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ROLE OF *WAAL* AND *UMUDC* IN *ERWINIA AMYLOVORA* EA1189
IN OXIDATIVE STRESS AND ULTRAVIOLET RADIATION
SURVIVAL

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ROLE OF *WAAL* AND *UMUDC* IN *ERWINIA AMYLOVORA* EA1189 IN OXIDATIVE STRESS AND ULTRAVIOLET RADIATION SURVIVAL

By

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Abstract

ROLE OF *WAAL* AND *UMUDC* IN *ERWINIA AMYLOVORA* EA1189 IN OXIDATIVE STRESS AND ULTRAVIOLET RADIATION SURVIVAL

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Bacteria are exposed to many stresses throughout their life cycle, including ultraviolet radiation (UV) radiation and oxidative stress. Oxidative stress and ultraviolet radiation were focused on specifically because *Erwinia amylovora* has been observed to induce an oxidative burst in host plants, and UV radiation stress was tested because few studies to date have explored the role of UV sensitivity and virulence. A forward genetics approach was used to identify *E. amylovora* Ea1189 gene mutations that resulted in an increased sensitivity to hydrogen peroxide. Of the mutants identified, further study focused on one mutant with a defective *waaL* gene, which is responsible for attaching O-antigen to the lipopolysaccharide (LPS) layer. Other studies have shown that deficiencies in the LPS layer can lead to different phenotypes including decreased virulence, decreased motility, and increased sensitivity to antibiotics. Prior to the work discussed here, a relationship between a truncated LPS layer and increased sensitivity to hydrogen peroxide had not been discovered. Complementation of the *waaL* gene on the plasmid pMCB3 restored the mutant to near WT levels in hydrogen peroxide sensitivity as well as the other phenotypes mentioned. A reverse genetics approach was used to study the response of *E. amylovora* Ea1189 to UV radiation. When compared to other Gram-negative bacteria, *E. amylovora* had a higher survival and mutability rate. Survival was reduced in an *umuDC* knockout strain, whose gene product is responsible for mutagenic DNA repair. Mutability was greatly reduced in the *umuDC* knockout strain, but both phenotypes were restored when complemented with plasmids pJJK25 and pJJK27 which carry the *umuDC homolog rulAB*, and carry *umuDC* respectively.

To my Mom and Dad

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Chapter 1

Literature Review

Introduction

Fire blight, a disease of the *Rosaceae* family including apple and pear, was first observed in 1782 in North America (Bonn & van der Zwet, 2000). Early hypotheses on the causal agent of fire blight included poisoning of the plant by insects (Skinner, 1829), sap freezing, and lightning (Arthur, 1886); later observations led to the discovery of the bacterium that causes fire blight (Arthur, 1886). This bacterium, *Erwinia amylovora,* is a Gram-negative plant pathogen that initiates infections in the spring when bacteria, present in ooze from overwintering cankers, are spread via rain and insects to flowers or open wounds on the plant (Thomson, 2000). Systemic migration of bacterial cells from infected flowers throughout the host occurs via the xylem (Vanneste & Eden-Green, 2000). Symptoms of fire blight include water soaking, necrosis, and wilting of infected tissue (Jones & Aldwinckle, 1990).

Fire blight causes economic losses every year, and, during epidemics, monetary losses of millions of dollars can occur (Vanneste, 2000; Norelli et al., 2003). Difficulty in managing this disease contributes to the yearly losses. Use of the bactericides streptomycin and copper represents the main control strategy utilized for flower infection, but both treatments have limitations. Streptomycin resistance in *E. amylovora* was first reported in the 1970's and continues to become more prevalent over time (Jones & Schnabel, 2000). Other antibiotics, such as oxytetracycline have been tested but are less efficacious than streptomycin (McManus et al., 2002). Copper bactericides are effective against *E. amylovora*, but can damage the plant, including the fruits, which reduces crop

value (McManus et al., 2002). Subsequently, studying the life cycle, various chemical treatments, and host-pathogen interactions should yield valuable insights that could result in novel methods of managing this disease.

Defenses Used by Plants to Ward off Pathogen Invasion

Plants use a variety of responses to ward off pathogen invasion such as basal defenses, which include physical and chemical barriers as well as non-specific and specific defenses. The cuticle is the first physical barrier that a plant possesses, and is difficult to penetrate because it is composed of waxes and cutin (Baker & Martin, 1963; Chassot et al., 2007). The stomata are another potential physical barrier to pathogenesis, and impede pathogen invasion by closing. However, some plant pathogenic bacteria, for example *Pseudomonas syringae*, bypass this barrier by secreting a chemical mimic of jasmonic acid that causes the stomata to open (Melotto et al., 2006).

Basal plant defenses, such as modification of the cell wall, occur at the cellular level. Alterations in cell wall composition exclude the pathogen from entry into the cell, but this response can be suppressed by the pathogen (Vorwerk et al., 2004; Yun et al., 2006). Basal defenses are triggered by pathogen associated molecular patterns, which are conserved features shared among microbial pathogens such as flagella and lipopolysaccharides (Chisholm et al., 2006). For example, the survival of *P. syringae* pv. tomato DC3000 was inhibited when *Arabidopsis thaliana* plants were pre-treated with flagellin, but WT plants without pre-treatment were susceptible to infection (Zipfel et al., 2004). Likewise, the lipopolysaccharide layer (LPS), the outermost layer of the Gram-negative bacterial membrane to be detailed later, triggered the production of reactive

oxygen species (ROS) and induced the production of defense related genes in rice (Desaki et al., 2006).

Plant defenses are classified into non-specific microbe recognition and those that recognize specific elicitors released by the pathogen during infection (Jones & Dangl, 2006). Pathogen associated molecular patterns elicit a non-specific response from the host. In contrast, the recognition of a specific pathogen effector (virulence and pathogenesis determinants that facilitate infection in the host) by a resistance protein triggers the hypersensitive response (HR), which results in the release of phytoalexins, production of ROS, host cell death, and the activation of other defense responses (Nürnberger & Brunner, 2002; Chisholm et al., 2006; Jones & Dangl, 2006; Shetty et al., 2008). One of the best known examples of a resistance protein-effector interaction is AvrPto-Pto. AvrPto acts to suppress the innate immunity response in the host if the host lacks Pto (Xiang et al., 2008). However, if the host plant encodes *pto*, then the Pto protein will directly interact with AvrPto inducing the expression of defense genes and the HR (Nomura et al., 2005; Xiang et al., 2008). The previous two relationships represent compatible and incompatible interactions, respectively. For an incompatible interaction to occur, the host plant must recognize an effector secreted by the pathogen, which triggers the HR resulting in cell death (Gürlebeck et al., 2006; Zhao et al., 2006). If the pathogen lacks the recognized effector or the host lacks the resistance protein that recognizes the effector, a compatible interaction results, in which pathogenesis occurs and the HR is suppressed (Gürlebeck et al., 2006; Zhao et al., 2006).

A compatible interaction does not appear to occur between *E. amylovora* and the host because *E. amylovora* induces the host to produce the HR, which is similar to an

incompatible interaction (Venisse et al., 2001; Venisse et al., 2002). As examples, *E. amylovora* elicits an incompatible interaction in Tobacco (not a host of *Erwinia*), and in pear (a host of *Erwinia*), evidenced by the production of superoxide (Venisse et al., 2001) (Figure 1.1).

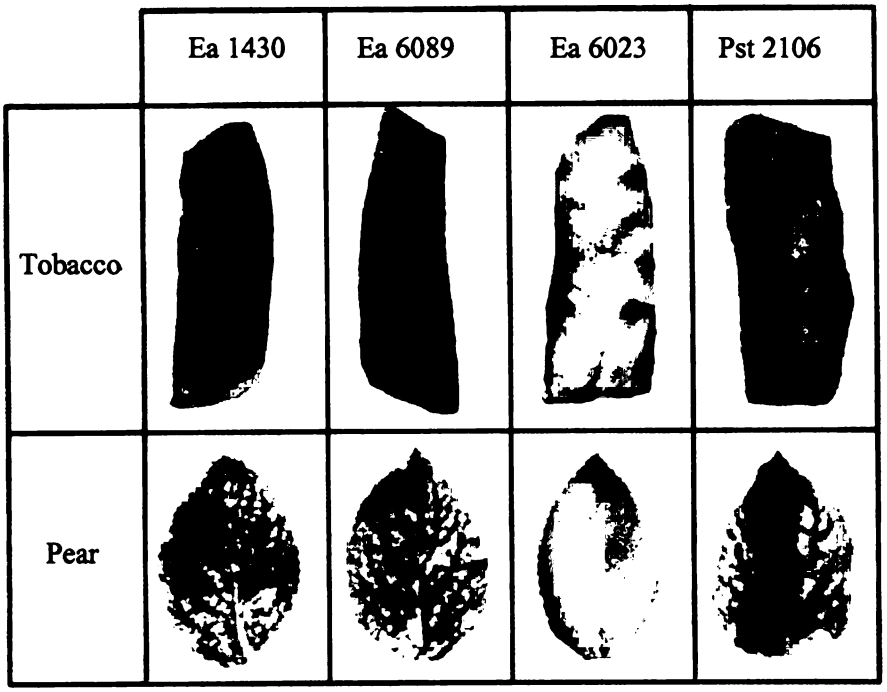

Figure 1.1. Staining of superoxide in tobacco and pear respectively. Nitroblue tetrazolium staining in tobacco and pear leaves 12 and 8 h, respectively, after infiltration of *E. amylovora* 1430 (WT), *E. amylovora* 6089 (*ams*$^-$), *E. amylovora* 6023 (*hrp*$^-$), and *Pseudomonas syringae* pv. tabaci 2106 (WT). The black staining indicates the presence of O_2^- (Figure taken from Venisse et al., 2001).

Other work by Venisse et al. (2001; 2002) showed that the production of superoxide and suppression of plant antioxidants preceded invasion by *E. amylovora* (Figure 1.2).

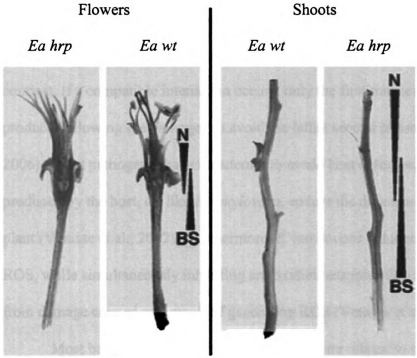

Figure 1.2. Superoxide anion O_2^- generation bioassay in apple flowers and shoots of the susceptible genotype MM106. Flowers were sampled 4 days after deposit of a drop of bacterial suspensions (10^7 CFU/ml) into the hypanthium. Shoots were sampled 10 days after deposit of a drop of bacterial suspensions (10^7 CFU/ml) onto a fresh cut made on young developed leaves. Samples were vacuum infiltrated with a 0.5% solution of nitroblue tetrazolium and photographed 30 min later. Spreading of blue staining (BS), which indicates qualitatively the presence of O_2^-, and of necrosis (N) are indicated by triangles. Ea wt = wild-type strain of *E. amylovora* CFBP1430; Ea *hrp* = *E. amylovora hrp* secretion mutant PMV6023 derived from CFBP1430 (Figure taken from Venisse et al., 2002).

Production of Reactive Oxygen Species and Genes Involved in ROS Resistance

The production of ROS, a pivotal component of plant defense systems, is triggered during the HR, considered one of the first lines of defense of the host (Lamb & Dixon, 1997; Venisse et al., 2001; Inzé & Montagu, 2002; Venisse et al., 2002). In plants, microbes can elicit either a compatible or incompatible reaction from the host. An incompatible reaction involves a biphasic accumulation of ROS (the oxidative burst) where the first phase is a small transient increase of ROS followed by a second phase

consisting of a more intense continuous production of ROS (Torres et al., 2006). The second phase of the oxidative burst is responsible for killing the invading pathogen. In contrast, if a compatible interaction occurs, only the first transient burst of ROS is produced allowing the pathogen to avoid the lethal second phase of ROS (Torres et al., 2006). Plant pathogenic bacteria attempt to evade host defenses, detoxify compounds produced by the host, or, like *E. amylovora*, endure the defenses produced by the host plant (Venisse et al., 2002). Furthermore, *E. amylovora* induces the host to produce ROS, while simultaneously inhibiting antioxidant enzymes that could protect the plant from damage created as a result of generating ROS (Venisse et al., 2002).

Most bacteria encode a suite of genes that are related to oxidative stress susceptibility. The two largest sets of genes involved in oxidative stress survival are regulated by OxyR and SoxRS. The OxyR regulon includes the catalase-peroxidase genes, *ahpC*, and peroxiredoxin (Charoenlap et al., 2005; Mongkolsuk & Dubbs, 2005; Hishinuma et al., 2006). Catalase and peroxidase both inactivate hydrogen peroxide (Charoenlap et al., 2005; Mongkolsuk & Dubbs, 2005; Hishinuma et al., 2006) producing water and oxygen as end products (Inzé & Montagu, 2002). AhpC and peroxiredoxin work together to detoxify ROS with AhpC becoming oxidized as it detoxifies and peroxiredoxin reducing AhpC so it regains function (Charoenlap et al., 2005; Mongkolsuk & Dubbs, 2005; Hishinuma et al., 2006). Another gene regulated by OxyR is *dps* (DNA binding protein in stationary phase). The Dps protein does not directly inactivate ROS, but instead protects the bacterium from DNA damage by binding to chromatin, and also serves a preventative role by binding iron, which can be used to generate ROS (Halsey et al., 2004). Sequestering iron from the host is an important

preventative function because iron, in the presence of hydrogen peroxide and superoxide, can yield hydroxyl radicals, the most damaging of the ROS (Inzé & Montagu, 2002). This reaction recycles iron so that it can be used repeatedly to generate more hydroxyl radicals in a process known as the Haber-Weiss reaction (Inzé & Montagu, 2002) (Figure 1.3).

(1) the Fenton reaction, resulting in the production of $OH^{\bullet}$ from H_2O_2:

$$H_2O_2 + Fe^{+2} \longrightarrow OH^{\bullet} + OH^{-} + Fe^{+3}$$

(2) recycling of ferrous ion by superoxide, which acts as a reductant, allowing reaction (1) to continue:

$$O_2^{\bullet -} + Fe^{+3} \longrightarrow + O_2 + Fe^{+2}$$

(3) the net sum of reactions (1) and (2) is the so-called Haber-Weiss reaction:

$$H_2O_2 + O_2^{\bullet -} \longrightarrow O_2 + OH^{\bullet} + OH^{-}$$

Figure 1.3. Molecular formula illustrating the Haber-Weiss reaction. Iron atoms are not consumed in this reaction but recycled as hydroxyl radicals are formed. (Figure taken from Inzé & Montagu, 2002).

Whereas OxyR is involved in hydrogen peroxide stress, SoxRS regulates genes involved with superoxide stress survival (Wu & Weiss, 1992; Inzé & Montagu, 2002). SoxRS regulates the transcription of many genes including *sodA*, *fur*, and *nfo* (Pomposiello & Demple, 2001). Superoxide dismutase (*sodA*), one of the most studied of the genes regulated by SoxRS, when expressed, detoxifies superoxide by converting two superoxide molecules into hydrogen peroxide and oxygen (Inzé & Montagu, 2002). SoxR, once activated, initiates the transcription of *soxS*, which then regulates the other genes in the regulon (Wu & Weiss, 1992). Genes regulated by SoxS are not transcribed as frequently when *soxR* is knocked out (Wu & Weiss, 1992).

DNA damage is the most destructive result of exposure to ROS. One example of this is shown by Greenberg & Demple (1988), where *Eschericia coli* lacking a functional *oxyR* gene resulted in a phenotype of increased spontaneous mutations by 80-fold. When exogenous catalase or alkyl hydroperoxide reductase was added, the rate of spontaneous mutations decreased by 10 to 20-fold compared to the mutant (Greenberg & Demple, 1988). This result would suggest that a bacterium unable to mount defenses to hydrogen peroxide stress is damaged to an extent where repair was necessary to survive, which could explain the evolutionary retention of oxidative stress related genes.

