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Characterization of a RTX-Like Toxin and an Alpha-2-Macroglobulin in *Pantoea stewartii* subsp. *stewartii*, Causal Agent of Stewart's Wilt of Sweet Corn

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Microbiology

by

Kayla Marie Williams

December 2014

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The Thesis of Kayla Marie Williams is approved:

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GENERAL INTRODUCTION

Lifecycle of *Pantoea stewartii* subsp. *stewartii*

P. stewartii subsp. stewartii DC283 (formally *Erwina* stewartii) is a motile, non-sporulating, Gram-negative rod shaped bacterium that is roughly 0.4-0.7 x 0.9-2.0 μm in size and belongs to the *Enterobacteriaceae* (Bradbury, 1967; Wang *et al.,* 2012). Colonies on nutrient agar (NA) are light yellow in color and vary in colony morphology from raised, flat, or convex (Bradbury, 1967; Pepper, 1967)

P. stewartii is the causal agent of Stewart's wilt, which is an important bacterial pathogen of sweet corn. *P. stewartii* colonizes the digestive tract (specifically the midgut and hindgut lumen) of the insect vector, *Chaetocenema pulicaria* (Melsheimer), commonly known as the corn flea beetle. The bacterium gains access to the apoplast and the xylem through wounds inflicted by the feeding corn flea beetle (Correa, 2012; Braun, 1982; Braun 1990). The bacteria are present on the body and feces of the insect and the exact mode of transmission is unclear.

Once *P. stewartii* enters the apoplastic space of the sweet corn leaf it incites water-soaked (WS) lesions, which is the initial phase of infection commonly referred to as leaf blight. The later phase of infection results in wilting through obstruction of the xylem by bacterial colonization and stewartan exopolysaccharide (EPS)-encased biofilm formation (Ham *et al.,* 2006; Roper, 2011).

The bacteria can be found in all parts of a systemically infected corn plant including the stalks, roots, leaves, tassels, cobs, kernels, and husks (Munkvold, 2001). Seed transmission of the bacteria is very rare (0.02 percent) (Munkvold, 2001). The

severity of bacterial infection on the corn plants can in part be due to the environment that the plant is growing. Certain growing conditions affect the susceptibility of the corn plant, such as soil composition (Munkvold, 2001). Soils with higher concentrations of nitrogen, ammonium, or phosphorus can increase the likelihood of infection (Munkvold, 2001).

Disease Cycle

The adult corn flea beetles emerge from overwintering and transmit *P. stewartii* to young sweet corn seedlings. This first cycle is the most detrimental because the immature sweet corn seedlings are vulnerable during the developmental stage and are more susceptible to disease (Pataky, 2003; Roper, 2011). These infected seedlings succumb to both the leaf blight and wilting. The second cycle requires the bacteria be transferred from the infected sweet corn plants to the summer generation of the corn flea beetles. This infection cycle is not as detrimental to the newly infected corn plants because at this point in the growing season the mature plants are able to tolerate the bacterial infection. These plants display the leaf blight symptoms but not the wilting symptom of infection (Pataky, 2003; Roper, 2011). The last corn flea beetle generation of the season acquires the disease from infected mature corn plants and this becomes the overwintering population. This allows the cycle of bacterial transmission to repeat again in the spring (Pataky, 2003; Roper, 2011).

Geographical Distribution

Stewart's wilt disease of sweet corn has been reported in many parts of the world, including Europe, Asia, North and South America, but the disease is endemic to North America, where the majority of sweet corn is grown. Stewart's wilt was first reported in New York in 1897 by F. C. Stewart (Claflin, 2000; Stewart, 1897) and is now a prevalent disease from the Northern Pacific coast to the Midwest (Rand & Cash, 1921; Robert, 1955).

Stewart's wilt was the most influential bacterial disease of corn in the United States during the first half of the 20th century. It was economically important until resistant hybrid cultivars were introduced into the market. Outbreaks still can occur, as seen in the 1990's with repeated seasons of favorable weather (Nutter *et al.,* 1998; Esker & Nutter 2001; Nutter *et al.,* 2002). There have also been strict guidelines on imported seed trade when an area was known to have the pathogen present. The largest contributor to increased outbreaks is warmer temperature that is more favorable for the corn flea beetle, which carries and distributes the pathogen (Stevens, 1934; Elliot, 1935; Metcalf *et al.,* 1962; Dill, 1979).

Stewart's wilt is not found in South or Central America, where maize was initially domesticated. It is suggested that genetic manipulation by the growers for more favorable traits caused the plants to become susceptible to disease in other parts of the world. By domesticating corn in new geographical locations, pests native to the new locations, such as the corn flea beetle, were able to feed on the new plants and transmit the disease (Pataky *et al*., 2000).

Disease Forecasting

Once a corn flea beetle has acquired *P. stewartii*, the insect will carry the bacteria in it's digestive tract and will transmit disease throughout the insect's life (Dill, 1979). The corn flea beetle is capable of carrying the disease locally, from grasses near cornfields to sweet corn seedlings.

The infection levels of Stewart's wilt disease can be predicted based on weather and overwintering potential of the insect vector. The overwintering survival rate of the corn flea beetle directly relates to the severity of Stewart's wilt disease in the cornfield in the spring. Disease severity can be predicted by the sum of the mean temperatures from December, January and February as described in Table 1 (Agrios, 2005; Claflin, 2000; Stevens, 1934; Roper, 2011). Years with the mean temperatures between 32-38°C have higher rates of corn flea beetle survival due to the mild winter conditions. Increased number of beetles that survive the winter directly correlates to a more severe incidence of Stewart's wilt disease in the spring. In comparison, winters that are especially cold (mean temperature is 20-24°C) decrease the likelihood of disease during the next growing season. The pathogen require many years to get established in a field and this allows corn growers to predict if Stewart's wilt will be worse this year based on disease persistence from the previous year (Elliott, 1941; Bradbury, 1967; Pepper, 1967; Robert, 1967; Heichel et al., 1977; Shurtleff, 1980; EPPO quarantine pest data sheet; Roper, 2011).

Stevens-Boewe forecast for Stewart's wilt. Developed by N. E. Stevens in the 1930's and revised in the 1940's by G.H. Boewe at the Illinois Natural History Survey, University of Illinois

Winter Temp Index	Seedling wilt phase	Leaf blight phase
37.7 or more	Destructive	Severe
32.2 to 37.7	Light to severe	Severe
29.4 to 32.2	Nearly absent	Moderate
26.6 to 29.4	Nearly absent	Light
Below 26.6	Nearly absent	Trace

Winter temperature index = sum of the average temperature (°C) for December, January, and February.

