaa, 2006).
Lastly, it would be interesting to see if *E. amylovora* can obtain and utilize other amino acids than arginine from the host tissue. In order to answer this question, other amino acid biosynthesis pathways need to be disrupted and studied.

Finally, it would be interesting to test if the *argD* mutant could be developed as a biocontrol. The *argD* mutant could compete for space with the wild-type as well as stimulate host defense in apple. Fig. 5-2 shows that the *argD* mutant was able to cause hypersensitive response (HR) in non host tobacco leaves. This is preliminary data suggests that the *argD* mutant might be able to induce host defenses in non-host plants and possibly promote fire blight resistance in host plants.

![Fig. 5-2 The *argD* mutant was able to cause a hypersensitive response in non-host tobacco.](image)

Regarding the *pyrC* mutant, it would be interesting to further examine if *E. amylovora* usually obtains uracil from the host or not during the normal disease interaction. A pyrimidine or specifically a uracil membrane transporter mutant in *E. amylovora* could help answer this question. This could help clarify what exactly are *E. amylovora* nutrient demands during
infection and after infection. It would also be interesting to study if *E. amyllovora* can only obtain sufficient uracil from the host or the bacteria can get any kind of pyrimidines and purines from the host. In order to answer this question, other pyrimidines and purine basic metabolic pathways should be disrupted and studied.

REFERENCES


APPENDIX A

DELETION OF THE $\text{argD}$ GENE IN *ERWINIA AMYLOVORA*
Targeted gene deletions are not routine in *E. amylovora*, so it would be of great use for us to develop a clean deletion protocol. I succeeded in obtaining a clean deletion of the *argD* gene. The purpose of this appendix is to describe this protocol. A clean deletion of the *argD* gene in *Erwinia amylovora* strain 6p1nalR is also another way to confirm that the *argD* gene is required for *E. amylovora* to cause disease.

**Protocol:**

1. Create the primers: Here shown as A, B, C, and D

2. First two PCRs: In order to get the AB and CD fragments separately.
The primers A and B were created and used to get the DNA segment AB. Segment AB includes most of the \textit{yhfK} gene and part of the \textit{pabA} gene. The primers C and D were created and used to get the DNA segment CD. Segment CD includes most of the \textit{pabA} gene and part \textit{yhfK}.

**Chemicals used:**
- Pfu buffer: 2.5 µl
- dNTP: 0.4 µl
- Template: 0.34 µl
- Primers: 0.5 µl each
- Pfu Turbo enzyme: 1 µl
- Water: 19.3 µl

3. Overlap PCR: In order to get AD fragment
Fig. Appendix A 1-2 Primers A and D were used to get an AD segment, the segment included *yhfK* and *pabA* genes.

4. Clone the overlap PCR in pCR2.1

5. Transform Top10 cells with the plasmid pCR2.1-overlap PCR
6. Plasmid Isolation of the pCR2.1-overlap PCR from Top10 cells
7. Cut the pCR2.1-overlap PCR and suicide plasmid pDS132 with XbaI.
8. CIP treatment both
9. Ligate the overlap PCR to the suicide plasmid pDS132
10. Transform DH5α λpir cells with pDS132-overlap PCR
11. Plasmid isolation of the pDS132-overlap PCR from DH5α λpir cells
12. Transform SM10 λpir cell with pDS132-overlap PCR
13. Conjugation between SM10 λpir cell pDS132-overlap PCR and 6p1<sup>nalR</sup>.
14. Second cross-over in LB with no salt and 5% sugar.
15. Plating 6p1<sup>nalR</sup> in LB supplemented with 25 µg/ml nalidixic acid and 25µg/ml of chloramphenicol
16. Characterize ∆argD mutant
Using the primers P1 and P2, Advantage 2 polymerase reaction mix and a thermal cycler program of 2 minutes at 95°C followed by 12 cycles of 30 seconds at 95°, 30 seconds at 60°C, and 90 seconds at 72°C we got a 1,400bps product from the wild-type and a 150bps product from the ΔargD mutant, as expected.
The ΔargD mutant was not able to grow in M9TN media supplemented with sorbitol, while the wild-type grew almost 4 fold.

The ΔargD mutant as well as the wild-type grew in M9TN media supplemented with sorbitol and arginine.
Fig. Appendix A 1-6 The ΔargD mutant did not grow in immature apple fruits as well as the wild-type grew. The ΔargD gene pCR2.1-argD grew as well as the wild-type did in the immature apples, demonstrating the argD gene was deleted in the ΔargD mutant.

The ΔargD mutant and argD1000::Tn5 had the same phenotype in M9TN media supplemented with sorbitol, in M9TN media supplemented with sorbitol and arginine and in immature apples fruits.
APPENDIX B

*ynfF* GENE
**yrfF gene in *Erwinia amylovora***

The *yrfF* gene was one the most interesting genes I worked with during my PhD. Unfortunately, I could never complement it; therefore I am describing all the experiments I did with it for reference and future work.