Correlation Between Reactive Oxygen Species and Virulence Efficacy

One factor that affects the virulence of a plant pathogen is the concentration of ROS produced by the plant (Wu et al., 1995; Hu et al., 2003). If ROS cannot be detoxified, avoided, or prevented from being formed, then the bacterium can be killed. This is well represented in work by Wu et al. (1995), in which transgenic potato plants that produced higher concentrations of hydrogen peroxide than WT inhibited growth and virulence of the potato pathogens, *Erwinia carotovora* subsp. carotovora (now called *Pectobacterium carotovorum* subsp. carotovorum) and *Aspergillus niger* . Another finding of note is that the increased production of hydrogen peroxide did not result in spontaneous lesions in the transgenic potato when compared to the wild type plant (Wu et al., 1995). Generating transgenic plants that produce a higher concentration of hydrogen peroxide has been attempted in other plant systems such as sunflowers with similar results (Hu et al., 2003).

E. amylovora, like some pathogens, forms a biofilm, an extracellular matrix that a group of cells form in culture and *in planta* (Koczan et al., 2008). Work by Elkins et al.

(1999) provides an example of how the impact of ROS is altered by the presence of a biofilm. Susceptibility to hydrogen peroxide was increased by 100 fold in individual cells compared to cells in a biofilm (Elkins et al. 1999). In addition, *E. amylovora* lacking the *ams* operon, necessary for formation of a biofilm, is incapable of establishing infection in the host (Koczan et al., 2008). Formation of a biofilm could be a component a pathogen uses to survive oxidative stress in the host.

Introduction to the Lipopolysaccharide Layer

The lipopolysaccharide layer (LPS) comprises the outer layer of the outer membrane and can represent up to 90% of the outer layer in Gram-negative bacteria (Rosenfeld & Shay, 2006). This layer can be separated into three components (Hitchcock et al., 1986) (Figure 1.4).

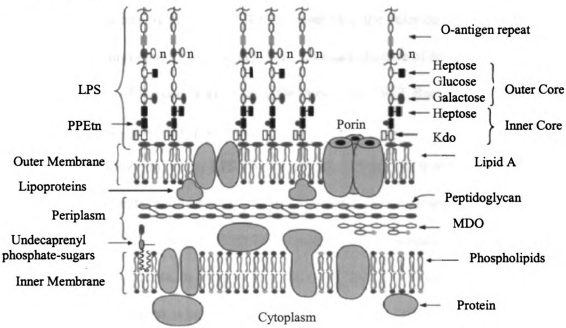

Figure 1.4. Model of the inner and outer membranes of *E. coli* K-12. (Figure taken from Raetz & Whitfield, 2002).

The first component (lipid A) is attached to the phospholipid layer of the outer membrane, is well conserved, and is a requirement for survival by most bacteria (Fraser et al., 1998; Raetz & Whitfield, 2002) (Figure 1.4). Conservation of the lipid A portion can be seen in different species of bacteria including *E. amylovora* (Hitchcock et al., 1986; Ray et al., 1986).

The second component, which is attached to the lipid A, is the core oligosaccharide, which is further divided into two subsections, the inner and outer core (Newman et al., 2007) (Figure 1.4). The inner core is mainly composed of KDO (keto-3-deoxyoctanate), is well conserved in bacteria, and is required for survival similar to lipid A (Hitchcock et al., 1986; Raetz & Whitfield, 2002; Newman et al., 2007) (Figure 1.4). The outer core can be composed of multiple sugars, including heptose, galactose, glucose, and fucose among others. Unlike the inner core, the outer core is more variable among bacteria, and not necessary for survival, although absence of an outer core results in decreased fitness of the cell (Ray et al., 1986; Ray et al., 1987; Raetz & Whitfield, 2002; Newman et al., 2007) (Figure 1.4).

The last component of the LPS layer is the O-antigen, which is the most variable of the three components, and is not necessary for survival of the bacterium (Hitchcock, 1986). Typically the O-antigen is composed of repeats of a monosaccharide, which can be rhamnose, xylose, fucose, or others (Newman et al., 2007). To highlight the variability of the O-antigen, when LPS from *E. coli* and *Salmonella* were compared, only three O-antigen types were shared by both species after comparing 173 and 50 O-antigen types, respectively (Reeves et al., 1996). Strains can also be serotyped by the type of O-antigen produced, and, in some cases, the O-antigen can be used as a vaccine (Goldberg

& Pier, 1996). This portion of the LPS layer is not as prevalent in bacteria outside of the enteric family (Hitchcock et al., 1986), but serves as a virulence factor when the O-antigen is present (Bengoechea et al., 2004; Lapaque et al., 2005; Plainvert et al., 2007). O-antigen acts as a virulence factor through masking the more conserved inner portions of the LPS layer, and can also conceal receptors used by bacteriophage to initiate infection (Whitfield et al., 1997).

The LPS layer has other properties that have not been extensively researched but should be noted. Up to 90% of the outer leaflet of the outer membrane is composed of LPS (Rosenfeld & Shay, 2006), and of that, up to 50% of the LPS molecules in the cell can exhibit a rough (classified as an LPS layer lacking O-antigen or the terminal portion of the core oligosaccharide) or semirough (an LPS layer with no more than one O-antigen molecule attached) phenotype (Hitchcock et al., 1986, Raetz & Whitfield, 2002). In other words, in any given wild type cell, the LPS layer can be composed of molecules with variable lengths of O-antigen repeats or no O-antigen. This variability of the O-antigen could explain its role as a virulence factor. Another notable property of the LPS layer is that when any of the genes responsible for assembling the core are disrupted, subsequent steps of LPS biosynthesis do not occur indicating that each sugar needs the previous one to attach to the molecule, and that there is no compensatory mechanism that takes the place of the disrupted gene so that biosynthesis can continue.

Alterations to the Lipopolysaccharide Layer in Response to Stress

The LPS layer is modified in response to stress in some bacteria. In *Pseudomonas aeruginosa*, an opportunistic pathogen that typically infects the lungs of Cystic Fibrosis patients, two types of LPS known as the A-band and B-band exist

(Goldberg & Pier, 1996; Sabra et al., 2003). While the A-band is constitutively expressed during all stages of infection, B-band LPS is present at its highest concentration upon initial infection and decreases once the infection becomes chronic (Goldberg & Pier, 1996; Sabra et al., 2003). In addition, B-band LPS is expressed at higher oxygen concentrations, but not below 3% (microaerophilic) conditions (Sabra et al., 2003). Although not directly related to oxidative stress, this work indicates that the LPS layer can change in response to stress.

Klebsiella pneumoniae is another bacterium where different forms of LPS exist. There have been at least two core types found in *K. pneumoniae*, type I and type II (Regué et al., 2005). The difference in the two cores is explained by type I cores encoding different genes (*wabI* and *wabJ*) than type II cores (*wabK* and *wabM*), but in both cores, the two genes are found flanking *waaL* (Regué et al., 2005). Although a strain of *K. pneumoniae* with both core types has not yet been discovered, it has been demonstrated that different core types display different levels of virulence in mouse models (Regué et al., 2005). It has yet to be explored why the more virulent type II core is not more prevalent in populations of *K. pneumoniae,* where it composes only 19% of the collection of 100 isolates obtained by the Regué lab. A recent study by Patil et al. (2007) highlights the variability of the LPS layer between different bacterial species. Amongst the genes explored in this work, only two genes were conserved across all eight of the *Xanthomonads* studied.

The Lipopolysaccharide Layer and its Contribution to Virulence

Many studies over the last few decades demonstrate the relationship between bacterial virulence and the presence of the LPS layer (Newman et al., 2001; Erbs &

Newman, 2003; Bengoechea et al., 2004; Lapaque et al., 2005; Plainvert et al., 2007). These studies have covered animals, plants, and humans, typically showing a correlation between truncation of the LPS layer and a decrease in virulence. Mutations that cause truncations in the LPS layer result in a number of phenotypes including sensitivity to antibiotics and reduced motility. In *Erwinia carotovora* subsp. *atroseptica*, for example, loss of O-antigen resulted in a deficiency in motility, decrease in virulence, and reduced production of exoenzymes used by *E. carotovora* for virulence (Toth et al., 1999). In *E. coli* and *Salmonella* strains, truncated cores resulted in an increased sensitivity to hydrophobic antimicrobials and leakage of periplasmic enzymes into the extracellular space (Heinrichs et al., 1998). Another study in *Salmonella* showed that a strain with a mutation in *waaP*, a gene responsible for adding phosphate to the first heptose residue of the inner core, was more sensitive to the antibiotics novobiocin, polymyxin, and SDS, and was also unable to infect mouse models (Yethon et al., 2000). In contrast, all of the mice infected with WT *Salmonella* died (Yethon et al., 2000). Further studies with *K. pneumoniae* detailed effects of mutations in the outer core of the LPS layer on virulence in mice (Izquierdo et al., 2003). Four mutants were explored with mutations in *waaC, waaF, wabG,* and *waaL* (Izquierdo et al., 2003). With the exception of *waaL,* which attaches the O-antigen to the lipid A core, these genes are responsible for attaching different sugars onto the Lipid A core Multiple organs were tested in mouse models to observe bacterial colonization. While *waaL* was the only one of the four mutants that could survive as well as WT in one of the organs tested (the lung), all of the mutants tested could not survive in the other organs tested (Izquierdo et al., 2003). Also, the mutant strains were more susceptible than WT to different antibiotics including SDS and

polymyxin B (Izquierdo et al., 2003). This correlation between core truncation and sensitivity to antibiotics is also observed in *Burkholderia cenocepacia*, in which a mutant that produced a truncated core lost the ability to infect the lung of rat models (Loutet et al., 2006). As previously mentioned, purified LPS can be used as a vaccine in animal models, but this effect is seen in plants as well. In pepper, localized resistance can last up to 30 hours post LPS inoculation, but in tobacco, systemic resistance has been demonstrated (Newman et al., 2001). This resistance involves processes such as the oxidative burst, cross-linking of cell walls, and increased transcription of defense genes. As shown by an *Arabidopsis* microarray, treatment with LPS caused the induction and repression of many genes, specifically stress and defense genes at the site of inoculation (Zeidler, 2004). This effect was also seen systemically although expression diminished over distance (Zeidler, 2004).

The Lipopolysaccharide Layer and Mutualism

Although most of the work that has focused on the LPS layer examines the correlation between deficiencies in the LPS layer and virulence, some work has also focused on how mutualistic relationships are affected by LPS deficiencies. Numerous species of *Rhizobium* and *Bradyrhizobium* are incapable of establishing a population in host plants when genes responsible for LPS biosynthesis are knocked out. Eight LPS mutants were investigated by Cava et al. (1989), all of which were lacking all or most of the O-antigen component of the LPS layer. These mutants were not able to survive in the bean plant and could only produce what is called an abortive infection thread (Cava et al., 1989). In *Rhizobium tropici* CIAT899, three mutants were explored that were missing O-antigen with one of the three also truncated in the outer core of LPS (Ormeño-Orrillo et

al., 2008). The truncated core mutant CIAT899-E3 could not produce nodules in the host, and the two O-antigen mutants produced nodules but could not sustain populations in the host (Ormeño-Orrillo et al., 2008). Other studies with *Rhizobium* have produced contradictory results however. Work by D'Antuono et al. (2005) demonstrated that the bacterium *Mesorhizobium loti* sustained normal infection in the host with a truncated or non-existent O-antigen, but the mutants were less fit than WT in a competition assay (D'Antuono et al., 2005). Similarly, competition experiments performed by Ormeño-Orrillo et al. (2008) showed that LPS mutants were less fit than the WT strain. Whether this new finding has to do with the specific strain or host plant involved, or if this finding will be observed by other researchers in the field, has yet to be determined.

Conclusions

The LPS layer serves many important roles during the bacterial life cycle. Because the LPS layer is important for Gram-negative bacterial survival, it is recognized as a pathogen associated molecular pattern and triggers basal immune responses. This importance is also observed when the LPS layer is truncated due to disruption of LPS biosynthesis genes, which increases a cell's susceptibility to antibiotics and decreases motility and virulence. As the next chapter will demonstrate, a defective LPS layer also exhibits a phenotype of sensitivity to ROS.

Introduction to Ultraviolet Light Stress and DNA Repair

Almost every known organism is exposed to ultraviolet radiation (UV) at some point in its life cycle. UV light causes direct and indirect damage to cells (Joux et al., 1999; Kim & Sundin, 2001; Qiu et al., 2004; Zenoff et al., 2006). UV-B (280-315 nm) is

a higher energy radiation than UV-A (315-400 nm) and damages DNA which is lethal if not repaired (Sinha & Hader, 2002). UV-A can indirectly damage the cell by generating ROS, which can then damage cells in ways previously mentioned in this review (Sinha & Hader, 2002). There is also a third type of UV light, UV-C (<280 nm), but it is absorbed by the atmosphere before reaching the Earth's surface (Sinha & Hader, 2002). Because UV light is a ubiquitous part of most environments and damages cells, almost every organism, from bacteria to humans, has evolved mechanisms to combat UV-induced damage. Coping with UV damage can involve avoidance using negative phototaxis, or absorption of light by compounds like flavenoids in plants, melanin in humans, or other compounds (Sinha & Hader, 2002). If UV induced damage does occur, the cell employs a suite of enzymatic systems that can repair damaged DNA resulting from UV-light absorption by the cell.

Genes involved in UV tolerance are found in most organisms including those that are not typically exposed to UV light. Isolates identified by Arrage et al. (1993) that were shielded from UV light for millions of years were resistant to UV light similarly to isolates from the soil surface. Of the 70 isolates discovered, 31% of the isolates in the soil were resistant to UV light whereas 26% on the soil surface were resistant (Arrage et al., 1993). From this same work, two trends were identified: pigmented cells and Gram-positive cells were more resistant to UV light than non-pigmented cells and Gram-negative cells (Arrage et al., 1993).

DNA Photolyase and Photoreactivation

There are many gene products responsible for resistance to UV light in bacteria (Sinha & Hader, 2002). One of the best characterized is DNA photolyase, encoded by

the *phr1* gene (Yasui & Chevallier, 1983; Kim & Sundin 2001). The Phr protein repairs UV damage in a light dependent reaction, compared to other repair mechanisms that can function in the dark (Kim & Sundin 2001; Sinha & Hader, 2002). DNA photolyase repairs cyclobutane-pyrimidine dimers such as thymine-thymine (T-T) or thymine-cytosine (T-C), which are commonly formed after exposure to UV light (Brash et al., 1985; Kim & Sundin, 2001).

Nucleotide Excision Repair

In addition to DNA photolyase, nucleotide excision repair (NER) and base excision repair (BER) are important in repairing DNA damage. Whereas base excision repair specifically removes individual damaged bases, NER is responsible for removing a larger section of DNA that disrupts the overall structure of the double helix. NER is more complex than photoreactivation requiring around 30 genes to function (Sinha & Hader, 2002). In *Shewanella oneidensis*, NER is not efficiently expressed, and this difference makes *S. oneidensis* sensitive to UV light (Qiu et al., 2004). After exposure to 15 J m^{-2}, cell survival was less than 0.1% (Qiu et al., 2004). In comparison, the survival of *E. amylovora* Ea1189 does not drop to 0.1% until cells are exposed to nearly 150 J m^{-2} UV-C light. (Berry, unpublished). Because NER is coordinated by more than one gene, different phenotypes can be exhibited depending on the gene that is defective. At least three different diseases in humans are linked to defective components of NER. Each of these diseases has a phenotype of sunlight sensitivity (de Boer & Hoeijmakers, 2000).