Table 1. Stevens-Boewe Forecast for Stewart's wilt. The sum of the average temperature for December, January, and February can predict the severity of the seedling wilt phase and the leaf blight phase of Stewart's wilt disease. Winters with lower averages during these three months have an extreme decrease in disease development, almost non-existent. Years with warmer winter temperatures during these three months see severe disease development (adapted from Pataky, 2004).

P. stewartii Detection and Control

Visual confirmation of *P. stewartii* infection can be determined by lacerating the infected corn stem or leaves. Large quantities of yellow, mucus-like bacterial biofilm can be extracted from the laceration. The bacteria can also be visualized by microscopy in the presence of water, which will induce *P. stewartii* to stream from the apoplastic space within the leaf. Additional pathogen detection methods include enzyme–linked immunoabsorbent assay (ELISA), immunofluorescence, and biochemical tests to determine the presence of *P. stewartii* (Lamka *et al.,* 1991; Block *et al.*, 1999; Roper, 2011).

Current control mechanisms include planting cultivars resistant to Stewart's wilt, spraying insecticides and the use of disease-free seeds for replanting (Pepper, 1967). There are varieties of corn that demonstrate significant levels of resistance to Stewart's wilt and prevent systemic infection, for example varieties Ambrosia, Lancelot, and Sweet Sue. *P. stewartii* is transmitted to these corn varieties by the corn flea beetle but bacterium infection is localized to the point of inoculation and the pathogen is unable to become systemic in the host plant (Freeman & Pataky, 2001; Pataky, 2004; Pataky *et al.,* 2008). However, some varieties of sweet corn that are more profitable on the market are genetically manipulated to maturation faster or have higher starch content remains highly susceptible (Pataky *et al.,* 1997; Pataky *et al.,* 2000; Roper, 2011).

Economic Impact

Stewart's wilt has a significant impact on the economic market in three ways: (1) total crop yield loss due to leaf blight and seedling death from wilting in both sweet and dent cultivars, (2) moderate crop loss in dent corn due to leaf blight in mature plants, and (3) limitations of seed trading within the corn seed market (Munkvold, 2001). *P. stewartii* subsp. stewartii DC283 is under quarantine restrictions to prevent dispersion to uninfected geographical areas. This limits the markets where growers can participate in seed trade (Lamka *et al.,* 1991; Michener *et al.,* 2002; Roper, 2011) Expensive testing, such as ELISA, is required to guarantee that the seed destined for export markets is not infested with *P. stewartii* subsp. stewartii (Lamka *et al*., 1991; Michener *et al.,* 2002; Roper, 2011). The overall cost of seed testing in the competitive export market negatively affects the seed corn industry.

Virulence Factors

Known virulence factors within *P. stewartii* that contribute to Stewart's wilt disease development include (1) a type III secretion system (Hrp T3SS), (2) stewartan exopolysaccharide (EPS), (3) an endoglucanase enzyme, and (4) a siderophore-mediated iron uptake system.

Two Type III Secretion Systems in *P. stewartii*

Gram-negative pathogens of both plant and animals typically contain type III secretion systems (T3SS). The T3SS is a specialize mechanism that allow bacterial

virulence effector proteins to be injected into the plant host cells. Specific examples of phytopathogens that have a T3SS include *Pantoea, Ralstonia, Erwinia,* and *Xanthomonas*. These phytopathogens are able to colonize the plants, and cause cellular death of the host plant during the disease development (Alfano & Collmer, 2004).

The T3SS apparatus has three parts that include (1) the needle complex, which crosses the inner and outer membranes and protrudes from the cell, (2) the basal body, which forms a base that penetrates the two bacterial membranes and the periplasmic space, and lastly (3) the export gate that controls what specific proteins are secreted through the needle complex (Gazi *et al*., 2012). Effector proteins expressed in the cytoplasmic space of the bacteria are transported through the needle complex to the cytosol of the eukaryotic host cell where they will carry out their functions in manipulating the host defense system.

Pathogenesis *in planta* involves many complex steps for the bacterium to gain entry into the host. These steps include suppression of the initial plant defenses, suppression of pathogen-specific hypersensitive response (HR) defense detection, bacterial growth within the host, and lastly production of characteristic disease symptoms (Alfano & Collmer, 2004). The T3SS pathway is encoded by two vital gene clusters: hrp (hypersensitive response and pathogenicity) and hrc (hypersensitive response and conserved) that are broken up into two families which are Hrc-Hrp1 and Hrc-Hrp2 (Alfano & Collmer, 2004; Correa *et al.,* 2012). Hrc-Hrp1 and Hrc-Hrp2 synthesize pili that are able to gain access past the plant cell wall, which allows the bacteria to colonize the host (Correa *et al.,* 2012). Previous research determined that the water-soaked (WS)

lesions observed in the initial phase of infection by *P. stewartii* on immature corn seedlings are dependent on the Hrp-Hrc1 family T3SS (von Bodman & Farrand, 1995; Coplin *et al.,* 1992; Dolph *et al.*, 1988; Roper, 2011; Correa *et al.,* 2012).

P. stewartii has an additional T3SS required for bacterial survival in the corn flea beetle and contributes to transmission from the insect gut to the sweet corn plants. This specific T3SS necessary for bacterial survival in the insect vector is called the *Pantoea* secretion island 2 (PSI-2), which is part of the Inv-Mxi-Spa T3SS family (Correa *et al.,* 2012; Merighi, *et al.,* 2003). The Inv-Mxi-Spa T3SS family is well recognized as a requirement for bacterial invasion of animal cells that most likely occurred through horizontal gene transfer from the host to the pathogen (Trisfontaines & Cornelis, 2005; Correa *et al.,* 2012). The gene *psaN* located in the PSI-2 encodes an ATPase necessary for secretion of the T3SS effectors by the needle complex. When a mutation was made in *psaN*, it resulted in decreased *P. stewartii* colonization of the corn flea beetle because the effectors were unable to be secreted. Interestingly, this mutation did not affect the colonization in the corn plant (Correa *et al.,* 2012). This is suggestive that the PSI-2 T3SS is necessary for colonization of the insect, but not the corn plant. *P. stewartii* is not alone in containing multiple T3SS proteins; other bacteria with multiple T3SS proteins include *Yersinia, Salmonella,* and *Sodalis* subspecies (Correa *et al.,* 2012). However unlike other pathogenic systems, additional T3SS proteins often contribute to the infection of a single host and not multiple hosts, as observed in *P. stewartii* (Correa *et al.,* 2012).