In the human pathogen *Salmonella enterica*, the *igaA* gene, which is a homologous gene of *yrfF* gene in *Erwinia amylovora*, is an essential gene that encodes an outer membrane protein (Cano, 2002). In *S. enterica*, *igaA* regulates the expression of the *rcs* gene through a negative control system, which means that when *igaA* is expressed, *rcs* gene are not expressed (Domínguez-Bernal et al., 2004; Tierrez and Garcia-del Portillo, 2004; Mariscotti and Garcia-del Portillo, 2009; Mariscotti and Garcia-del Portillo, 2008; Garcia-Calderon et al., 2009; Cano et al., 2002). The Rcs protein participates in the production of the colanic acid capsule, which helps the bacteria to attach to the host. Rcs also participates in the synthesis of exopolysaccharides and flagella. Rcs regulates swarming, cell division, biofilm formation, and resistance to cell envelope stress in *S. enterica* (Garcia-Calderon et al., 2009). The *igaA* gene is related to virulence since it controls Rcs protein expression, which is related to all these important processes in *S. enterica*. In *E. amylovora*, *yrfF* might be involved in the attachment of *E. amylovora* to the host, movement in general, cell division, biofilm production, and the synthesis of exopolysacharides. Therefore, the *yrfF* gene could be a pathogenicity gene in *E. amylovora*, too. The *yrfF* gene has never been described before in a plant pathogenic organism.
Fig. Appendix B 1-1 The *yrfF* mutant did not cause disease in immature apple fruits while the wild-type caused fire blight symptoms at 7 days post inoculation.

Fig. Appendix B 1-2 The Tn5 insertion in the *yrfF* gene is shown in green and purple.
Auxotrophy assay

The $yrf^F$ mutant could grow in M9 minimal media supplemented with thiamine, nicotinic acid (M9TN media) and sorbitol as the carbon source. Therefore the $yrf^F$ mutant was a prototroph and therefore the $yrf^F$ was expected to be a pathogenicity gene in *E. amylovora*.

Fig. Appendix B 1-3 Photo of the $yrf^F$ mutant growing in M9TN media supplemented sorbitol.

The photo was taken two days after inoculating the media with the $yrf^F$ mutant.
Unsuccessful efforts to complement the \textit{yrfF} mutant

In an effort to complement the \textit{E. amylovora yrfF} mutant, we used the high copy number plasmid pCR2.1 and the low copy number plasmid pCPP9. All four different genomic segments that were used to try to complement the \textit{yrfF} mutant were cloned into these two different plasmids, for a total of 8 different plasmids. None of the below DNA segments complemented the \textit{yrfF} mutant, neither when cloned into pCR2.1 or pCPP9.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig Appendix B 1-4.png}
\caption{The first genomic DNA (gDNA) segment used to try to complement the \textit{yrfF} mutant was the \textit{yrfF} gene alone, with a very short promoter segment before the \textit{yrfF} gene.}
\end{figure}
The second gDNA segment used to try to complement the yrfF mutant was the yrfF operon but with a very short promoter segment before the yrfF gene.

The third gDNA segment used to try to complement the yrfF mutant with the yrfF gene and part of the yrfE gene, which included the entire yrfF promoter region.
Fig. Appendix B 1-7 The fourth gDNA segment used to complement the yrfF mutant was yrf operon with part the yrfE and pckA genes, which included the entire yrfF promoter region.
REFERENCES – Appendix B


MOLECULAR GENETIC STUDY OF *ERWINIA AMYLOVORA*: *rpoN*, *argD* AND *pyrC* GENES

**Abstract**

Fire blight is the most destructive bacterial disease of apples and pears worldwide. Apples are the second most consumed fresh fruit in the United States. The causal agent of fire blight is *Erwinia amylovora*. Except for streptomycin, there is no registered product that can effectively prevent *E. amylovora* from infecting apple and pear orchards. Unfortunately, in the United States, streptomycin-resistant strains of *E. amylovora* have been found. A better understanding in general of the molecular genetics of the bacteria could lead not only to a better comprehension of the disease process but also the development of additional reliable management tools. In this research, three *E. amylovora* genes were studied: a regulatory gene called *rpoN*; and two metabolic genes called *argD* and *pyrC*. I found that the *rpoN* gene was essential for pathogenicity, for the hypersensitive response in non-host tobacco, and for the expression of the HrpN protein. The *rpoN* mutant was prototroph, indicating that the *rpoN* mutant non-pathogenic phenotype was not due to a defect in basic metabolism or growth. The *argD* gene is essential for the biosynthesis of the amino acid arginine in bacteria, including *E. amylovora*. *E. amylovora argD* mutant was an arginine auxotroph and did not cause disease or grow in host tissue. This study suggests that *E. amylovora* cannot obtain enough arginine from the host during infection. The *pyrC* gene is indispensable for the biosynthesis of pyrimidines. Surprisingly, even though the *E. amylovora pyrC* mutant was a uracil auxotroph, it was able to obtain uracil from the host, grow and cause disease. This study suggests that *E. amylovora* can obtain and utilize sufficient uracil from the host. The study of the last two genes gave us an insight about the nutrition requirements that the bacteria have during infection and after infecting the host. This knowledge could help develop potential fire blight controls.

**Keywords**

Erwinia amylovora, fire blight, *argD, pyrC, rpoN* and plasmid

**Files**

https://psu.box.com/s/0qha4xdog7kvoopkt3jb