Mutagenic DNA Repair

umuDC and its homologs belong to the mutagenic DNA repair family of genes and are part of the SOS system of repair, which means that these genes are regulated by

RecA and LexA (Witkin, 1976). LexA represses SOS response genes from being transcribed until RecA cleaves LexA releasing it from the DNA and allowing transcription to occur (Bagg et al., 1981). RecA is activated by the presence of single stranded DNA, which is present when a DNA mutation occurs that stalls the replication machinery (Lee et al., 1996). Mutagenic repair is a last resort of the cell to stay alive after other repair mechanisms fail to correct DNA damage (Sinha & Hader, 2002). Mutagenic repair is error prone, occasionally causing a mutation at the site of damage, but allowing replication to continue ensuring cell survival.

umuDC is present throughout the enterobacteriaciae, but varies in its efficiency (Sedgwick et al., 1991). Differences in mutability between strains have been observed to be 200-fold even in strains with the same gene pair (Sedgwick et al., 1991). *umuDC* and its homologs such as *rulAB, rumAB, samAB*, *impCAB*, and *mucAB* function in mutagenic repair. Differences in efficiency and mutations caused by UmuDC and its homologs vary (Szekeres Jr. et al., 1996). For example, *umuDC* is the most inefficient at DNA repair compared to other UV repair homologs (Woodgate & Sedgwick, 1992; Kim & Sundin, 2000). This is because UmuD is cleaved in a RecA-mediated reaction generating UmuD', which forms a homodimer that complexes with UmuC (Woodgate & Sedgwick, 1992; Kim & Sundin, 2000). *umuDC* produces transversions, specifically T to A, five times more often than T to C transitions (Szekeres Jr. et al., 1996). In *rumAB* and *mucAB*, the opposite is true with the same transversion occurring five times less frequently than the same transition seen in *umuDC* strains (Szekeres Jr. et al., 1996). Differences in mutability are variable depending on the cell in which the gene pair is expressed. For example, when *mucAB* was inserted into a *E. coli umuDC*- cell,

mutability was increased. Similarly, the same experiment with *rulAB* resulted in increased mutability compared to *umuDC* (Kim & Sundin, 2000). When the three gene pairs were placed in *P. aeruginosa*, the mutabilities of the three strains were different than when in *E. coli* (Kim & Sundin, 2000). For this reason it is hard to assign an order of which gene pair makes the cell more mutable because efficiency, in part, is also dictated by the environment (the cell) where the gene pair exists (Kim & Sundin, 2000). However, when studies in culture are compared to *in planta* studies the amount of viable cells found after UV light exposure are similar. RulAB functioned similarly in the cell whether it was grown in LB or in a plant host (Kim & Sundin, 2000). One similarity that the different gene homologs have in common is genomic location in the cell. *umuDC* specifically is usually found on conjugative plasmids making horizontal transfer likely and the observation of its presence in many bacterial species better understood, however, the gene pair can also be found on chromosomal DNA (Woodgate & Sedgwick, 1992).

Conclusions

DNA repair is important to a bacterial cell because it allows replication to continue. Without DNA repair, cells would amass mutations detrimental to survival causing the cell to die. In some instances, the commonly used mechanisms of repair are not sufficient to correct damage caused by UV radiation. Without mutagenic repair, the cell would likely die in this instance, but mutagenic repair corrects damage that otherwise could not be fixed allowing replication to continue although the cost is an increased mutation rate.

The LPS layer and DNA repair represent ways that bacteria cope with stress. It could be argued that bacteria are especially adapted to resisting stress because they have

had much more time to deal with a stress than another organism that has not been around as long, evolutionarily speaking. For this reason, bacteria successfully inhabit almost every environment on Earth.

References

Abeyrathne PD, Daniels C, Poon KKH, Matewish MJ & Lam, J S (2005) Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. *J Bacteriol* **187:** 3002-3012.

Arrage AA, Phelps TJ, Benoit RE & White DC (1993) Survival of subsurface microorganisms exposed to UV radiation and hydrogen peroxide. *Appl Environ Microbiol* **59:** 3545-3550.

Arthur JC (1886) History and biology of pear blight. *Proc Natl Acad Sci U S A* **38:** 322-341.

Bagg A, Kenyon CJ & Walker GC (1981) Inducibility of a gene product required for UV and chemical mutagenesis in *Escherichia coli. Proc Natl Acad Sci U S A* **78:** 5749-5753.

Baker EA & Martin JT (1963) Cutin of plant cuticles. *Nature* **199:** 1268-1270.

Bengoechea JA, Najdenski H & Skurnik M (2004) Lipopolysaccharide O antigen status of *Yersinia enterocolitica* O:8 is essential for virulence and absence of O antigen affects the expression of other *Yersinia* virulence factors. *Mol Microbiol* **52:** 451-469.

Bonn WG & van der Zwet T Distribution and economic importance of fire blight. Vanneste JL, ed. (2000) Fire blight the disease and its causative agent *Erwinia amylovora*. New York NY: CABI Publishing. 37-54.

Brash DE, Franklin WA, Sancar GB, Sancar A & Haseltine WA (1985) *Escherichia coli* DNA photolyase reverses cyclobutane pyrimidine dimers but not pyrimidine-pyrimidone (6-4) photoproducts. *J Biol Chem* **260:** 11438-11441.

Cava JR, Elias PM, Turowski DA & Noel KD (1989) *Rhizobium leguminosarum* CFN42 genetic regions encoding lipopolysaccharide structures essential for complete nodule development on bean plants. *J Bacteriol* **171:** 8-15.

Charoenlap N, Eiamphungporn W, Chauvatcharin N, Utamapongchai S, Vattanaviboon P & Mongkolsuk S (2005) OxyR mediated compensatory expression between *ahpC* and *katA* and the significance of *ahpC* in protection from hydrogen peroxide in *Xanthomonas campestris*. *FEMS Microbiol Lett* **249:** 73-78.

Chassot C, Nawrath C & Métraux J-P (2007) Cuticular defects lead to full immunity to a major plant pathogen. *Plant J* **49:** 972-980

Chisholm S, Coaker G, Day B & Staskawicz B (2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124:** 803-814.

D'Antuono AL, Casabuono A, Couto A, Ugalde RA & Lepek VC (2005) Nodule development induced by *Mesorhizobium loti* mutant strains affected in polysaccharide synthesis. *Mol Plant Microbe Interact* **18:** 446-457

de Boer J & Hoeijmakers JHJ (2000) Nucleotide excision repair and human syndromes. *Carcinogenesis* **21:** 453-460.

Desaki Y, Miya A, Venkatesh B, Tsuyumu S, Yamane H, Kaku H, Minami E & Shibuya N (2006) Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells. *Plant cell physiol* **47:** 1530-1540.

Elkins, JG, Hassett, DJ, Stewart, PS, Schweizer, HP & McDermott, TR (1999) Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Appl Environ Microbiol* **65:** 4594-4600.

Erbs G & Newman M-A (2003) The role of lipopolysaccharides in induction of plant defence responses. *Mol Plant Pathol* **4:** 421-425.

Fraser CM, Norris SJ, Weinstock GM, White O, Sutton GG, Dodson R, Gwinn M, Hickey EK, Clayton R, Ketchum KA, Sodergren E, Hardham JM, McLeod MP, Salzberg S, Peterson J, Khalak H, Richardson D, Howell JK, Chidambaram M, Utterback T, McDonald L, Artiach P, Bowman C, Cotton MD, Fujii C, Garland S, Hatch B, Horst K, Roberts K, Sandusky M, Weidman J, Smith HO & Venter JC (1998) Complete Genome Sequence of *Treponema pallidum* the Syphilis spirochete. *Science* **281:** 375-388.

Goldberg JB & Pier GB (1996) *Pseudomonas aeruginosa* lipopolysaccharides and pathogenesis. *Trends Microbiol* **4:** 490-494.

Greenberg JT & Demple B (1988) Overproduction of peroxide-scavenging enzymes in *Escherichia coli* suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR*- mutants. *EMBO J* **7:** 2611-2617.

Gürlebeck D, Thieme F & Bonas U (2006) Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interaction with the host plant. *J Plant Physiol* **163:** 233-255.

Halsey TA, Vazquez-Torre A, Gravdahl DJ, Fang FC & Libby SJ (2004) The ferritin-like Dps protein is required for *Salmonella enterica* serovar typhimurium oxidative stress resistance and virulence. *Infect Immun* **72:** 1155-1158.

Heinrichs DE, Yethon JA & Whitfield C (1998) Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. *Mol Microbiol* **30:** 221-232.

Hishinuma S, Yuki M, Fuimura M & Fukumori F (2006) OxyR regulated the expression of two major catalases, KatA and KatB, along with peroxiredoxin, AhpC in *Pseudomonas putida*. *Environ Microbiol* **8:** 2115-2124.

Hitchcock PJ, Leive L, Mäkelä PH, Rietschel ET, Strittmatter W, Morrison DC (1986) Lipopolysaccharide nomenclature-past, present, and future. *J Bacteriol* **166:** 699-705.

Hu X, Bidney DL, Yalpani N, Duvick JP, Crasta O, Folkerts O & Lu G (2003) Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiol* **133:** 170-181.

Inzé D, Van Montagu M (2002) Oxidative Stress in Plants. Boca Raton, FL: CRC Press. 1-32.

Izquierdo L, Coderch N, Piqué, N, Bedini E, Corsaro M M, Merino S, Fresno S, Tomás J M & Regué, M (2003) The *Klebsiella pneumoniae wabG* gene: Role in biosynthesis of the core lipopolysaccharide and virulence. *J Bacteriol* **185:** 7213-7221.

Jones AL & Aldwinckle HS (1990) Compendium of Apple and Pear Diseases. St. Paul, MN: APS Press. 61-63

Jones AL & Schnabel EL The development of streptomycin-resistant strains of *Erwinia amylovora*. Vanneste JL, ed. (2000) Fire blight the disease and its causative agent *Erwinia amylovora*. New York NY: CABI Publishing. 235-252.

Jones JDG & Dangl JL (2006) The plant immune system. *Nature* **444:** 323-329.

Joux F, Jeffrey WH, Lebaron P & Mitchell DL (1999) Marine bacterial isolates display diverse responses to UV-B radiation. *Appl Environ Microbiol* **65:** 3820-3827.

Kim JJ & Sundin GW (2000) Regulation of the *rulAB* mutagenic DNA repair operon of *Pseudomonas syringae* by UV-B (290 to 320 nanometers) radiation and analysis of *rulAB*-mediated mutability in vitro and in planta. *J Bacteriol* **182:** 6137-6144.

Kim JJ & Sundin GW (2001) Construction and analysis of photolyase mutants of *Pseudomonas aeruginosa* and *Pseudomonas syringae*: contribution of photoreactivation, nucleotide excision repair, and mutagenic DNA repair to cell survival and mutability following exposure to UV-B radiation. *Environ Microbiol* **67:** 1405-1411.

Koczan J, McGrath M, Zhao Y & Sundin GW (2008) Biofilm formation in *Erwinia amylovora*: implications in pathogenicity. *Acta Horticulturae.* **793**: 67-72.

Lamb C & Dixon RA (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* **48:** 251-275.

Lapaque N, Moriyon I, Moreno E & Gorvel J-P (2005) *Brucella* lipopolysaccharide acts as a virulence factor. *Curr Opin Microbiol* **8:** 60-66.

Lee MH, Guzzo A & Walker GC (1996) Inhibition of RecA-mediated cleavage in covalent dimers of UmuD. *J Bacteriol* **178:** 7304-7307.

Loutet SA Flannagan RS, Kooi C, Sokol PA & Valvano MA (2006) A complete lipopolysaccharide inner core oligosaccharide is required for resistance of *Burkholderia cenocepacia* to antimicrobial peptides and bacterial survival in vivo. *J Bacteriol* **188:** 2073-2080.

Martin JT (1964) Role of cuticle in the defense against plant disease. *Annu Rev Phytopathol* **2:** 81-100

McManus PS, Stockwell VO, Sundin GW & Jones AL (2002) Antibiotic use in plant agriculture. *Annu Rev Phytopathol* **40:** 443-465.

Melotto M, Underwood W, Koczan J, Nomura K & He SY (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell* **126:** 969-980.

Mongkolsuk S & Dubbs J M (2005) Chemical modulation of physiological adaptation and cross-protective responses against oxidative stress in soil bacterium and phytopathogen *Xanthomonas*. *J Ind Microbiol Biotechnol* **32:** 687-690

Newman M-A Dow JM & Daniels MJ (2001) Bacterial lipopolysaccharides and plant-pathogen interactions. *Eur J Plant Pathol* **107:** 95-102.

Newman M-A Dow JM, Molinaro A & Parrilli M (2007) Priming induction and modulation of plant defence responses by bacterial lipopolysaccharides. *J Endotoxin Res* **13:** 69-84.

Nomura K, Melotto M & He S-Y (2005) Suppression of host defense in compatible plant-*Pseudomonas syringae* interactions. *Curr Opin Plant Biol* **8:** 361-368.

Norelli JL, Holleran HT, Johnson WC, Robinson TL & Aldwinckle HS (2003) Resistance of Geneva and other apple rootstocks to *Erwinia amylovora*. *Plant Dis* **87:** 26-32.

Nürnberger T & Brunner F (2002) Innate immunity in plants and animals: emerging parallels between the recognition general elicitors and pathogen-associated molecular patterns. *Curr Opin Plant Biol* **5:** 318-324.

Ormeño-Orrillo E, Rosenblueth M, Luyten E, Vanderleyden J & Martínez-Romero E (2008) Mutations in lipopolysaccharide biosynthetic genes impair maize rhizosphere and root colonization of *Rhizobium tropici* CIAT899. *Environ Microbiol* **10:** 1271-1284.

Patil PB, Bogdanove AJ & Sonti RV (2007) The role of horizontal transfer in the evolution of a highly variable lipopolysaccharide biosynthesis locus in xanthomonads that infect rice citrus and crucifers. *BMC Evol Biol* **7:** 243-255.

Plainvert C, Bidet P, Peigne C, Barbe V, Medigue C, Denamur E, Bingen E et al. (2007) A new O-antigen gene cluster has a key role in the virulence of *Escherichia coli* meningitis clone O45:K1:H7. *J Bacteriol* **189:** 8528-8536.

Pomposiello PJ & Demple B (2001) Redox-operated genetic switches: the SoxR and OxyR transcription factors. *Trends Biotechnol* **19:** 109-114.

Qiu X, Sundin GW, Chai B & Tiedje JM (2004) Survival of *Shewanella oneidensis* MR-1 after UV radiation exposure. *Appl Environ Microbiol* **70:** 6435-6443.

Raetz, CRH & Whitfield C (2002) Lipopolysaccharide Endotoxins. *Annu Rev Biochem* **71:** 635-700.

Ray TC, Smith ARW, Carter KJ & Hignett RC (1986) Composition of lipopolysaccharides from four strains of *Erwinia amylovora*. *J Gen Microbiol* **132:** 3159-3167.

Ray TC, Smith ARW, Wait R & Hignett RC (1987) Structure of the sidechain of lipopolysaccharide from *Erwinia amylovora* T. *Eur J Biochem* **170:** 357-361.

Reeves PR, Hobbs M, Valvano MA Skurnik M, Whitfield C, Coplin D, Kido N, Klena J, Maskell D, Rates CRH & Rick P D (1996) Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* **4:** 495-503.

Regué, M, Izquierdo L, Fresno S, Piqué, N, Corsaro MM, Naldi T, De Castro C, Waidelich Merino S & Tomás JM (2005) A second outer-core region in *Klebsiella pneumoniae* lipopolysaccharide. *J Bacteriol* **187:** 4198-4206.

Rosenfeld Y & Shay Y (2006) Lipopolysaccharide (Endotoxin)-host defense antibacterial peptides interactions: Role in bacterial resistance and prevention of sepsis. *Biochem Biophys Acta* **9:** 1513-1522.

Sabra W, Lünsdorf H, Zeng & A-P (2003) Alterations in the formation of lipopolysaccharide and membrane vesicles on the surface of *Pseudomonas aeruginosa* PAO1 under oxygen stress conditions. *Microbiology* **149:** 2789-2795.

Sedgwick SG, Ho C & Woodgate R (1991) Mutagenic DNA repair in enterobacteria. *J Bacteriol* **173:** 5604-5611.

Sinha RP & Häder D-P (2002) UV-induced DNA damage and repair: a review. *Photochem Photobiol Sci.* **1:** 225-236.

Skinner JS (1829) American Farmer. St. Paul MN: John D Toy. 212.

Somerville Jr JE, Cassiano L, Bainbridge B, Cunningham MD & Darveau RP (1996) A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J Clin Invest* **97:** 359-365.

Szekeres Jr ES, Woodgate R & Lawrence CW (1996) Substitution of mucAB or rumAB for umuDC alters the frequencies of the two classes of mutations induced by a site-specific T-T cyclobutane dimmer and the efficiency of translesion DNA synthesis. *J Bacteriol* **178:** 2559-2563.

Thomson SV Epidemiology of fire blight. Vanneste JL, ed. (2000) Fire blight the disease and its causative agent *Erwinia amylovora*. New York NY: CABI Publishing. 9-36.

Torres MA Jones J DG & Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiol* **141:** 373-378.

Toth IK, Thorpe CJ, Bentley SD, Mulholland V, Hyman LJ, Perombelon MCM & Salmond GPC (1999) Mutation in a gene required for lipopolysaccharide and enterobacterial common antigen biosynthesis affects virulence in the plant pathogen *Erwinia carotovora* subsp. atroseptica. *Mol Plant Microbe Interact* **12:** 499-507.