Stewartan Exopolysacchride

Stewartan exopolysacchride (EPS) is a well-documented cell density-dependent pathogenicity factor in *P. stewartii*. It is thought that Stewartan EPS causes occlusions of the xylem water transport system in sweet corn seedlings, preventing disruption of water transport in the plant, which leads to the wilting phase (Beck *et al.*, 1995; Dolph *et al*., 1988; Roper, 2011). Stewartan EPS is comprised of three polysaccharides; glucuronic acid, glucose and galactose, at a ratio of 1:3:3 (Nimtz *et al.,* 1996; Yang *et al.,* 1996; Roper, 2011). The gene clusters responsible for EPS production in *P. stewartii* include *wceI*, *wceII,* and *wceIII* (Dolph *et al.,* 1988; Carlier *et al*., 2009; Roper, 2011) and are controlled by two pathways (1) a quorum sensing system (EsaI/EsaR) and (2) a signal transduction system called regulator of capsular polysaccharide synthesis (Rcs) (Gottesman *et al.,* 1985; Majdalani & Gottesman, 2005). Rcs is an environmental monitoring system found in the *Enterobacteriaceae* (Majdalani & Gottensman, 2005). This system has two sensor kinases (RcsC and RcsD), two co-activators of transcription (RcsA and RcsB), and a lipoprotein that is bound in the outer membrane (RcsF) as demonstrated in Figure 1 (Majdalani *et al.*, 2005).

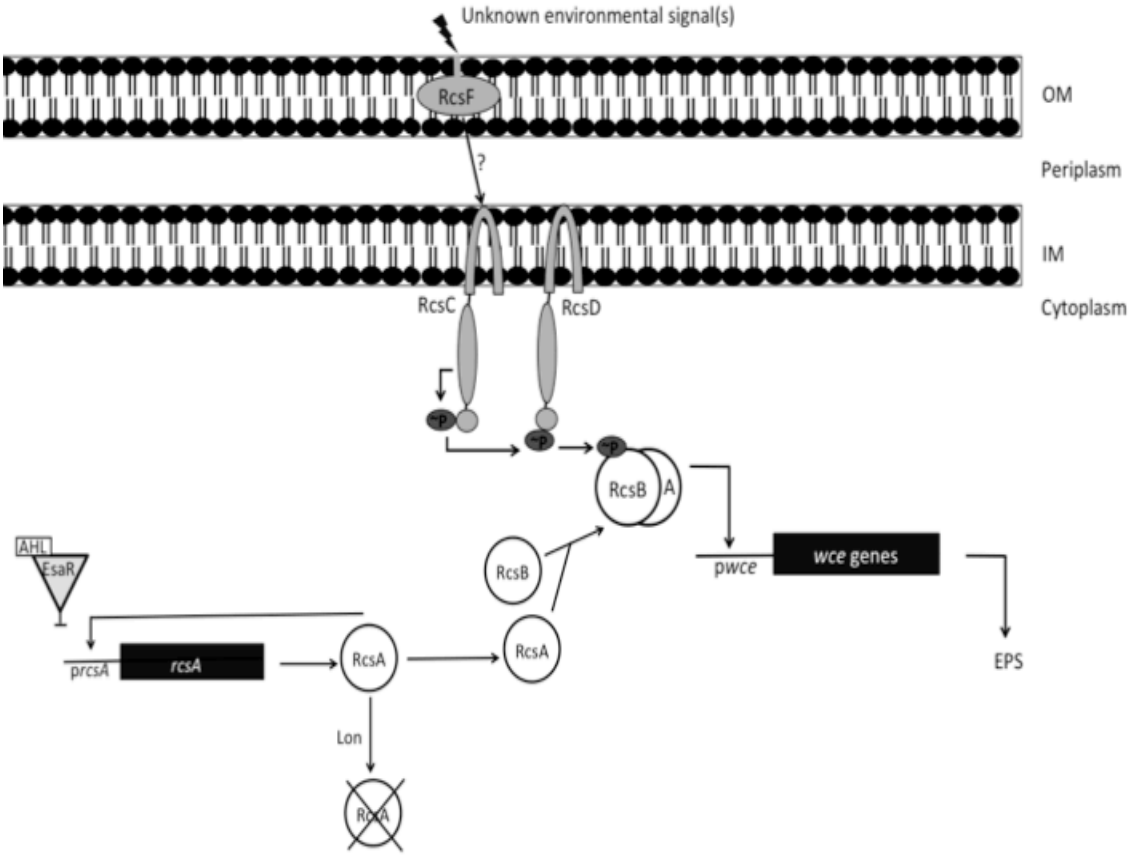


Figure 1. The environmental signaling pathway Rcs controls stewartan EPS production. It is hypothesized that the *P. stewartii* Rcs phosphorelay system begins with unknown environmental signal (s) recognized by RcsF. RcsF then is thought to act as a trigger for the phosphorelay cascade, phosphorylating RcsC which phosphorylates RcsD. RcsA binds to RcsB to create the transcriptional activation complex that then activates expression of the *wce* gene cluster among other loci. When the *wce* gene cluster is activated, stewartan EPS is synthesized. This can only occur when cell densities are high, when EsaR is bound to the autoinducer, AHL. This allows production of RcsA to occur. Lon degrades some of the RcsA protein that is produced, but the remaining RcsA is able to form the RcsAB complex and activate gene expression of the *wce* gene cluster, resulting in EPS production (adapted from Roper, 2011).

The quorum sensing system is tightly regulated by the EsaI/R cell density sensing system. EsaI is the signal synthase that produces small signaling molecules that act as autoinducers and EsaR is the response regulator (Carlier *et al.,* 2009). When bacterial cell densities are low, the overall concentration of AHL is low; however, when cell densities are high, the concentration of AHL is high, which binds to EsaR. EsaR acts as the responsive transcription factor that dimerizes and binds to DNA when EsaI is absent, acting as a repressor (Carlier & von Bodman, 2006; Roper, 2011). EsaR indirectly controls the *wce* gene system that includes 12 genes that encode functions necessary for biosynthesis and translocation of EPS in *P. stewartii* (Carlier & von Bodman, 2006). The *wceI* gene cluster is regulated by the Rcs phosphorelay system, specifically the co-activator *rcsA,* which controls the quorum-sensing system in *P. stewartii* (Torres-Cabassa *et al.,* 1987; Carlier & von Bodman, 2006). When cell densities are low and EsaR is not bound to EsaI, EsaR binds to the promoter region for *rcsA* and decreases transcription. The concentration of RcsA present under these conditions is not sufficient to form the RcsA/RcsB complex necessary to activate transcription of the *wceI*, *wceII,* and *wceIII* gene clusters; therefore EPS production is halted (Beck *et al*., 1998; Carlier *et al.,* 2009; Roper, 2011).