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

Vanneste JL & Eden-Green S Migration of *Erwinia amylovora* in host plant tissues. Vanneste JL, ed. (2000) Fire blight the disease and its causative agent *Erwinia amylovora*. New York NY: CABI Publishing. 73-84.

Venisse J-S, Gullner G & Brisset M-N (2001) Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol* **125:** 2164-2172.

Venisse JS, Malnoy M, Faize M, Paulin JP & Brisset MN (2002) Modulation of defense responses of *Malus* spp. during compatible and incompatible interactions with *Erwinia amylovora*. *Mol Plant Microbe Interact* **15:** 1204-1212.

Vorwerk S, Somerville S & Somerville C (2004) The role of plant cell wall polysaccharide composition in disease resistance. *Trends Plant Sci* **9:** 203-209

Whitfield C, Amor PA & Köplin R (1997) Modulation of the surface architecture of Gram-negative bacteria by the action of surface polymer: lipid A-core ligase and by determinants of polymer chain length. *Mol Microbiol* **23:** 629-638.

Witkin EM (1976) Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. *Microbiol Mol Biol Rev* **40:** 869-907.

Woodgate R & Sedgwick SG (1992) Mutagenesis induced by bacterial UmuDC proteins and their plasmid homologues. *Mol Microbiol* **6:** 2213-2218.

Wu G, Shortt B, Lawrence EB, Levine EB, Fitzsimmons KC & Shah DM (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. *Plant Cell* **7:** 1357-1368.

Wu J & Weiss B (1992) Two-stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli*. *J Bacteriol* **174:** 3915-3920.

Xiang T, Zong N, Zou Y, Wu Y, Zhang J, Xing W, Li Y et al. (2008) *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Curr Biol* **18:** 74-80.

Yasui A & Chevallier M-R (1983) Cloning of photoreactivation repair gene and excision repair gene of the yeast *Saccharomyces cerevisiae*. *Curr Genet* **7:** 191-194.

Yethon JA, Gunn JS, Ernst RK, Miller SI, Laroche L, Malo D & Whitfield C (2000) *Salmonella enterica* Serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence in vivo. *Infect Immun* **68:** 4485-4491.

Yun MH, Torres PS, Oirdi ME, Rigano LA, Gonzalez-Lamothe R, Marano MR, Castagnaro AP, Dankert MA et al. (2006) Xanthan induces plant susceptibility by suppressing callose deposition. *Plant Physiol* **141:** 178-187.

Zeidler D, Zähringer U, Gerber I, Dubery I, Hartung T, Bors W & Hutzler P (2004) Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proc Natl Acad Sci U S A* **101:** 15811-15816

Zenoff VF, Siñeriz F & Farias ME (2006) Diverse responses to UV-B radiation and repair mechanisms of bacteria isolated from high-altitude aquatic environments. *Appl Environ Microbiol* **72:** 7857-7863.

Zhao Y, He S-Y & Sundin GW (2006) The *Erwinia amylovora avrRpt2*$_{Ea}$ gene contributes to virulence on pear and AvrRpt2$_{Ea}$ is recognized by Arabidopsis RPS2 when expressed in *Pseudomonas syringae*. *Mol Plant-Microbe Interact* **19:** 644-654.

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G & Boller T (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* **428:** 764-767.

Chapter 2

Effect of a *waaL* mutation on lipopolysaccharide composition, oxidative stress survival, and virulence in *Erwinia amylovora*[*]

Abstract

Erwinia amylovora, the causal agent of fire blight, is an enterobacterial pathogen of Rosaceous plants including apple and pear. We have been studying the response of *E. amylovora* to oxidative stress because, during infection, the bacterium elicits an oxidative burst response in host plants. During the screening of a transposon mutant library for hydrogen peroxide sensitivity, we identified a mutant carrying an insertion in *waaL*, a gene involved in lipopolysaccharide (LPS) biosynthesis that was more sensitive to hydrogen peroxide than the parental wild-type strain. We also confirmed that a *waaL* mutant of *Pseudomonas aeruginosa* exhibited an increased sensitivity to hydrogen peroxide compared to the wild-type strain. The *E. amylovora waaL* mutant also was reduced in virulence, showed a decrease in twitching motility, and was more sensitive to polymyxin B than the wild-type. Each of these phenotypes was complemented by the cloned *waaL* gene. Our results highlight the importance of the LPS layer to virulence in *E. amylovora* and the unexpected finding of an additional function of LPS in protection from oxidative stress in *E. amylovora* and *P. aeruginosa*.

Introduction

The enterobacterial plant pathogen *E. amylovora* is the causal agent of fire blight, an economically important disease of apple and pear trees, and other hosts in the family *Rosaceae*. *E. amylovora* infects multiple host organs including blossoms and actively-growing shoots, and uses a type III secretion system to directly inject effector proteins

such as DspA/E into host cells and initiate pathogenesis (Oh & Beer, 2005). The exopolysaccharide amylovoran is also a pathogenicity factor (Bugert & Geider, 1995), and other virulence factors include the type III effector $AvrRpt2_{Ea}$ (Zhao *et al.*, 2006), the iron-binding siderophore desferrioxamine (Dellagi *et al.*, 1998) and sorbitol-utilization genes (Aldridge *et al.*, 1997). Infection is predominantly systemic in host plants with the bacterium migrating through the xylem or water-conducting vascular system of the host (Suhayda & Goodman, 1981).

Plant pathogen/host interactions have been termed "molecular arms races" due to constant refinement of effector genes by the pathogen and resistance genes by the host. Elicitors of defense responses include general determinants such as flagella, that are common among most bacterial pathogens, and specific determinants such as protein effectors secreted via type III secretion that are recognized by the products of plant resistance genes. An integral aspect of the plant defense response against pathogens is the occurrence of an oxidative burst in which reactive oxygen species (ROS) are produced following pathogen recognition (Shetty *et al.*, 2008). Avirulent or unsuccessful pathogens often induce a biphasic response consisting of a transient first phase of ROS accumulation of lower intensity, followed by a continuous phase of much higher intensity (Torres *et al.*, 2006). Most virulent pathogens are capable of avoiding or suppressing host defenses during disease initiation and only induce the transient first phase of ROS accumulation (Bolwell *et al.*, 2002).

E. amylovora is one of only a few known plant pathogens that induces an oxidative burst similar to that of a plant resistance response prior to successful pathogenesis (Venisse *et al.*, 2001). This host response is mediated by the recognition of

the type III effectors HrpN and DspA/E (Venisse *et al.*, 2003). *E. amylovora* cells survive the resulting oxidative burst, and nutrients released by host cells killed in this process provide the energy for subsequent pathogen buildup and systemic invasion of the vascular system (Venisse *et al.*, 2003). While determinants such as superoxide dismutase and catalase enzymes would be predicted to be involved in the survival of *E. amylovora* exposed to oxidative stress, to date, only the siderophore desferrioxamine has been demonstrated to contribute to the survival of *E. amylovora* in the presence of elevated levels of hydrogen peroxide (Venisse *et al.*, 2003).

Lipopolysaccharide (LPS) is composed of complex glycolipids and is the major molecular component of the outer membrane of gram-negative bacteria. The LPS can be divided into three structural regions: the lipid A that binds the LPS to the outer membrane, the core which is an oligosaccharide attached to lipid A, and the O-antigen which is distal to the outer membrane (Raetz & Whitfield, 2002). Because of its external location, the LPS is important for interaction with the environment and also with potential host organisms. The O-antigen of LPS plays an important role in surface phenomena including swarming motility (Toguchi *et al.*, 2000), and a role in flagella biogenesis (Abeyrathne *et al.*, 2005). The LPS is thought of as a physical barrier that protects the bacterium from antibacterial agents such as peptides (Rosenfeld & Shai, 2006). LPS plays multiple roles during bacterial pathogenesis and plant host response; for example, LPS is recognized by plants and elicits a defense response (Erbs & Newman, 2003), and LPS can prime plants to respond more rapidly to subsequent attack by bacterial pathogens (Newman *et al.*, 2002). The LPS may also contribute to the

protection of infecting bacteria from antimicrobial substances produced by plants (Newman *et al.*, 2001).

Because *E. amylovora* survives the oxidative burst response of the host during infection, we hypothesized that the organism would exhibit tolerance to ROS and that genes encoding ROS tolerance would be important virulence factors. As part of this work, we screened a transposon-insertion mutant library of the pathogenic strain *E. amylovora* Ea1189 for mutants reduced in virulence and with decreased survival in the presence of hydrogen peroxide. Sequence identification of one such mutant revealed the unexpected result of an insertion in *waaL*, a gene involved in LPS biosynthesis. In this work, we characterized the *waaL* gene of *E. amylovora* and detailed the involvement of LPS in oxidative stress survival and virulence of the fire blight pathogen.

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions

The wild-type virulent strain *E. amylovora* Ea1189 (Burse *et al.*, 2004) was used in all experiments. Cloning experiments were done using *Escherichia coli* DH5α (Sambrook *et al.*, 1989). For comparative characterization analyses of the Ea1189 *waaL* mutant, we obtained *Pseudomonas aeruginosa* PAO1 and PAO1 *waaL*::IS*phoA/hah* (Jacobs *et al.*, 2003) from the Department of Genome Sciences at the University of Washington. All bacteria were grown in Luria-Bertani (LB) broth or solidified media (1.5% agar). Ampicillin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) were added to media when necessary. All bacteria were grown at 28°C except *Escherichia coli* and *Pseudomonas aeruginosa*, which were grown at 37°C

Peroxide sensitivity assay

Overnight cultures were pelleted, washed, and resuspended in 0.5x PBS to an optical density ($OD_{600\ nm}$) of 0.1. To determine the LD_{50} of hydrogen peroxide of *E. amylovora* Ea1189, hydrogen peroxide ([30% solution] J.T. Baker; Phillipsburg, NJ) was added to cells in various concentrations, and the cell suspensions were incubated from one to 20 minutes at 25°C After incubation, 25 µl samples from appropriate serial dilutions were plated on LB medium. Plates were incubated at 28°C for 48 hr prior to bacterial enumeration. The concentrations of hydrogen peroxide used to screen wild type and mutant strains of *E. amylovora* and *P. aeruginosa* for LD_{50} determination were 250 µM and 600 µM, respectively. The sensitivity of *E. amylovora* strains to a range (250 µM-750 µM) of hydrogen peroxide concentrations (15 min exposure) was also examined.

Transposon mutant library screen for hydrogen peroxide sensitive mutants

E. amylovora Ea1189 was grown overnight in LB broth at 28°C, subcultured in LB broth, and grown to exponential phase (OD_{600} = 0.8). Cells were pelleted, then made electrocompetent (Sambrook *et al.*, 1989) and stored at –80°C One µl of the EZ::TN <KAN-2> Tnp transposome (Epicentre; Madison, Wisconsin) was added to the electrocompetent cells, and electroporation was performed using a Gene Pulser (Biorad; Hercules, California) according to the manufacturer's recommendations. Electroporated cells were immediately recovered by adding 1 ml of SOC medium (Sambrook *et al.*, 1989) and then transferred to a sterile tube and incubated on a shaker at 28°C for 2 hr. Transformants were then plated on LB with kanamycin (LB_{Km}) and, after 48 hr, individual colonies were stored in 96-well plates containing LB broth with 10% glycerol and kanamycin. The randomness of transposon insertion and confirmation of single

insertions was assessed for 20 randomly-selected mutants using Southern hybridization of genomic DNA preparations digested with EcoRI, an enzyme that does not have any recognition sites within the EZ::TN <KAN-2> Tnp transposon. Over 6,100 random clones were recovered and stored.

Screening for the sensitivity of mutants in the library to hydrogen peroxide was done in flat bottom 96-well plates (Evergreen Scientific; Los Angeles, CA). Mutants were grown overnight in 250 µl LB broth at 19.5°C A sample of 2 µl of each culture was then inoculated into new 96-well plates containing LB broth or LB broth amended with 250 µM H_2O_2. These two 96-well plates were also incubated at 19.5°C for 24 hr following which the OD_{600} of each culture was determined using a Safire microplate reader (Tecan; Research Triangle Park, NC). The lower temperature of incubation was used to decelerate the breakdown of hydrogen peroxide. The absorbance data were then converted to cell numbers using a standard curve previously generated for *E. amylovora* (M. Berry, unpublished).

Identification of transposon insertion sites

To identify the genes that were interrupted by the EZ::Tn transposon, the random amplification of transposon ends (RATE) PCR method of Ducey and Dyer (2002) was utilized with slight modifications. The boiled cells were diluted ten-fold before use in the PCR reaction, and the $MgCl_2$ concentration was doubled to 30 mM. PCR products were purified with a PCR Purification Kit (Qiagen; Gaithersburg, MD), and the resulting DNA was sequenced at the Michigan State University Research Technology Support Facility. All sequences were compared using BlastX to the closed *E. amylovora* genome sequence available at (http://www.sanger.ac.uk/Projects/E_amylovora/), followed by comparison

with the NCBI database to identify homologs. The *waaL* gene was identified in this manner and oligonucleotide primers WaaL 2 For (5'-ATGCGATGCTGCCGGAATTCTGTTGTGAG-3") and WaaL 2 Rev (5'-ATGCCCGCGGGTCCCACCAATGCTGCTATCC-3') were used to amplify and clone the full-length *waaL* coding sequence (also including approximately 200 bp upstream and downstream) into pGem5zf (Promega Corp., Madison, WI) downstream of the pGem5zf *lac* promoter, creating the plasmid pMCB3.

Virulence assays

We used an immature pear fruit assay for virulence assessment of *E. amylovora* strains (Zhao *et al.*, 2005); this type of assay has been routinely used to examine virulence of the fire blight pathogen. Bacterial inocula were grown overnight, pelleted, and resuspended in 0.5x phosphate-buffered saline (PBS; Sigma-Aldrich Inc.; St. Louis, MO) to an OD_{600} of 0.1. Ten µl of bacterial cells at a concentration of 10^8 cells/ml (or 0.5x PBS as a control) were then applied to the surface of each of 10 pears used per strain after which a No. 2 insect pin was pushed through the bacterial droplet into the pear to a depth of ~0.5 cm. Following inoculation, the pears were placed in a covered humidified chamber for nine days at 28°C Lesion size was monitored daily and bacterial cell counts were also determined in some experiments by sampling pear cores taken from the site of inoculation using a #4 cork borer. Each core was hand ground with a sterile plastic mortar in 500 µl of 0.5x PBS, and appropriate dilutions in 0.5x PBS were plated onto LB medium. Cells were enumerated after incubation at 28°C for two days. A total of five experiments were done with Ea1189 and four experiments were done with GS1 and GS1/pMCB3.

Visualization of LPS

Crude cell pellets of *E. amylovora* Ea1189 and GS1 were extracted twice with in 11 ml 90% ethanol for 1 hr, once with 5 ml acetone and once with 2 ml diethyl ether for 30 min at 25°C Dry cell masses were suspended in 0.5 ml 10 mM tricine (pH 8.0) and digested with 0.5 mg proteinase K overnight. A modification of the phenol/water extraction procedure of Westphal and Jann (1965) was then used to extract LPS. Briefly, cells were suspended in 6 ml water and heated to 65°C; 6 ml of 65°C phenol was then added, and the cells were incubated at 65°C for 0.5 hr with stirring after which they were centrifuged to separate phases. Aqueous phases were taken and interfacial/phenol phase material was reextracted with water. Gels were fixed and stained using the SilverSNAP stain kit II (Pierce; Rockford, IL) following the manufacturer's instructions. LPS extraction and analysis was done at the Complex Carbohydrate Research Center at the University of Georgia.

Polymyxin B sensitivity assay

Previous studies have shown that a truncated LPS layer is correlated with an increase in sensitivity to antimicrobial peptides including polymyxin B (Yethon *et al.*, 2000). We used the protocol of Loutet *et al.* (2006) to assess the sensitivity to polymyxin B of *E. amylovora* Ea1189 and GS1. Polymyxin B (Sigma; St. Louis, MO) was added to a final concentration of 0.25 $\mu g\ ml^{-1}$ in LB broth which contained *E. amylovora* at an OD_{600} of 0.1. The cells were incubated at 28°C for 2 hr prior to plating to assess cell survival.