A strain lacking the *esaI* gene in *P. stewartii* does not produce wild type (WT) levels of EPS or cause disease when infecting the sweet corn plant host. Without EsaI, the signal synthase, the bacteria are unable to induce the signal (AHL) necessary to initiate EPS production. On the other hand, if a mutation is created in *esaR*, the EPS production is constitutively expressed and results in large quantities of EPS secreted by

the bacteria. This phenotype is observed because EsaR is not available to repress expression of *rcsA*. RcsA/RcsB under these conditions are able to easily form and activate transcription of the gene clusters *wceI*, *wceII,* and *wceIII* that will continue producing stewartan EPS. The *esaR* mutants are unable to cause disease at the same rate as WT (Koutsoudis *et al.,* 2006; Roper, 2011). Mutations in both *esaI* and *esaR* affect the rate of systemic colonization in the plant xylem tissue and maturation of biofilm formation compared to WT (Koutsoudis *et al*., 2006).

Traditionally, biofilm development can be broken down into five stages; (1) individual cells attach to a solid surface, (2) irreversible attachment (3) development of microcolonies, (4) development of complex 3-dimensional biofilms, and lastly (5) dispersion from the biofilm (Kokare *et al.,* 2009). Initial surface adhesion is a key step in the initiation of biofilm formation. A deletion mutation to *esaI* affects the ability to release the quorum-sensing signal (AHL) based on cellular density changes. However, a deletion mutation to the receptor, EsaR could prevent the bacterium from initiating surface adhesion required for development into mature biofilms (von Bodman *et al.,* 2003; Roper, 2011).

Endoglucanase activity

In maize, selectively permeable pit membranes separate the xylem transport system into individual vessels. These membranes prevent microorganisms, air embolisms, and other particulates from migrating from one xylem vessel to another (Buchanan *et al.,* 2000; Gibeaut & Carpita, 1994). The pit membrane is composed of

cellulose microfibrils in a polysaccharide matrix of hemicellulose and pectin (Buchanan *et al.,* 2000; Gibeaut & Carpita 1994), which closely resembles the composition of the plant cell wall. In order to cause a systemic infection these pit membranes must be broken or degraded to allow the pathogen to move from vessel to vessel (Huang &Allen 2000; Roper *et al*., 2007). Cell-wall-degrading enzymes (CWDE) are important virulence factors in other xylem-dwelling bacteria that break down cellulose, proteins, pectin, and hemicellulose from the plant cell wall and pit membranes (Carpita & Gibeaut 1993; Keegstra *et al*., 1973; Mohammadi *et al.*, 2011).

In a recent study (Mohammadi *et al.*, 2011), EGase was identified as a hydrolytic enzyme in *P. stewartii*, encoded by *engY*. EGase is capable of degrading β-1, 4 glucosyl and β-1, 4 xylosyl linkages similar to those found in the cell walls and pit membranes of sweet corn plants. A mutation in *engY* rendered EGase inactive and prevented systemic infection of the bacteria throughout the xylem vessels. This demonstrated the importance of EGase in systemic infection of *P. stewartii*.

Siderophore production

Iron is an essential nutrient for many microorganisms (Miethke & Marahiel, 2007; Burbank *et al.,* 2014). However, biologically available iron can be limited and difficult to acquire from the environment. The limitation of this important resource can lead to competition between the host and pathogen as well as other microorganisms present. Siderophores are ferric iron-chelating compounds secreted in times of iron starvation that form stable and tight associations with ferric iron (Miethke & Marahiel, 2007). In order

for the bacteria to utilize the iron from the environment, they must produce and secrete the siderophore to the environment. Iron binds to the siderophore, the iron-siderophore complex is transported into the bacteria cytoplasm and then the iron is released from the siderophore to the bacterium. (Miethke & Marahiel, 2007). Excess iron can lead to toxic hydroxyl radicals within the bacterial cells. Thus iron homeostasis is vital and needs to be tightly regulated (Zheng *et al.,* 1999; Burbank *et al.,* 2014; Miethke & Marahiel, 2007).

A recent study demonstrated that siderophore production is essential for full virulence in *P. stewartii* (Burbank *et al.,* 2014). *P. stewartii* colonizes the apoplast and the xylem tissues which are presumed to be environments with low concentrations of iron. In *P. stewartii,* proteins needed for synthesis and secretion of siderophores are encoded on the *iucABCDiutA* operon (Burbank *et al.,* 2014). *iucA* encodes an enzyme called aerobactin synthase which synthesizes the sideophores and *iutA* encodes a siderophore receptor in the bacteria (Burbank *et al.,* 2014). A global iron regulator (Fur) represses the *iucABCDiutA* operon. Fur binds to the Fur recognition site of the *iucABCDiutA* operon when the corepressor (Fe II) binds to the regulatory site on Fur, thus repressing siderophore iron uptake (Bagg & Neilands, 1987; Miethke & Marahiel, 2007; Burbank *et al.,* 2014). When a mutation was made in *iucA*, complete loss of siderophore production was observed (Burbank *et al.,* 2014). On the other hand, when a mutation was made in *iutA*, siderophores were over-produced likely because the bacterial cells were unable to transport the siderophore-iron complex back into the cell (Burbank *et al.,* 2014). Alterations to the operon resulted in decreased surface motility and decreased virulence when in the sweet corn seedling host (Burbank *et al.,* 2014).

RTX toxin

Cytotoxins such as repeats-in toxin (RTX) have been studied in other pathogenic systems including *Escherichia coli*, *Vibrio choleara*, and *Bordatella pertussis* (Short & Kurtz, 1971; Hanski & Farfel, 1985: Welch, 2001; Linhartova *et al.,* 2010). Genome-wide analysis proved *P. stewartii* has two putative *rtx* genes, one of which is an important virulence factor in the water-soaked lesion phase of Stewart's wilt disease (Chapter 1) (Roper and von Bodman, in preparation). The RTX family of proteins are characterized by the asparate and glycine (D-G) rich repeats on the C-terminus for calcium ion binding and the protein is export through a type I secretion system (T1SS) (Linhartova *et al.,* 2010). The calcium ion binding only occurs once RTX has been secreted because the concentration of calcium outside of the bacterial cell is greater than the calcium concentration within the intracellular cytoplasm (Gangola & Rosen, 1987; Linhartova *et al.,* 2010). Initial studies determined RTX as a toxin, but more recent studies have demonstrated that RTX proteins can also be virulence factors such as secreted lipases and proteases that cause tissue damage to the host (Linhartova *et al.,* 2010).

The RTX protein family can be broken up into two subgroups: (1) pore-forming leukotoxins and (2) multifunctional autoprocessing RTX toxins (MARTX). The pore-forming leukotoxin subgroup contains a hydrophobic domain that aids in pore formation of the host cell membrane. It requires post-translational activation, is exported through a type I secretion system to the extracellular environment and activated once calcium ions binding to the D-G repeats. Once thought to be host and cell-type specific (ex. leukotoxins and hemolysins), it was later determined that RTX could be classified more

generally as cytotoxins (Welch, 1991; Coote, 1992; Linhartova *et al.,* 2010). The second subgroup of RTX proteins, referred to as MARTX has been identified in several *Vibrio* species (Satchell, 2007). MARTX is different from the pore-forming RTX because it has an additional N-terminal repeat sequence (19 to 20-residues, specifically) and is much larger, with a molecular mass of 485 kDa. Very little is known about the MARTX subgroup of RTX proteins and further investigation is required.

Colonization factors

For a bacterial pathogen to cause infection within the host, it must be able to attach to the host surface and colonize. Colonization factors allow the pathogenic bacteria to remain attach to a host even in the presence of extreme environmental challenges (ex. xylem sap velocity or friction-forces). Bacteria are able to adhere to host cells because of specific proteins on their extracellular surface, for example adhesins. Specifically, ashesins are found on the ends of pili or fimbriae (hair-like appendages) (Jones *et al.,* 1995) and act as virulence factors in host colonization. Depending on the phase of bacterial infection and environmental signals, specific adhesins will be produced for that particular environment (ex. plant tissue or insect digestive track) (Klemm & Schembri, 2000). Little is known about adhesins in *P. stewartii* but these colonization factors aid the bacteria in colonization of the plant tissue to establish mature biofilms.

A recently suggested colonization factor in numerous members of the *Enterobacteriaceae* family is an alpha-2-macroglobulin protein (α_2M) (Budd *et al,* 2004). In invertebrates and vertebrates, α_2M is a protease inhibitor and a part of the innate

immune system that catches and degrade pathogens. However, through horizontal gene transfer, Gram-negative pathogens have obtained α_2M. In the genome of these Gram-negative pathogens, the *α_2M gene* is commonly found in close proximity to *pbp,* a penicillin-binding protein (PBP). The two genes typically co-occur and are often adjacent to one another in the same operon as predicted by Budd *et al*., (2004). PBP is a cell wall repair protein found in Gram-negative bacteria that functions to repair the peptidoglycans of the cell wall by catalyzing transglycosylation and transpeptidation processes (Tipper & Strominger, 1965; Sauvage *et al.,* 2008).

The co-transcription of *α_2M* and *pbp* suggests they work together (Budd *et al*., 2004). The α_2M protein is hypothesized to protect the bacteria from non-self proteases and the PBP repairs the damaged peptidoglycan that results from perturbations in the cell envelope (Neves *et al.,* 2012). These two proteins working together could potentially aid in colonization of the pathogen in the host. Genome-wide analysis has determined *P. stewartii* has two putative *α_2M* genes in close proximity to a putative *pbpC*. The function of α_2M and PBP has yet been determined in Gram-negative bacteria and further investigation is required.

This study aims to characterize the factors that contribute to Stewart's wilt disease. This disease remains a highly infectious agent of corn, an economically important crop. Investigating and characterizing the function of potential *P. stewartii* virulence factors, including RTX and α_2M, provides a greater understanding of the molecular mechanisms that lead to the disease. In this thesis, I proposed RTX and α_2M contribute to disease progression by independent methods. By knowing how the pathogen

causes disease in the host, we can identify methods to counteract the bacteria infection and improve crop quality.

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Chapter 1: Characterization of an RTX-like Toxin in Virulence of *Pantoea stewartii* subsp. *stewartii*

ABSTRACT

Pantoea stewartii subsp. stewartii DC283, the causal agent of Stewart's wilt, is an important bacterial pathogen of sweet corn. *P. stewartii* colonizes the apoplastic space and xylem tissue, resulting in characteristic water-soaked (WS) lesions and wilting. A gene encoding a putative RTX-like toxin, *rtx2*, has been identified in *P. stewartii*. RTX toxins belong to the pore-forming toxin family and have lytic properties in animal systems. Little is known about the role of RTX toxins in plant pathogenesis. Interestingly, a *P. stewartii Δrtx2* mutant was noticeably unable to incite WS lesions i*n planta* implicating it in the cell lysis associated with lesion formation. In addition, *rtx2* is genetically linked to components of the Rcs phosphorelay; a global regulatory system of critical virulence factors in *P. stewartii*. This suggests a possible functional relationship, particularly because they are transcribed from a single promoter. The RTX2 domain structure is consistent with an autotransported protein and contains conserved transmembrane domains suggesting it translocates itself to the cell surface. The signal relay pathway that stimulates the Rcs phosphorelay is unknown, but membrane perturbation plays a key role in stimulating the Rcs system. We hypothesize that RTX2 contributes to the membrane dynamics that are being sensed by the Rcs system, thus modulating activation of signal transduction through the system.

INTRODUCTION

Pantoea stewartii subsp. stewartii DC283 (syn. *Erwinia stewartii*) is a serious plant pathogen of sweet corn and the causal agent of Stewart's wilt disease (Bradbury, 1967; Wang *et al*., 2012). This Gram-negative bacterium is transmitted by *Chaetocnema pulicaria* Melsheimer, commonly known as the corn flea beetle (Braun, 1982). *P. stewartii* causes disease in sweet corn by colonizing the apoplast and xylem tissues. Successful colonization and infection leads to the development of disease symptoms, including water-soaked (WS) lesions and wilting (Pataky & Ikin, 2003; Ham *et al.,* 2006; Roper, 2011). Thus far, there are several types of virulence factors identified in the *P. stewartii* system that include (1) a type III secretion system (T3SS), (2) stewartan exopolysaccharide (EPS), (3) an endoglucanase enzyme, and (4) a siderophore-dependent iron uptake system (von Bodman & Farrand, 1995; Coplin *et al.,* 1992; Dolph *et al.*, 1988; Roper, 2011; Merighi, *et al.,* 2003; Carlier *et al*., 2009; Majdalani *et al.*, 2005; Bernhard *et al*., 1996; Mohammadi *et al*., 2011; Burbank *et al.,* 2014). Each of these virulence factors contribute to different phases of bacterial invasion. Identifying colonization or virulence factors within *P. stewartii* is vital to understanding how this pathogen promotes disease in sweet corn and in developing a means to combat Stewart's wilt.

A genome wide analysis of *P. stewartii* identified two orthologs of a repeats-in toxin (RTX) protein. These proteins are cytotoxins that have been studied in other pathogenic systems including *Escherichia coli*, *Vibrio cholera*, and *Bordatella pertussis* (Short & Kurtz, 1971; Hanski & Farfel, 1985: Welch, 2001; Linhartova *et al.,* 2010).