Twitching and swimming assay

In *P. aeruginosa*, a *waaL* mutant was impaired in motility because of a reduction in number of flagella per cell (Abeyrathne *et al.*, 2005). We assessed swimming and twitching motility of *E. amylovora* Ea1189 and GS1 using a 0.3% agarose medium and a stab assay, respectively. Distance traveled from the initial point of inoculation was measured using analog calipers. Additional protocol information and results scoring were as according to Rashid and Kornberg (2000).

Results and Discussion

Pleiotropic phenotypes of the *E. amylovora waaL* mutant: increased sensitivity to hydrogen peroxide, alterations in virulence, sensitivity to polymyxin B, and motility

The LD_{50} exposure regime (250 μM H_2O_2) was used for screening the EZ::TN transposon mutant library of *E. amylovora* Ea1189, and yielded 45 mutants with differences in peroxide sensitivity relative to the wild type *E. amylovora* Ea1189. RATE-PCR was used to map transposon insertion sites, and one of the mutants, GS1, had an insertion in a gene homologous to *waaL*, which functions in the ligation of the O-antigen to the lipid A core during LPS biosynthesis (Abeyrathne *et al.*, 2005). A translation of the *waaL* gene from *E. amylovora* Ea273 (http://www.sanger.ac.uk/Projects/E_amylovora/) shared closest amino acid identity with the corresponding translated proteins from *E. tasmaniensis*, *Serratia marscescens*, and *Klebsiella pneumoniae* (data not shown). The other 44 EZ::TN insertion mutants will be characterized elsewhere.

The *E. amylovora* Ea1189 *waaL* mutant strain GS1 exhibited a two fold reduction in survival following 10, 15, and 20 min exposure to 250 μM H_2O_2 (Figure 2.1A).

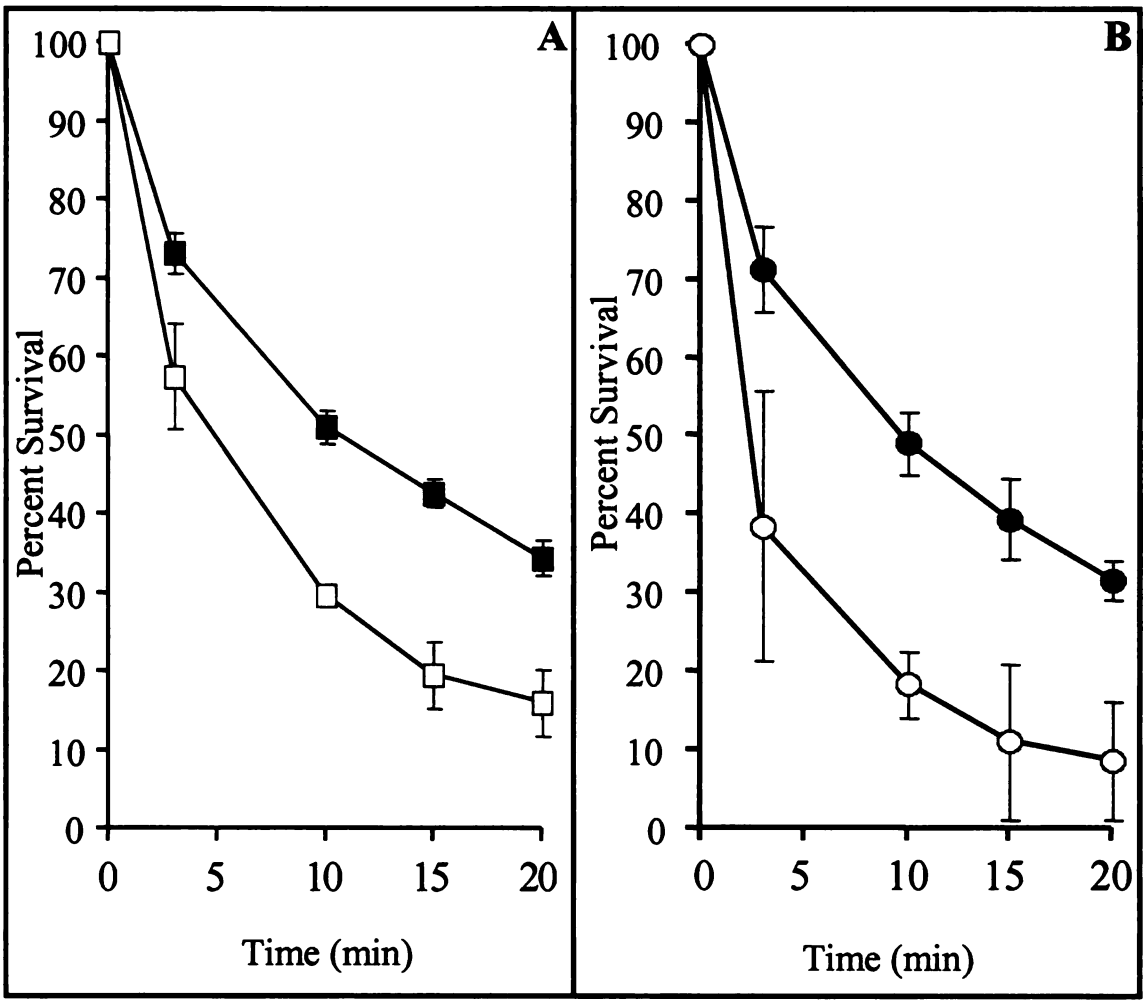

Figure 2.1. Sensitivity to hydrogen peroxide in *Erwinia amylovora* and *Pseudomonas aeruginosa* strains. (A) *E. amylovora* Ea1189 (closed squares) and GS1 (open squares) were exposed to 250 μM H_2O_2 for various durations, before cells were plated. (B) The same was done for *P. aeruginosa* PAO1 (closed circles), and *P. aeruginosa waaL*::ISphoA/hah (open circles) with 600 μM H_2O_2. Results shown with standard error and represented as a mean of the four replicates for *E. amylovora* Ea1189, three replicates for *E. amylovora* GS1, and two replicates for the *P. aeruginosa* strains tested.

Because bacterial LPS has not previously been implicated in hydrogen peroxide sensitivity, we sought to confirm this result using another organism. An analysis using *Pseudomonas aeruginosa* PAO1 and the corresponding transposon mutant *P. aeruginosa waaL*::IS*phoA/hah* also revealed differential sensitivity with four fold reductions in

survival observed after 15 and 20 min exposure to 600 μM H_2O_2 (Figure 2.1B). Exposure of Ea1189 and GS1 to increased concentrations of hydrogen peroxide revealed differences in sensitivity as large as 123 fold. The sensitivity of the GS1 mutant strain increased dramatically upon exposure to higher doses (Figure 2.2).

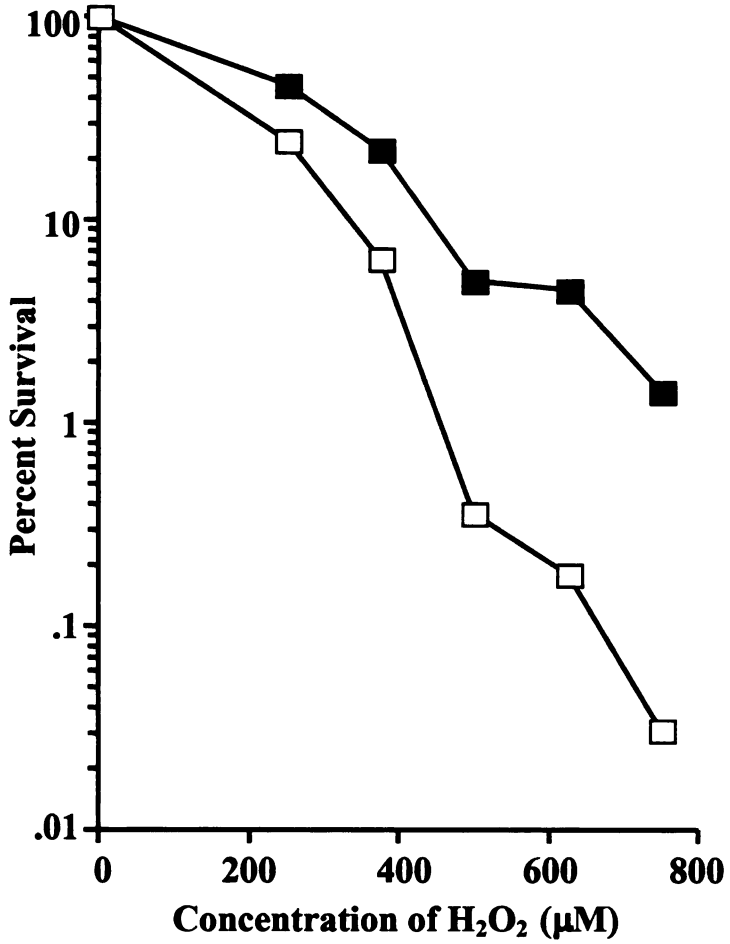

Figure 2.2. Survival curve of *E. amylovora* Ea1189 and GS1 after exposure to increasing concentrations of hydrogen peroxide. Ea1189 (closed squares) and GS1 (open squares) were challenged with various concentrations of hydrogen peroxide from 250 μM-750 μM for 15 minutes before plating. Standard error bars have been omitted because they were smaller than the size of the symbols in the figure. A representative experiment of three experimental replicates is shown.

Complementation of GS1 with pMCB3 restored wild type levels of hydrogen peroxide sensitivity (data not shown).

Analysis of the LPS produced by the wild type strain Ea1189 revealed a typical ladder-like pattern produced as a result of varying lengths of O-antigen attached to the Lipid A core (Figure 2.3; Lane 1).

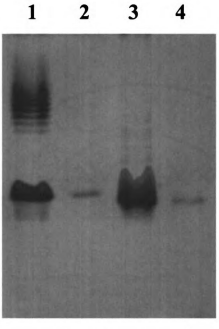

Figure 2.3. Polyacrylamide gel electrophoresis analysis of the lipopolysaccharide layer in *Erwinia amylovora* strains. Lane 1, *E. amylovora* Ea1189 aqueous phase; lane 2, *E. amylovora* Ea1189 phenol phase; lane 3, *E. amylovora* GS1 aqueous phase; lane 4, *E. amylovora* GS1 phenol phase

The O-antigen side chains were absent in the *waaL* mutant GS1 (Figure 2.3; Lane 3), although very faint bands of a smaller molecular weight than the O-antigen bands from Ea1189 were present. These bands could be a contaminant or could possibly represent a low level of O-antigen addition by a Wzy-independent pathway (Raetz & Whitfield, 2002). Electrophoresis of the phenol phase from the LPS extractions demonstrated that the phenol phase harbored small amounts of LPS that was similar to the main LPS of the

aqueous phase (Figure 2.3, Lanes 2 and 4). The ladder-like pattern of O-antigen lengths was restored in the complemented strain GS1/pMCB3 (data not shown).

In an immature pear assay, *E. amylovora* GS1 was markedly reduced in virulence compared to the wild type strain Ea1189 and GS1/pMCB3 as evidenced by a decreased lesion size (Figure 2.4).

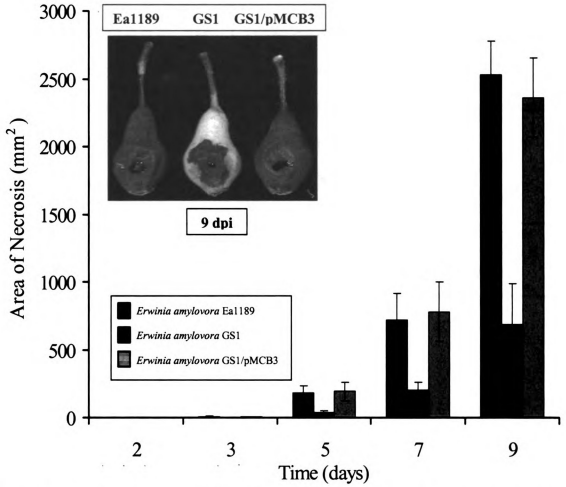

Figure 2.4. Virulence assays. Necrotic lesion size in immature pear following inoculation with *Erwinia amylovora* strains. This experiment was performed over nine days with measurements taken at days 2, 3, 5, 7, and 9. A total of 10 immature pears were used for each strain in each experiment. A total of five experiments were conducted with *E. amylovora* Ea1189, and four experiments were conducted with *E. amylovora* GS1 and GS1/pMCB3. Results shown are the average of experimental replicates and are shown with standard error. Inset: Representative pears at day 9. Strains inoculated, from left to right, were *E. amylovora* Ea1189, GS1, and GS1/pMCB3.

Differences in lesion size were notable by five days post inoculation and more distinctive over the remainder of the experiment. By day 9, a nearly 3.5 fold reduction in lesion size was seen in *E. amylovora* GS1 compared to the other two strains tested. Compared to Ea1189, GS1 exhibited a 7.6 to 45.6 fold reduction in population over a four day period following inoculation in immature pears (data not shown). These population reductions are similar to those observed with other *E. amylovora* virulence mutants such as the *avrRpt2* mutant (Zhao *et al.*, 2006). Complementation of GS1 with the *waaL* gene on pMCB3 restored population to wild-type levels (data not shown).

After exposure to polymyxin B, a two log reduction was observed in the *waaL* mutant GS1 when compared to Ea1189, whereas there was little difference between Ea1189 and the *waaL*-complemented strain GS1/pMCB3 (Table 1).

Strain	Log_{10} Starting cfu/mL	Log_{10} cfu/mL after PBS Exposure	Log_{10} cfu/mL after Polymyxin B Exposure	Percent Survival after Polymyxin B Exposure
E. amylovora Ea1189	8.6	8.6	8.3	52.6
E. amylovora GS1	8.6	8.5	6.2	0.4
E. amylovora GS1/pMCB3	8.5	8.5	8.0	29.9

Table 2.1. Comparative survival of *E. amylovora* Ea1189 and the *waaL* mutant GS1 following exposure to polymyxin B The bacteria were exposed to either polymyxin B (diluted in 0.5x PBS) or an equivalent volume of 0.5x PBS as a control for 2 hr in LB broth prior to plating. This test was done in triplicate, and the means of the three experimental replicates are shown.

No reductions in cell number were observed following exposure to PBS in control cultures (Table 1). A significant reduction ($P < .01$) in twitching motility was observed in the *waaL* mutant GS1 compared to Ea1189 (Table 2).

Strain	Average distance (mm)	*P* value when compared to *E. amylovora* Ea1189	*P* value when compared to *E. amylovora* GS1
E. amylovora 1189	0.53 (0.04)	NA	0.00034
E. amylovora GS1	0.37 (0.02)	0.00034	NA
E. amylovora GS1/pMCB3	0.49 (0.03)	0.55761	0.00995

Table 2.2. Comparison of twitching motility in *E. amylovora* Ea1189, GS1, and GS1/pMCB3. A total of 42 replicates were performed with strains Ea1189 and GS1, and 15 replicates were performed with GS1/pMCB3. All results are shown as a mean of the replicates with standard error in parentheses.

This reduction in GS1 was restored to wild-type levels following complementation with the *waaL* gene on pMCB3 (Table 2). No significant differences in swimming motility were observed between GS1 and Ea1189 (data not shown).

The LPS covers more than 90% of the gram-negative bacterial cell surface and acts as a physical barrier in particular against antimicrobial peptides (Rosenfeld & Shai, 2006). In addition, deficiencies in LPS formation result in other pleiotropic phenotypes including reduction of swarming motility in *Salmonella enterica* (Toguchi *et al.*, 2000), and reductions in swimming and twitching motility and flagella production in *P. aeruginosa* (Abeyrathne *et al.*, 2005). However, an association between LPS and protection from oxidative stress has not, to our knowledge, been previously reported. There are other examples of a requirement of bacterial structural proteins for optimal

survival in the presence of hydrogen peroxide including porins in *E. coli* and a 59-kDa outer membrane protein in *S. enterica* serovar Typhimurium (Stinavage *et al.*, 1990; De Spiegeleer *et al.*, 2005). The differences in hydrogen peroxide sensitivity observed in the *waaL* mutants of *E. amylovora* and *P. aeruginosa* in this study highlight another function of LPS in bacterial physiology.