Two genes encoding putative RTX-like toxins are found in *P. stewartii* and designated *rtx1* and *rtx2* (Roper & von Bodman, in preparation) (Accession # ACV-0285926 and accession #ACV-0285925, respectfully).

In general, RTX proteins are exported to the extracellular environment by way of the bacterial type I secretion system (TISS) (Linhartova *et al.,* 2010). The toxin is extracellularly activated once calcium ions bind to the aspartate and glycine (D-G) rich repeats on the C-terminus (Linhartova *et al.,* 2010). Binding of calcium ions occurs when RTX has been secreted because the concentration of calcium in the environment outside of the bacterial cell is greater than the calcium concentration within the intracellular cytoplasm (Gangola & Rosen, 1987; Linhartova *et al.,* 2010). RTXs toxins were originally classified as host specific and cell-type specific toxins (ex. leukotoxins and hemolysins), but as the members of the RTX family of proteins expanded, RTXs proteins are more generally classified as cytotoxins (Welch, 1991; Coote, 1992; Linhartova *et al.,* 2010). RTX proteins also act as virulence factors and that cause tissue damage (Linhartova *et al.,* 2010).

Most interestingly, little is known about RTX proteins in plant pathogenic systems. In a previous study (Roper & von Bodman, in preparation), it was hypothesized that RTX1 and/or RTX2 were involved in the initial WS lesion development of Stewart's wilt disease. They hypothesized that these RTX toxins create pores in the plant cell membranes to promote the release of nutrients from the plant cells for *P. stewartii* to utilize as a nutrient source. Roper and von Bodman generated and evaluated *rtx1* and *rtx2* mutant *P. stewartii* strains for virulence. The study determined that RTX2 contributes to

the development of WS lesions, a characteristic of Stewart's wilting disease (Roper & von Bodman, in preparation).

My thesis focuses on expanding the characterization of RTX2 as a virulence factor. The predicted amino acid structure of RTX2 was analyzed using ProDom and InterPro Scan software by Roper & von Bodman (in preparation). They found that RTX2 has domains similar to those found in a hemolysin-like protein belonging to *Desulfovibrio vulgaris* (Heidelberg *et al.,* 2004). These domains include putative calcium binding domains that aid in pore formation and are required for biological function in other RTX toxins (Frey, 2006; Gentschev, 2003). Interestingly, RTX2 has several putative autotransport domains, suggesting that RTX2 is autotransported across the cell wall rather than being transported via the TISS as other canonical RTX toxins. The presence of five transmembrane domains at the C-terminus of RTX2 suggests that RTX2 is membrane bound after secretion.

Interestingly, both *rtx*1 and *rtx2* are co-transcribed with *rcsD* and *rcsB*, which encode a sensor kinase and a response regulator, respectively. These proteins are central components of the Rcs environmental sensing phosphorelay system found in the *Enterobacteriaceae* (Majdalani & Gottesman 2005; Schmoe *et al.,* 2011; Clarke, 2010; Roper, 2011). The Rcs phosphorelay system has been found to contribute to virulence, motility, and biofilm formation (Majdalani & Gottesman 2005; Schmoe *et al.,* 2011) in *E. coli*. In *E. coli,* this system has two sensor kinases (RcsC and RcsD), two co-activators of transcription (RcsA and RcsB), and a lipoprotein that is localized on the outer membrane (RcsF) (Majdalani & Gottesman 2005; Schmoe *et al.,* 2011; Roper, 2011). It is

hypothesized that the *P. stewartii* Rcs phosphorelay system behaves in the same manner as described in *E. coli*. The Rcs signal cascade in *E. coli* is activated by an unknown environmental signal(s). RcsF is required for perception of the signal and initiates the signal cascade. RcsC auto-phosphorylates and then phosphorylates RcsD. RcsD phosphorylates RcsB which binds to RcsA to create the transcriptional activation complex. The RcsA-B complex then activates expression of several loci, including the *wce* gene cluster. This gene cluster encodes the proteins necessary for EPS synthesis, a known virulence factor within *P. stewartii* (Majdalani & Gottesman 2005; Wang *et al.,* 2012; Carlier *et al*., 2009).

The co-transcription of *rtx2* and the Rcs phosphorelay system suggest a close relationship between the RTX proteins and the Rcs system. As demonstrated by Roper and von Bodman (in preparation), the *P. stewartii* Δ*rtx2* produced reduced EPS compared to the wild type bacterium and the complemented strain *Δrtx2*/*rtx2*+, suggesting an intimate link between RTX2 and stimulation of the Rcs phosphorelay. Interestingly, the *rtx2* mutant was also decreased in virulence *in planta*. Seedlings that were inoculated with Δ*rtx2* did not develop WS lesions, demonstrating that RTX2 is required for inciting WS formation (Roper & von Bodman, in preparation).

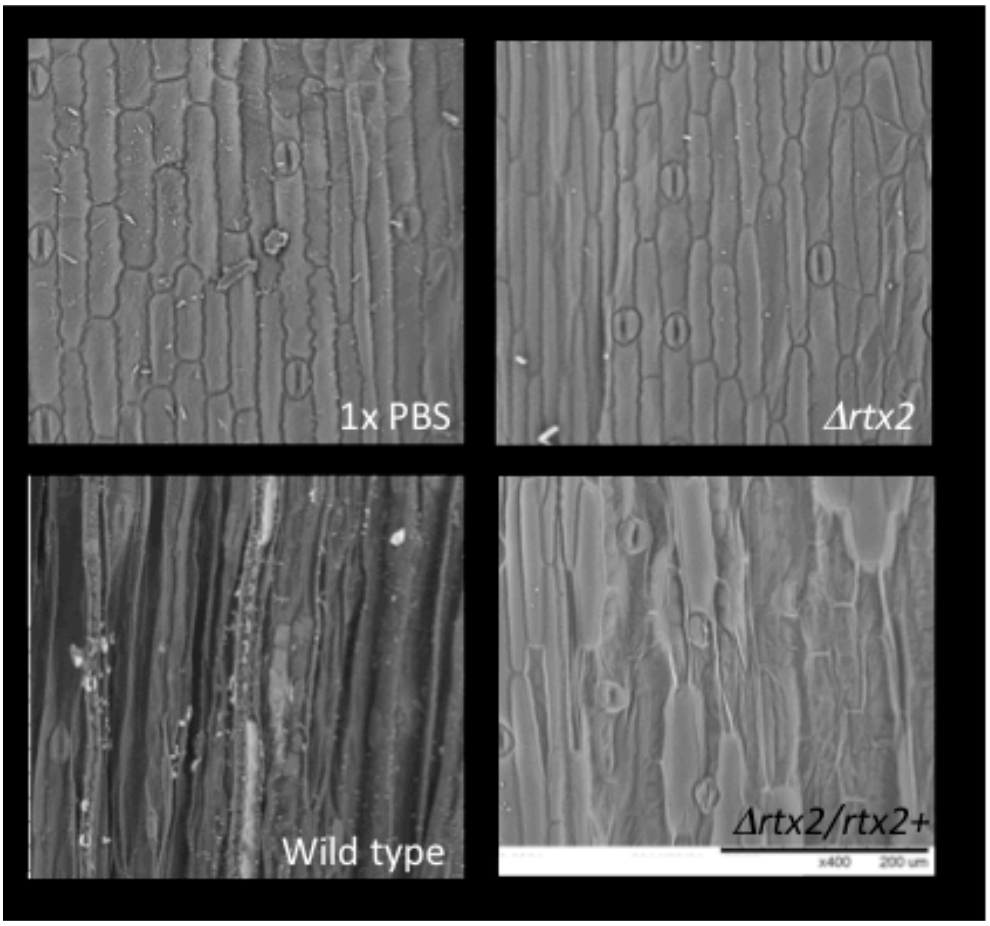
The goal of my thesis was to build on these findings and demonstrate that RTX2 mediates leakage of plant cells, further implicating it in the cell lysis associated with WS development. I also attempted to demonstrate that RTX2 localizes to the bacterial outer membrane.

RESULTS

The *Δrtx2* mutant does not incite plant cellular lysis. We hypothesize that the RTX2 protein is able to actively lyse plant cellular tissues to release nutrients into the apoplastic space for the bacterium to utilize. Determination of plant cellular lysis was determined by scanning electron microscopy (SEM) and trypan blue staining. Cells of sweet corn plants infected with wild type collapsed when compared to the 1x PBS control as seen by SEM (Figure 1. 1A). We reason that this collapse is due to lysis of plant cells. Leaf cells in plants inoculated with *Δrtx2* are turgid, maintain their original rectangular shape, and are indistinguishable from cells inoculated with 1x PBS. Cellular collapse was restored when *rtx2* was supplied *in trans* on plasmid pBBR1::*rtx2* (Figure 1.1B).

To confirm the SEM observations, trypan blue staining was used to visualize the cellular lysis (Heese *et al*, 2007). Trypan blue is a vital stain and an intact cellular membrane prevents entry of the stain into a healthy cell. Plant cells that have their membranes compromised absorb the stain, resulting in a prominent blue color. Leaf samples were imaged with a digital camera (Figure 1. 2A) and by light microscopy (Figure 1. 2B). The areas of water-soaked lesions in seedlings inoculated with wild type or *Δrtx2/rtx2*+ stained blue, indicating a compromised plant cell membrane. However, seedlings inoculated with *Δrtx2* or the 1x PBS-T negative control did not stain blue, indicating that these cells were not compromised. Taken together, these observations suggest that the *Δrtx2* mutant does not lyse the plant cells as compared to the wild type.

A. Top-down SEM images



B. Cross-section SEM images

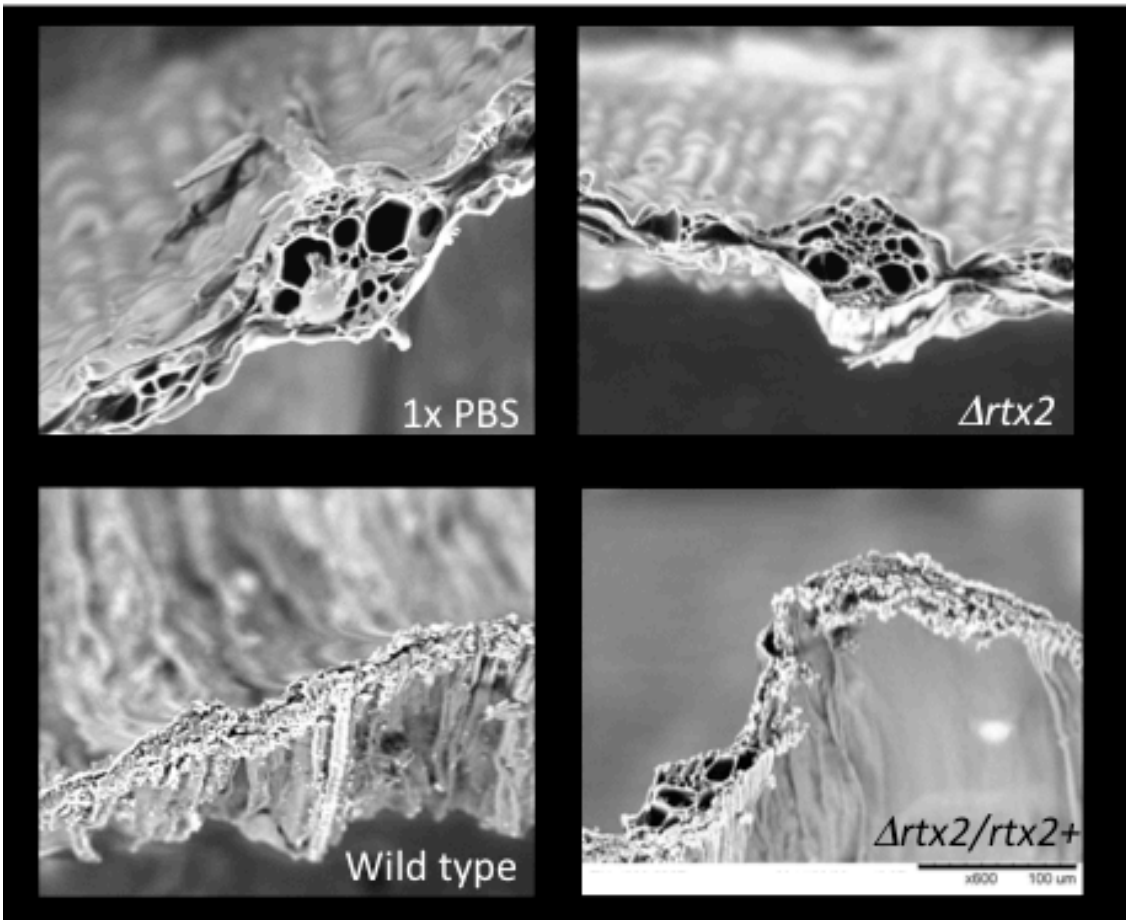
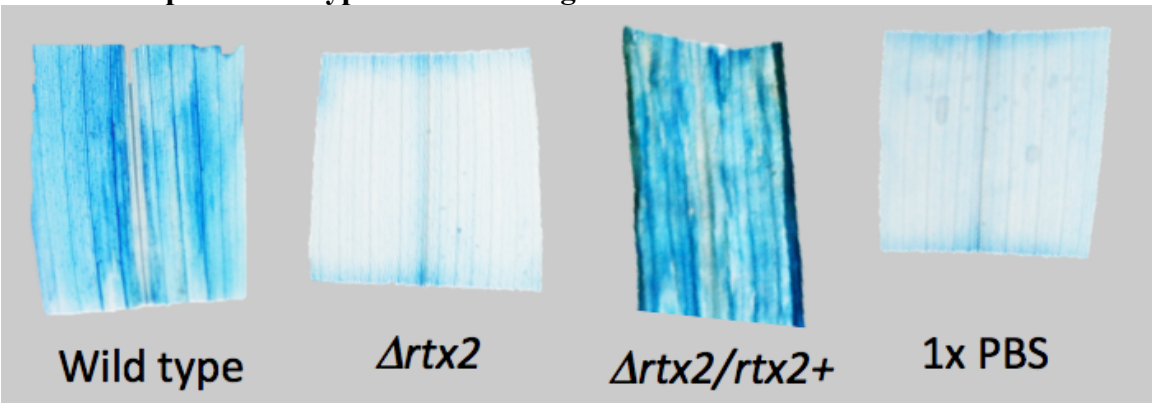