We demonstrated the importance of an intact LPS to virulence in *E. amylovora*. This was expected because an intact LPS layer is an important virulence determinant in many bacterial plant pathogens (for examples, see Schoonejans *et al.*, 1987; Dow *et al.*, 1995; Toth *et al.*, 1999). We also anticipated correlations between hydrogen peroxide sensitivity and reduced virulence because of the induction of an oxidative burst in plant hosts prior to successful infection by *E. amylovora*. What was unexpected, however, was that the LPS layer itself was involved in increased survival following hydrogen peroxide challenge. Because of its location on cell surfaces, LPS plays a key role in bacterial host-pathogen interactions. LPS is a pathogen-associated molecular pattern recognized by plants leading to an induction of defense responses in nonhost plants (Newman *et al.*, 2007). However, LPS can also prevent the induction of the hypersensitive response of plants and suppresses defense responses during nodulation by *Sinorhizobium meliloti* (Tellstroem *et al.*, 2007). Finally, other roles for LPS including cell-cell contact and protection from plant antimicrobials likely contributes to pathogen virulence in plants. In *E. amylovora*, the role of LPS in protection from plant antimicrobials including ROS is likely the prominent functional role of LPS in virulence since this organism elicits an oxidative burst defense response in its host during infection (Venisse *et al.*, 2001).

In summary, our results indicate that inactivation of *waaL* in *E. amylovora* Ea1189 resulted in a truncated LPS layer with consequences including decreased survival following hydrogen peroxide exposure and reduced virulence. This work confirms that the importance of the bacterial LPS is multifold, providing both protective functions and possibly aggressive functions during pathogenesis.

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References

Abeyrathne PD, Daniels C, Poon KKH, Matewish MJ & Lam JS (2005) Functional Characterization of *waaL*, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. J *Bacteriol* **187:** 3002-3012.

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

Bolwell GP, Bindschedler LV, Blee KA *et al.* (2002) The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *J Exp Bot* **53:** 1367-1376.

Bugert P & Geider K (1995) Molecular analysis of the ams operon required for exopolysaccharide amylovoran synthesis of *Erwinia amylovora*. *Mol Microbiol* **15:** 917-933.

Burse A, Weingart H & Ullrich MS (2004) NorM, an *Erwinia amylovora* multidrug efflux pump involved in vitro competition with other epiphytic bacteria. *Appl Environ Microbiol* **70:** 693-703.

Dellagi A, Brisset MN, Paulin JP & Expert D (1998) Dual role of desferrioxamine in *Erwinia amylovora* pathogenicity. *Mol Plant-Microbe Interact* **11:** 734-742.

De Spiegeleer P, Sermon J, Vanoirbeek K, Aertsen A & Michiels CW (2005) Role of porins in sensitivity of *Escherichia coli* to antibacterial activity of the lactoperoxidase enzyme system. *Appl Environ Microbiol* **71:** 3512-3518.

Dow JM, Osbourn AE, Wilson TJ & Daniels MJ (1995) A locus determining pathogenicity of *Xanthomonas campestris* is involved in lipopolysaccharide biosynthesis. *Mol Plant-Microbe Interact* **8:** 768-777.

Ducey TF & Dyer DW (2002) Rapid identification of EZ::TN™ transposon insertion sites in the genome of *Neisseria gonorrhoeae*. EPICENTRE Forum. **9:** 6-7.

Erbs G & Newman M (2003) The role of lipopolysaccharides in induction of plant defence responses. *Mol Plant Pathol* **4:** 421-425.

Jacobs MA, Alwood A, Thaipisuttikul I *et al.* (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100:** 14339-14344.

Loutet SA, Flannagan RS, Kooi C, Sokol PA & Valvano MA (2006) A complete lipopolysaccharide inner core oligosaccharide is required for resistance of *Burkholderia cenocepacia* to antimicrobial peptides and bacterial survival in vivo. *J Bacteriol* **188:** 2073-2080.

Maxson-Stein K, McGhee GC, Smith JJ, Jones AL & Sundin GW (2003) Genetic analysis of pathogenic *Erwinia* sp. isolated from pear in Japan. *Phytopathology* **93:** 1393-1399.

Newman M-A, Dow JM & Daniels MJ (2001) Bacterial lipopolysaccharide and plant-pathogen interactions. *Eur J Plant Pathol* **107:** 95-102.

Newman M-A, Dow JM, Molinaro A & Parrilli M (2007) Priming, induction and modulation of plant defence responses by bacterial lipopolysaccharides. *J Endotox Res* **13:** 69-84.

Newman M-A, von Roepenack-Lahaye E, Parr A, Daniels MJ & Dow JM (2002) Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria. *Plant J* **29:** 487-495.

Oh C-S & Beer SV (2005) Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. *FEMS Microbiol Lett* **253:** 185-192.

Raetz CR & Whitfield C (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71:** 635-700.

Rashid MH & Kornberg A (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **97:** 4885-4890.

Rosenfeld Y & Shai Y (2006) Lipopolysaccharide (endotoxin)-host defense antibacterial peptides interactions: role in bacterial resistance and prevention of sepsis. *Biochem Biophys Acta* **1758:** 1513-1522.

Sambrook J, Fritsch ER & Maniatis TA (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schoonejans E, Expert D & Toussaint A (1987) Characterization and virulence properties of *Erwinia chrysanthemi* lipopolysaccharide-defective, phi EC2-resistant mutants. *J Bacteriol* **169:** 4011-4017.

Shetty NP. Jorgensen HJL, Jensen JD, Collinge DB & Shetty HS (2008) Roles of reactive oxygen species in interactions between plants and pathogens. *Eur J Plant Pathol* **121:** 267-280.

Stinavage PS, Matrin LE & Spitznagel JK (1990) A 59 kilodalton outer membrane protein of *Salmonella typhimurium* protects against oxidative intraleukocytic killing due to human neutrophils. *Mol Microbiol* **4:** 283-293.

Suhayda CG & Goodman RN (1981) Early proliferation and migration and subsequent xylem occlusion by *Erwinia amylovora* and the fate of its extracellular polysaccharide (EPS) in apple shoots. *Phytopathology* **71:** 697-707.

Tellstroem V, Usadel B, Thimm O, Stitt M, Kuester H & Niehaus K (2007) The lipopolysaccharide of *Sinorhizobium meliloti* suppresses defense-associated gene expression in cell cultures of the host plant *Medicago truncatulata*. *Plant Physiol* **143:** 825-837.

Toguchi A, Siano M, Burkhart M & Harshey RM (2000) Genetics of swarming motility in *Salmonella enterica* serovar Typhimurium: critical role for lipopolysaccharide. *J Bacteriol* **182:** 6308-6321.

Torres MA, Jones JDG & Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiol* **141:** 373-378.

Toth IK, Thorpe CJ, Bentley SD *et al.* (1999) Mutation in a gene required for lipopolysaccharide and enterobacterial common antigen biosynthesis affects virulence in the plant pathogen *Erwinia carotovora* subsp. *atroseptica*. *Mol Plant-Microbe Interact* **12:** 499-507.

Venisse J-S, Barny M-A, Paulin J-P & Brisset M-N (2003) Involvement of three pathogenicity factors of *Erwinia amylovora* in the oxidative stress associated with compatible interaction in pear. *FEBS Lett* **537:** 198-202.

Venisse J-S, Gullner G & Brisset M-N (2001) Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol* **125:** 2164-2172.

Yethon JA, Gunn JS, Ernst RK *et al.* (2000) *Salmonella enterica* serovar typhimurium *waaP* mutants show increase susceptibility to polymyxin & loss of virulence in vivo. *Infect Immun* **68:** 4485-4491.

Westphal O & Jann K (1965) Bacterial lipopolysaccharides. *Methods Carbohydr Chem* **33:** 158-174.

Zhao YF, Blumer SE & Sundin GW (2005) Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. *J Bacteriol* **187:** 8088-8103.

Zhao Y, He S-Y & Sundin GW (2006) The *Erwinia amylovora avrRpt2*$_{Ea}$ gene contributes to virulence on pear and AvrRpt2$_{Ea}$ is recognized by Arabidopsis RPS2 when expressed in *Pseudomonas syringae*. *Mol Plant-Microbe Interact* **19:** 644-654.

Chapter 3

Survival and Mutability in Response to Ultraviolet Radiation in *Erwinia amylovora*

Abstract

Erwinia amylovora is a Gram-negative bacterial plant pathogen that infects members of the family *Rosacea* including apple and pear. This organism, like most on Earth, is exposed to ultraviolet (UV) radiation during the life cycle. Of the mechanisms used to repair damage caused by UV radiation, mutagenic repair is one of the last ones utilized since mutagenic repair is error prone and causes mutations in DNA. We discovered that *Erwinia* species survived UV light exposure and exhibited mutability in response to UV light at a higher rate when compared to *E. coli* and *Pseudomonas* strains. Survival and mutability were both decreased when the gene pair responsible for mutagenic repair, *umuDC*, was knocked out, but complementation with either *umuDC* from *E. coli*, or *rulAB*, a homolog of *umuDC*, from *Pseudomonas* was able to restore survival and mutability to wild type levels.

Introduction

Like most organisms on Earth, *E. amylovora* is exposed to ultraviolet (UV) stress during the life cycle. Repair mechanisms including photoreactivation, nucleotide excision repair, and recombinational repair exist in the bacterium that allow this pathogen to survive exposure to UV light and colonize the host (Yasui & Chevallier, 1983; Brash et al., 1985; Sinha & Hader, 2002). These systems have overlapping functions so that if one system is defective, the cell can survive with the repair systems that remain. Of the different repair mechanisms that exist, mutagenic repair is one of the last ones used to repair DNA damage because it is error prone and can cause a mutation at the site of DNA

damage (Sinha & Hader, 2002). Without mutagenic repair; some damage to DNA could not be corrected, which would stall replication to the point where the cell would die. In enteric bacteria, mutagenic repair is most commonly encoded by the gene pair *umuDC* (Sedgwick et al., 1991). This gene pair is part of the SOS response, which means it is regulated by two proteins, RecA and LexA (Witkin, 1976; Bagg et al., 1981). RecA detects damage induced by UV via the presence of single stranded DNA, after which RecA becomes activated and facilitates the autoproteolytic cleavage of LexA that functions as a repressor of SOS response genes including *umuDC* (Bagg et al., 1981; Lee et al., 1996). Once cleaved, LexA is released from its binding site allowing transcription of the SOS response genes to continue. *umuDC* is dicistronic with the two genes overlapping by 1 bp (Sedgwick et al., 1991). After translation, the UmuD protein is autoproteolytically cleaved at the 24^{th} amino acid (Woodgate & Sedgwick, 1992; Kim & Sundin, 2000). This form of UmuD is known as UmuD', which forms a homodimer before binding with UmuC (Woodgate & Sedgwick, 1992; Kim & Sundin, 2000). This complex then repairs DNA damage by inserting a base opposite the damaged base so that replication can proceed. Since the template's base is not used to determine which base is inserted, a mutation occurs when an incorrect base is incorporated opposite the original damaged base. Each step in the transcription of these SOS response genes is necessary for the genes to be transcribed at the appropriate level. In *P. syringae*, the absence of a functional *recA* gene results in cells that are over 10,000 fold more sensitive to UV radiation at the lowest dosage administered (Sundin, 1996).

Other homologs to *umuDC* include *mucAB*, *rulAB*, and *samAB*. While these are regulated and function similarly to *umuDC*, differences in efficiencies have been

observed in different systems (Sedgwick et al., 1991; Szekeres Jr. et al., 1996). These differences have been shown to be variable depending on the cell system that expresses the homologs. For example, differences of 100 fold have been observed in different species in the genus *Enterobacteriaciae* with *umuDC* (Sedgwick et al., 1991). Another study also noted differences in the *umuDC* homolog, *rulA*, at the amino acid level. Seven pathovars of *P. syringae* were compared for amino acid differences in the RulA protein, which yielded differences in every comparison between two pathovars of at least one amino acid and up to eight or more amino acids (Sundin, 2000). Although a bacterium can survive without mutagenic repair, a negative affect on survival and mutability is observed. In *P. syringae* FF5, loss of *rulAB* results in a 10 fold decrease in survival both in culture and in planta (Sundin, 1996; Sundin, 1999). Often, cells expressing *umuDC* are not as efficient as the other homologs, in mutagenic repair, because the step involving autoproteolytic cleavage of UmuD into UmuD' does not proceed as rapidly as in the other homologs (Woodgate & Sedgwick, 1992; Kim & Sundin, 2000). In this work, we sought to observe any differences in survival and mutability when comparing different Gram-negative strains as well as a knockout of *umuDC* and strains complemented with homologs of *umuDC*.

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions

All bacteria were grown in Luria-Bertani (LB) broth or solidified media (1.5% agar). Ampicillin (100 $\mu g\ mL^{-1}$), chloramphenicol (20 $\mu g\ mL^{-1}$), gentamicin (15 $\mu g\ mL^{-1}$), rifampicin (250 $\mu g\ mL^{-1}$), and spectinomycin (100 $\mu g\ mL^{-1}$) were added to media

when appropriate. All bacteria were grown at 28°C except *Escherichia coli*, which was grown at 37°C (Table 2.1)

Strain or Plasmid	Relevant Characteristics	Source or Reference
Strains-Experimental Screening		
Erwinia amylovora Ea1189	Wild type	Burse, 2004
Erwinia amylovora Ea273	Wild type	Bogdanove, 1998
Erwinia amylovora LebB66	Wild type	Foster, 2004
Escherichia coli DH5α	Strain used for cloning	G.W. Sundin
Escherichia coli K12	Enteric bacterium	ATCC 47076
Pseudomonas cichorii 302959	Plant pathogen	Zhang, 2004
Pseudomonas syringae B86-17	Plant pathogen	Legard, 1993
Plasmids		
pJJK25	2.45 kb *umuDC* promoter + *rulAB* as *SalI* and *BamHI* in pJB321	Kim & Sundin, 2000
pJJK27	2.45 kb *umuDC* promoter + *umuDC* as *SalI* and *BamHI* in pJB321	Kim & Sundin, 2000
Primers		
Cm + umuDC For	5′-ccgttatgccgcttatccgtccgctcgacattgattg ctccctgctacttgtgtaggctggagctgcttc -3′	This Study
Cm + umuDC Rev	5′-ccgcatcacttcgccacgggaagatcggcatagc gagtggtgtaagcgggcatatgaatatcctcctta -3′	This Study
umuDC For	5′-atggctggcctgctgttattc -3′	This Study
umuDC Rev	5′-ccatgcggttatctttctgttgc -3′	This Study

(ATCC) American Type Culture Collection

Table 3.1. Bacterial strains, plasmids, and oligonucleotide primers used in this study and their relevant characteristics

UV-C Survival Assay

Cells were grown overnight as described before centrifugation and resuspension in 0.85% saline. After measuring UV output of the lamp used (output around 1.46 J/m^2*s), bacteria were exposed to five 20 s doses of UV-C light and plated before the first dose and after each of the five doses applied. Following growth for two days at the appropriate temperature in darkness, cell counts were enumerated.

UV-C Mutability Assay

Cells were prepared and exposed to UV as described in the previous experiment except three 20 s doses were applied instead of five. Following exposure, one mL of the bacterial suspension was added to one mL of 2x LB broth and grown overnight in darkness at the appropriate temperature and plated on rifampicin (250 $\mu g\ mL^{-1}$) the following day. Two days after plating, rifampicin resistant colonies were counted.

Generation of *umuDC* Knockout

Methods for generating the *umuDC* knockout were adapted from the work by Datsenko (2000), in which a chloramphenicol resistance gene replaced the gene pair *umuDC*. The following changes were made to the protocol: cells were washed in ice cold water and incubation was at 28°C with heat shocking of cells not exceeding 37°C

Complementation of *umuDC*

umuDC was complemented with two different plasmids to assess differences in survival and mutability compared to the knockout. pJJK25, which encodes the gene pair *rulAB* and pJJK27, which encodes the gene pair *umuDC* (Kim & Sundin, 2000) were electroporated into *E. amylovora* Ea1189 *umuDC* generating *E. amylovora* Ea1189

umuDC/pJJK25 and *E. amylovora* Ea1189 *umuDC*/pJJK27 respectively. Both plasmids are driven by an *E. coli umuDC* promoter.

Hydrogen Peroxide Mutability Assay

Cells were grown overnight in appropriate conditions before exposure to hydrogen peroxide. Concentrations of hydrogen peroxide used were 0 μM, 250 μM, 500 μM, 750 μM, and 1 mM. Cells were exposed to the appropriate concentration of hydrogen peroxide for 15 minutes before one mL of cells was placed in one mL of 2x LB broth, which was then incubated overnight. The hydrogen peroxide was diluted out in the 2x LB broth, and any remaining hydrogen peroxide was deactivated by the incubation temperature. The overnight cultures were then plated on LB amended with rifampicin (250 μg mL^{-1}), and colony forming units (CFU) were enumerated two days later.

Results

UV-C Survival

To observe how well *E. amylovora* Ea1189 survives UV stress, a comparison was performed using bacteria from the genera *Erwinia*, *Escherichia*, and *Pseudomonas*. Of the six strains tested, the *Erwinias* (Ea1189, Ea273, and LebB66) had the highest rate of survival followed by *E. coli* K12, *P. syringae* B86-17, and *P. cichorii* 302959 (Figure 3.1).

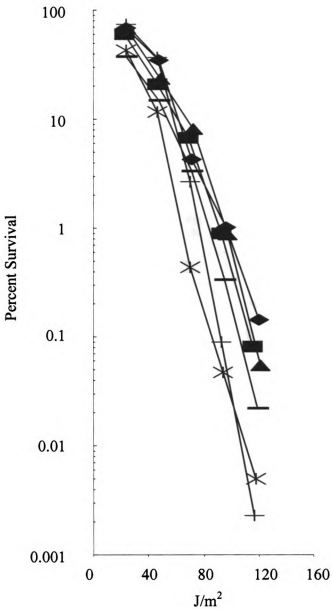

Figure 3.1. Comparison of survival in different species of Gram-negative bacteria after exposure to UV-C radiation. *E. amylovora* Ea1189 (closed diamonds) was compared to other *E. amylovora* strains Ea273 (closed squares) and LebB66 (closed triangles) as well as compared to other Gram-negative bacteria *Pseudomonas syringae* B86-17 (stars) *Pseudomonas cichorii* 302959 (crosses) and *E. coli* K12 (dashes) to assess survival in response to increasing doses of UV-C radiation. A representative of one of the three replicates is shown.

This trend was observed in each of the three replicates performed. This experiment was repeated using a strain of *E. amylovora* Ea1189 in which the *umuDC* gene pair was knocked out via gene replacement (called *E. amylovora umuDC*). The *umuDC* mutant was 1.5 to 32 fold more sensitive to UV light than the WT strain over the five doses administered (Figure 3.3).

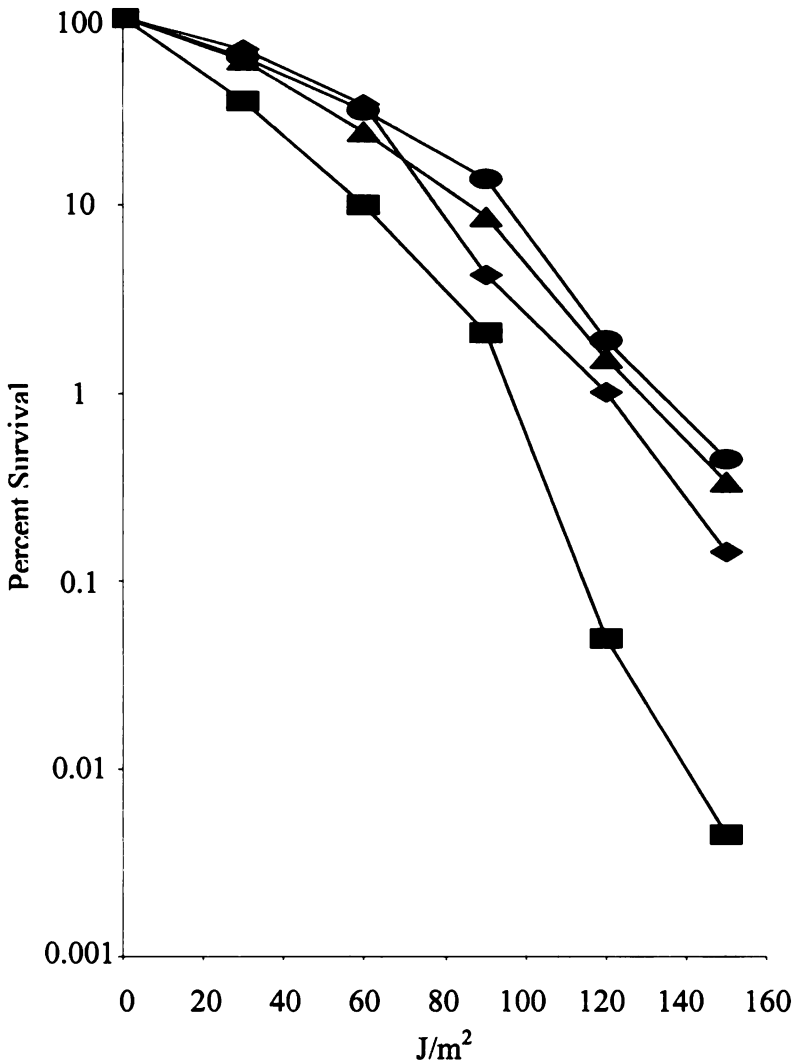

Figure 3.3. Comparison of survival in *E. amylovora* strains after exposure to UV-C radiation. *E. amylovora* Ea1189 (closed diamonds) was compared to Ea1189 *umuDC* (closed squares) and two complemented strains Ea1189 *umuDC*/pJJK25 (closed triangles) and Ea1189 *umuDC*/pJJK27 (closed circles) to assess mutability in response to increasing doses of UV-C radiation. A representative of one of the three replicates is shown.

This knockout was complemented with plasmids previously generated by Kim & Sundin (2000). These plasmids pJJK25 and pJJK27 restored the mutant to near WT levels where both plasmids were within 1.5 fold of the WT strain throughout the course of the experiment (Figure 3.3).

UV-C Mutability

Because there is a correlation between survival after UV exposure and an increase in mutability, the six strains from the previous experiment were compared for mutability on rifampicin plates (Figure 3.2).

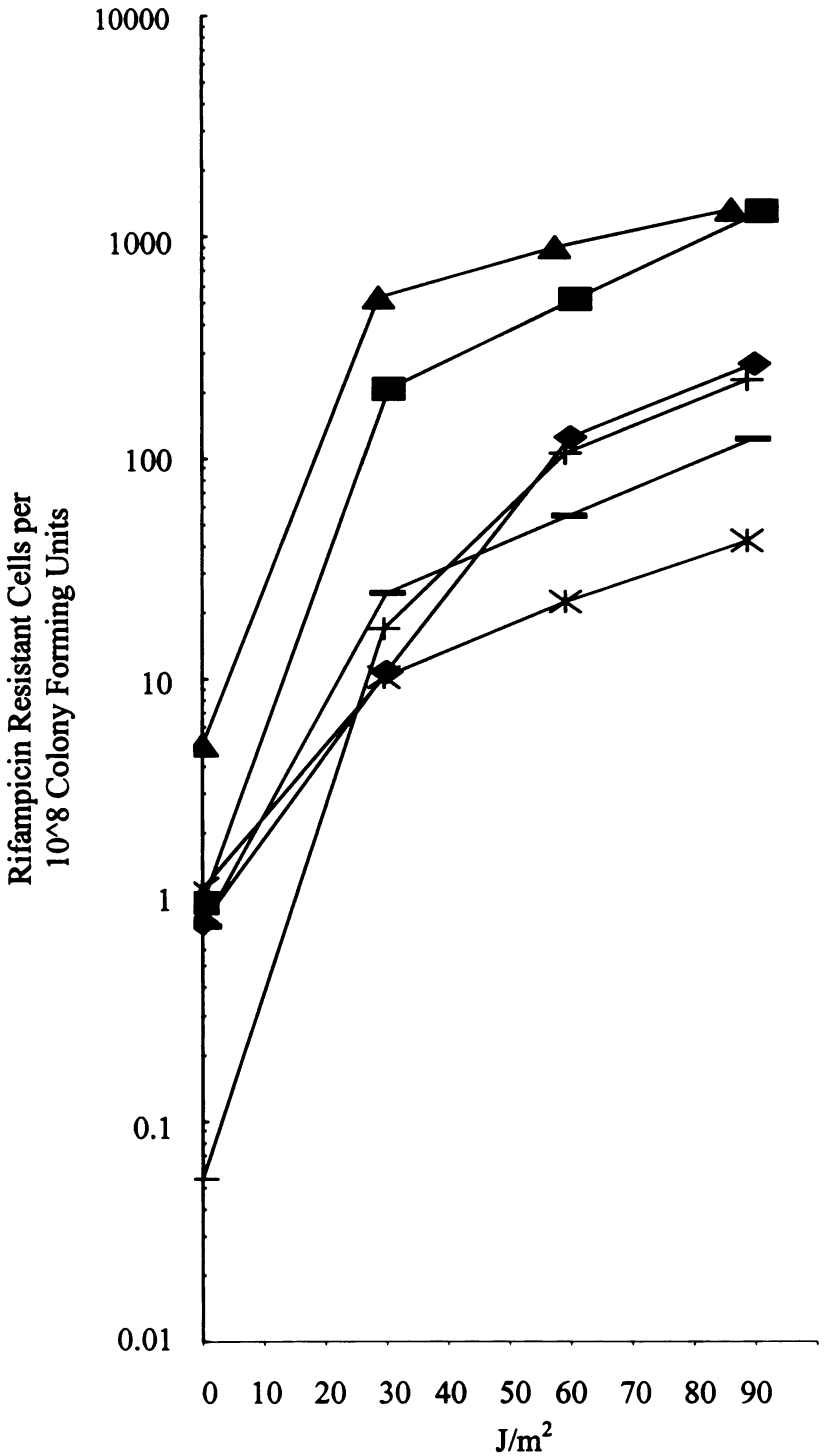

Figure 3.2. Comparison of mutability in different species of Gram-negative bacteria after exposure to UV-C radiation. *E. amylovora* Ea1189 (closed diamonds) was compared to other *E. amylovora* strains Ea273 (closed squares) and LebB66 (closed triangles) as well as compared to other Gram-negative bacteria *Pseudomonas syringae* B86-17 (X's) *Pseudomonas cichorii* 302959 (crosses) and *E. coli* K12 (dashes) to assess mutability induced by increasing doses of UV-C radiation. A representative of one of the three replicates is shown.

The relative mutability of the strains in response to UV was similar to their relative sensitivity to UV as assayed by cell survival. The *Erwinia* strains were the most mutable with strain LebB66 possessing the highest mutability followed by strain Ea273 and Ea1189. One difference from the UV-C survival experiment was that *P. cichorii* 302959 was more mutable than *E. coli* K12 and *P. syringae* B86-17 was the least mutable. As in the UV survival experiment, the *umuDC* mutant and the *umuDC* mutant complemented with either pJJK25 or pJJK27 were examined for differences in mutability (Figure 3.4).

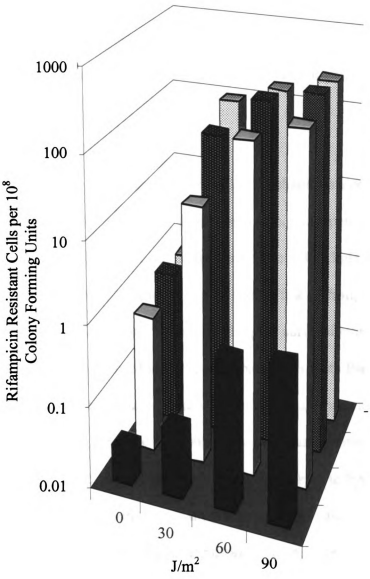

Figure 3.4. Comparison of mutability of *E. amylovora* strains after exposure to UV-C radiation. *E. amylovora* Ea1189 (white bars) was compared to the *umuDC* mutant Ea1189 *umuDC* (black bars) and two complemented strains Ea1189 *umuDC*/pJJK25 (black bars with white dots) and Ea1189 *umuDC*/pJJK27 (white bars with black dots) to assess survival in response to increasing doses of UV-C radiation. A representative of one of the three replicates is shown.

Compared to the WT strain, the mutant was 18 to 260 fold less mutable in response to UV light exposure (Figure 3.4). The two plasmid complements were able to restore mutability to near WT levels with a range of 1 to 4 fold difference in mutability (pJJK25)

and 1 to 6 fold difference (pJJK27) (Figure 3.4). Mutability in response to hydrogen peroxide exposure was also assessed in both Ea1189 and Ea1189 *umuDC*, but no difference was observed between the two strains at any of the concentrations tested (data not shown).

Discussion

Mutagenic DNA repair is one of the last mechanisms a cell can employ to repair DNA damage in order to survive. In *E. amylovora*, this repair is especially important since a portion of the life cycle can include exposure to UV light. *E. amylovora* is exposed to UV light when on the leaf surface or oozing from a fruit, canker, or other organ of the plant. *E. amylovora* strains exhibited higher survival rates when compared to the enteric bacterium *E. coli*, and two other plant pathogens, both Pseudomonads. This higher survival could be expected if any one of the repair mechanisms in *E. amylovora* are more rapidly induced or more efficiently processed than in other bacteria. It has been observed that *E. amylovora* can withstand exposure to hydrogen peroxide and actually induces the host to produce hydrogen peroxide as part of the infection process (Venisse et al., 2002). This survival after exposure to hydrogen peroxide is due, in part, because of different DNA repair mechanisms, which also repair damage caused by UV light. Perhaps in *Erwinia* these repair mechanisms are slightly more efficient based on the observations that *Erwinia* has higher survival and mutability after exposure to UV when compared to the other bacteria previously mentioned. Also, mutagenic repair genes demonstrate different efficiencies in bacteria and their products could be more efficiently processed in *E. amylovora*, than in the other bacteria tested here. A knockout of the gene pair responsible for mutagenic repair, *umuDC*, did not decrease survival to a noticeable

amount until higher doses of UV light, but these differences were small. This was expected because a cell has many DNA repair mechanisms that still function in an *umuDC* knockout background. Mutability, however, was greatly reduced in the *umuDC* knockout compared to the WT strain. This result could be interpreted to mean that *E. amylovora* only encodes *umuDC* as the mutagenic repair system in this organism. Had mutability been reduced to a lesser extent than was found in our research, it could be argued that, like some other organisms, a second gene pair homologous to *umuDC* was contributing to the mutability of this organism.

Ultraviolet radiation is a part of life for almost every organism on Earth. The ability to repair damage created by UV light is critical for survival. As was observed in this study, knocking out one of the mechanisms for repair does have an impact on survival. Because part of bacterial survival is the ability to adapt to harsh conditions, mutability was also tested. In *E. amylovora*, the gene pair *umuDC* is the only gene pair discovered that functions in mutagenic repair, but *umuDC* causes increased mutability compared to other bacterial strains in this study. Although causing mutations in the cell can be damaging to the organism, it can also provide an advantageous mutation that will benefit the cell during future stress, and for this reason, evolutionary retention of an error prone repair system can be explained.

References

Bagg A, Kenyon CJ & Walker GC (1981) Inducibility of a gene product required for UV and chemical mutagenesis in *Eschericia coli*. *Proc Natl Acad Sci U S A* **78**: 5749-5753.

Bogdanove AJ, Bauer DW & Beer SV (1998) *Erwinia amylovora* secretes DspE a pathogenicity factor and functional AvrE homolog through the Hrp (type III) secretion pathway. *J Bacteriol* **180:** 2244-2247.

Brash DE, Franklin WA, Sancar GB, Sancar A & Haseltine WA (1985) *Eschericia coli* DNA photolyase reverses cyclobutane pyrimidine dimers but not pyrimidine-pyrimidone (6-4) photoproducts. *J Biol Chem* **260:** 11438-11441.

Burse A, Weingart H & Ullrich MS (2004) NorM an *Erwinia amylovora* multidrug efflux pump involved in vitro competition with other epiphytic bacteria. *Appl Environ Microbiol* **70:** 693-703.

Datsenko KA & Wanner BL (2000) One-step inactivation of chromosomal genes in *Eschericia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97:** 6640-6645.

Foster GC, McGhee GC, Jones AL & Sundin GW (2004) Nucleotide sequence genetic organization and distribution of pEU30 and pEL60 from *Erwinia amylovora*. *Appl Environ Microbiol* **70:** 7539-7544.

Kim JJ & Sundin GW (2000) Regulation of the *rulAB* mutagenic DNA repair operon of *Pseudomonas syringae* by UV-B (290 to 320 nanometers) radiation and analysis of *rulAB*-mediated mutability in vitro and in planta. *J Bacteriol* **182:** 6137-6144.

Lee MH, Guzzo A & Walker GC (1996) Inhibition of RecA-mediated cleavage in covalent dimers of UmuD *J Bacteriol* **178:** 7304-7307.

Legard DE, Aquadro CF & Hunter JE (1993) DNA sequence variation and phylogenetic relationships among strains of *Pseudomonas syringae* pv. syringae inferred from restriction site maps and restriction fragment length polymorphism. *Appl. Environ. Microbiol.* **59:** 4180–4188.

Sedgwick SG, Ho C & Woodgate R (1991) Mutagenic DNA repair in enterobacteria. *J Bacteriol* **173:** 5604-5611.

Sinha RP & Häder D-P (2002) UV-induced DNA damage and repair: a review. *Photochem. and Photobiol. Sci.* **1:** 225-236.

Sundin GW & Murillo J (1999) Functional analysis of the *Pseudomonas syringae rulAB* determinant in tolerance to ultraviolet B (290-320 nm) radiation and distribution of *rulAB* among *P syringae* pathovars. *Environ Microbiol* **1:** 75-87.

Sundin GW, Kidambi SP, Ullrich M & Bender CL (1996) Resistance to ultraviolet light in *Pseudomonas syringae*: sequence and functional analysis of the plasmid-encoded *rulAB* genes. *Gene* **177:** 77-81.

Sundin GW, Jacobs JL & Murillo J (2000) Sequence diversity of *rulA* among natural isolates of *Pseudomonas syringae* and effect on function of *rulAB*-mediated UV radiation tolerance *Appl Environ Microbiol* **66:** 5167-5173.

Szekeres Jr. ES, Woodgate R & Lawrence CW (1996) Substitution of *mucAB* or *rumAB* for *umuDC* alters the frequencies of the two classes of mutations induced by a site-specific T-T cyclobutane dimmer and the efficiency of translesion DNA synthesis. *J Bacteriol* **178:** 2559-2563.

Witkin EM (1976) Ultraviolet mutagenesis and inducible DNA repair in *Eschericia coli*. *Microbiol Mol Biol Rev* **40:** 869-907.

Woodgate R & Sedgwick SG (1992) Mutagenesis induced by bacterial UmuDC proteins and their plasmid homologues. *Mol Microbiol* **6:** 2213-2218.

Venisse JS, Malnoy M, Faize M, Paulin JP & Brisset M N (2002) Modulation of defense responses of *Malus* spp. during compatible and incompatible interactions with *Erwinia amylovora*. *Mol Plant Microbe Interact* **15:** 1204-1212.

Yasui A & Chevallier M-R (1983) Cloning of photoreactivation repair gene and excision repair gene of the yeast *Saccharomyces cerevisiae*. *Curr Genet* **7:** 191-194.

Zhang S & Sundin GW (2004) Mutagenic DNA repair potential in *Pseudomonas* spp. and characterization of the *rulAB*$_{Pc}$ operon from the highly mutable strain *Pseudomonas cichorii* 302959. *Can J Microbiol.* **50:** 29-39.

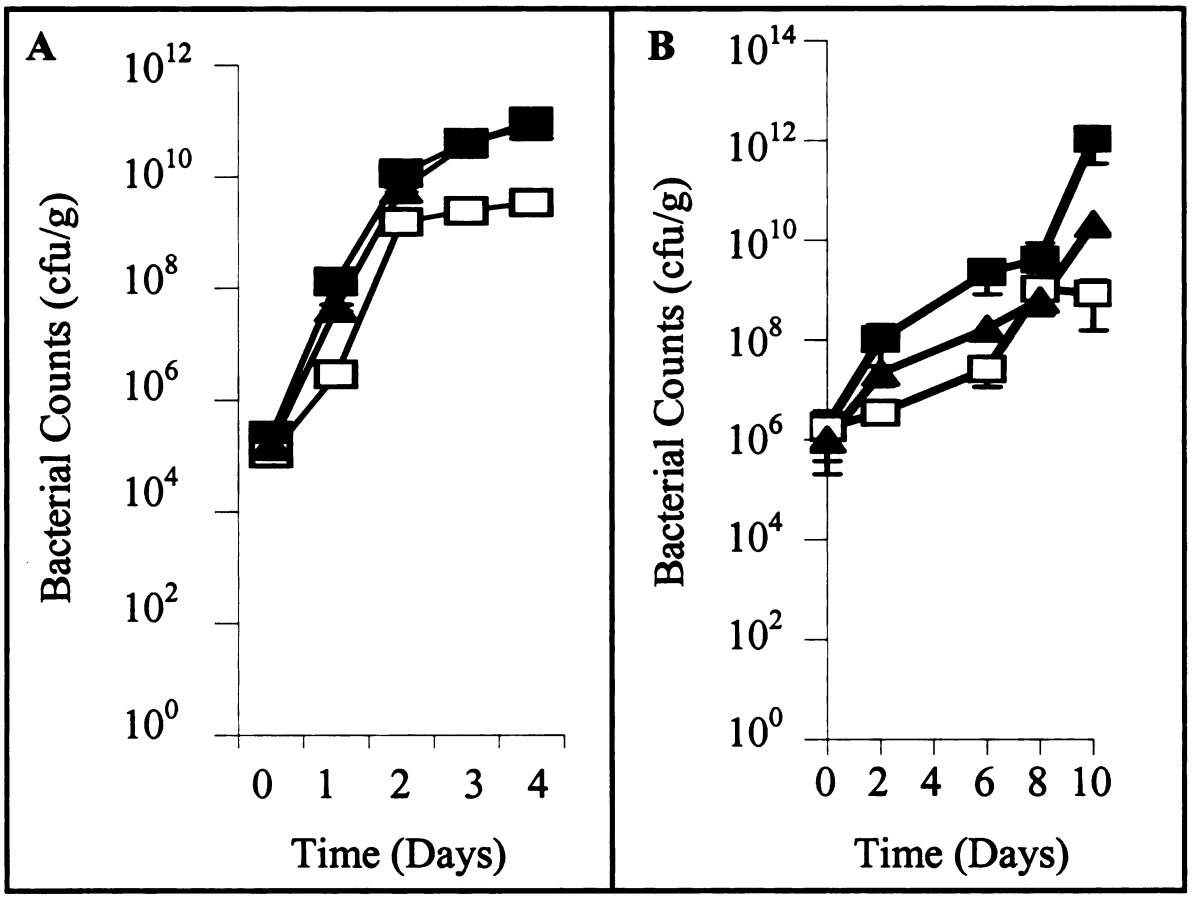

Figure A.1. Virulence assays in immature pears and apple seedlings. *Erwinia amylovora* Ea1189 (closed squares) was compared to the mutant strain *Erwinia amylovora* GS1 (open squares) and the *waaL* complement *E. amylovora* GS1/pMCB3 (triangles) in (A) immature pears and (B) apple seedlings. A representative of the two experimental replicates is shown for the apple seedling assay, while an average of the two experiment experimental replicates are shown for the pear assay. Each data point is shown with standard error unless the error bars could not be visualized on the graph due to the size of the symbol in which case they were removed for clarity.

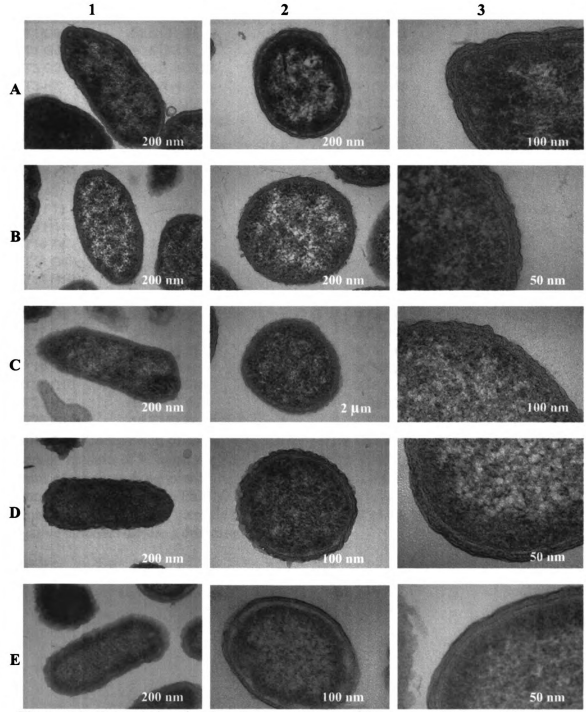

Figure A.2. Transmission electron microscopy images of *Erwinia amylovora* Ea1189, *E. amylovora* GS1, *E. amylovora* GS1/pMCB3, *Pseudomonas aeruginosa* PAO1, and *P. aeruginosa* PAO1 *waaL*::IS*phoA*/*hah*. Column1 represents the longitudinal view of the cell, column 2 represents the vertical view, and column three is a close up of the outer membrane. Bacteria observed include *E. amylovora* Ea1189 (A), *E. amylovora* GS1 (B), *E. amylovora* GS1/pMCB3 (C), *Pseudomonas aeruginosa* PAO1 (D), and *P. aeruginosa* PAO1 *waaL*::IS*phoA*/*hah* (E).

Mutant ID	Mean of three measurements	Mutant ID	Mean of three measurements
MT1 C9	-61.3 (31.8)	MT23 E9	-19.2 (18.2)
MT1 G6	-50.6 (27.3)	MT25 D10	-15.9 (43.2)
MT3 G9	+470.2 (265.2)	MT25 F12	-91.5 (17.4)
MT3 H7	+540.4 (447.4)	MT25 G9	-60.3 (8.0)
MT3 H11	+428.1 (373.8)	MT29 C3	-54.5 (31.1)
MT5 G7	+2223.6 (1328.4)	MT32 C3	+101.5 (121.1)
MT6 A3	+147.5 (107.7)	MT34 H4	-36.4 (28.9)
MT6 B8	+57.7 (113.8)	MT36 E9	-30.7 (30.9)
MT7 F12	+93.3 (121.4)	MT37 E2	-60.4 (34.1)
MT8 A3	-33.3 (7.5)	MT37 H12	-26.8 (17.6)
MT9 F11	+170.5 (129.1)	MT45 F1	+488.7 (505.2)
MT9 H1	-48.0 (27.6)	MT45 F7	+337.8 (397.1)
MT14 F4	-22.6 (24.5)	MT46 F8	-18.0 (18.8)
MT14 G4	-27.8 (17.5)	MT47 E8	-79.7 (45.4)
MT14 H10	-33.2 (30.5)	MT49 G6	-41.6 (19.0)
MT15 E7	-54.5 (29.1)	MT50 C2	-56.7 (14.9)
MT16 E6	-26.6 (17.6)	MT53 E8	-15.4 (12.8)
MT17 E3	-34.1 (22.0)	MT55 D11	+132.2 (125.2)
MT17 H6	+22.1 (39.8)	MT56 A6	-8.6 (25.3)
MT19 H11	+591.1 (593.3)	MT59 B2	-17.6 (15.6)
MT21 C10	+316.9 (304.6)	MT60 E11	+2.3 (59.1)
MT21 E12	+97.2 (37.0)	MT60 G4	-61.1 (29.0)
MT23 E8	-18.2 (15.2)	WT	0.0 (10.1)

Table A.1. Comparison of 45 mutants obtained from screening over 6,000 transposon insertion mutants of *E. amylovora* Ea1189 for survival in 250 μM of hydrogen peroxide. Means of the three experimental replicates are shown as a percent difference when compared to *E. amylovora* Ea1189 with the standard error in parentheses. Positive numbers denote a percent increase in survival compared to the WT strain, whereas negative numbers denote a decrease in survival.

Mutant Name	Percent Identity	Percent Positive	Amino Acid length	Bit Score	E-value	Organism	Gene Name
1G6	48	64	438	314	9e-84	*Thermobifida fusaca YX*	Histidyl-tRNA synthetase hisS
3G9	67	83	592	722	0	*Erwinia tasmaniensis* Et1/99	Type III secretion apparatus invG
3H7	46	66	374	276	2e-72	*Erwinia tasmaniensis* Et1/99	Type III secretion apparatus invE
5G7	29	55	125	52.8	9e-06	*Sodalis glossinidius* strain morsitans	Hypothetical Protein SG0553
6A3	53	71	1746	540	1e-151	*Erwinia tasmaniensis* Et1/99	Hypothetical Protein ETA_00070
6B8	81	91	414	626	1e-177	*Erwinia tasmaniensis* Et1/99	Lipid A core:surface polymer ligase *waaL*
7F12	71	83	298	433	8e-120	*Erwinia tasmaniensis* Et1/99	Putative Glycosyl Transferase wbbN
14F4	95	96	395	334	2e-90	*E. coli* HS	Phage Integrase Family Site Specific Recombinase
15E7	33	52	153	81.6	2e-14	*Erwinia tasmaniensis* Et1/99	Hypothetical Protein ETA_19030
17E3	70	82	334	330	4e-89	*Erwinia tasmaniensis* Et1/99	Hypothetical Protein ETA_30240
21C10	84	92	326	556	7e-157	*Erwinia tasmaniensis* Et1/99	Predicted Glycosyl Transferase, Family 2
34H4	53	70	565	581	6e-164	*Marimonas* sp. MED121	Hypothetical protein MED121_12715
36E9	84	93	630	1094	0	*Erwinia tasmaniensis* Et1/99	Glycosyl Transferase wbbM
37E2	98	99	205	421	1e-116	*Erwinia tasmaniensis* Et1/99	Manganese and iron superoxide dismutase
45F7	21	44	259	60.8	1e-7	*Sorgangium cellusom So ce 56*	Hypothetical Protein sce1197
46F8	83	91	371	536	1e-150	*Erwinia tasmaniensis* Et1/99	Export Protein for Peptides using the Type III Secretion System spaS2
49G6	63	81	254	257	5e-67	*Erwinia tasmaniensis* Et1/99	Surface Presentation of Antigens Protein, Type III Secretion Apparatus spaR2
50C2	84	93	630	1094	0	*Erwinia tasmaniensis* Et1/99	Glycosyl Transferase wbbM
53E8	48	63	636	245	4e-63	*Erwinia tasmaniensis* Et1/99	Cell Invasion Protein sipB1
55D11	57	77	214	174	3e-42	*Erwinia tasmaniensis* Et1/99	Hypothetical Protein ETA_19150
56A6	76	87	431	641	0	*Erwinia tasmaniensis* Et1/99	Type III secretion apparatus invC
59B2	76	87	435	641	0	*Erwinia tasmaniensis* Et1/99	Type III secretion apparatus invC

Table A.2. Sequence results for the mutants found in the hydrogen peroxide screen of the transposon library for *E. amylovora* Ea1189.

Bacterial Species and Strains	Percent Survival			
	3 min	10 min	15 min	20 min
Dickeya dadantii 3937	94.8 (7.7)	87.5 (3.1)	78.3 (1.7)	76.7 (6.2)
Pantoea agglomerans NCPPB 2971	90.2 (1.6)	85.9 (4.1)	80.4 (5.0)	67.2 (10.3)
Pseudomonas syringae FF5	91.3 (5.3)	72.5 (1.0)	58.3 (0.8)	53.8 (5.9)
Erwinia pyrifoliae 1/96	90.3 (4.2)	71.1 (9.4)	51.4 (24.2)	42.1 (20.5)
Erwinia sp. EJP556	90.0 (1.1)	57.2 (10.1)	56.8 (13.2)	26.5 (2.5)
***Erwinia amylovora* Ea1189**	**68.3 (4.0)**	**47.2 (6.4)**	**29.5 (4.7)**	**21.3 (3.0)**
Eschericia coli SY2	15.0 (1.9)	14.0 (4.6)	13.6 (5.9)	8.7 (4.4)
Xanthomonas campestris pv. phaseoli	5.7 (2.3)	2.2 (1.3)	2.7 (2.5)	2.8 (2.6)
Erwinia tracheiphila NCPPB 2452	9.8 (3.2)	5.8 (2.9)	1.9 (1.7)	1.2 (1.0)
Shewanella oneidensis MR-1	0.4 (0.4)	0.5 (0.5)	0.1 (0.1)	0.0 (0.0)

Table A.3. Comparative survival of different Gram-negative bacteria after exposure to 250 μM H_2O_2. All results shown as a mean of three replicates and with standard error in parentheses.