Figure 1. 1. Plant cellular collapse in water-soaked lesions is mediated by RTX2. Seedlings (var. Jubilee) were inoculated with wild type *P. stewartii* (DC283), *Δrtx2*, *Δrtx2/rtx2+*, or 1x PBS following the whorl inoculation method by (Ham *et al.,* 2006). The oldest leaf from each plant was excised and prepared for imaging 4 days post-inoculation. Images of water-soaked areas were taken using a Hitachi TM-1000 Tabletop SEM. (A) Top-down images of the plant leaf epidermis after infection with *P. stewartii* strains. (B) Cross-sections of the plant leaves infected with *P. stewartii* strains.

A. Leaf samples after trypan blue staining



B. 20x magnification of trypan blue stained, whorl-inoculated seedlings

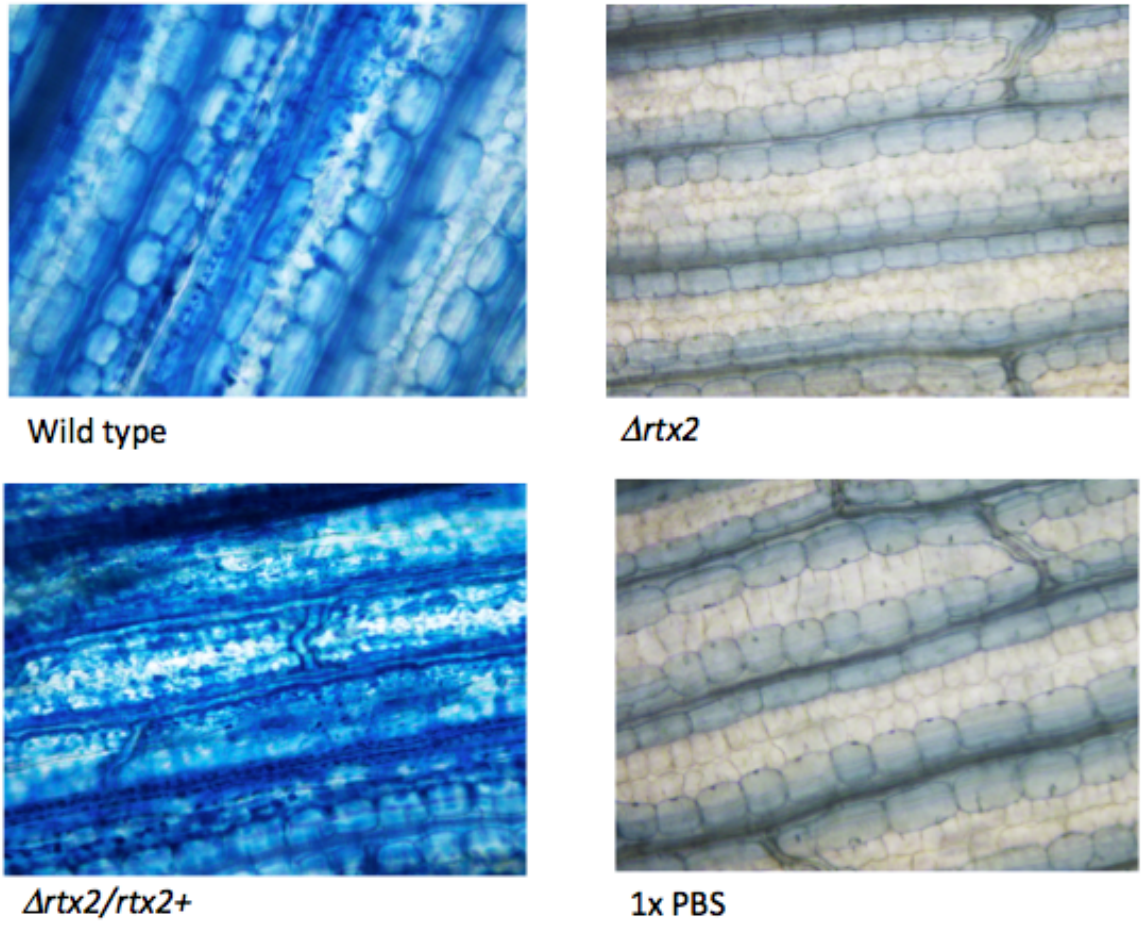


Figure 1. 2. Trypan blue staining of whorl-inoculated seedlings.
Seedlings (var. Jubilee) were inoculated with wild type *P. stewartii* (DC283), *Δrtx2*, *Δrtx2/rtx2+*, or 1x PBS following the whorl inoculation method by (Ham *et al.,* 2006) and were imaged 4 days post-inoculation. Leaf samples were stained for 10 min. following the trypan blue staining procedure from (Heese *et al*, 2007). Images of trypan blue stained areas were taken using (1) a digital camera and (2) a light-microscope DM4000, Leica Microsystems CMS (GmbH, Wetzlar, Germany). In the wild type and *Δrtx2/rtx2+*, the majority of the leaf tissue (Figure 2A) and the specific plant cells imaged at 20x magnification (Figure 2B) retained the blue stain, suggesting a compromised plant cell membrane. Leaf cells in plants inoculated with *Δrtx2* are indistinguishable from the 1x PBS control.

The apoplastic fluid of symptomatic leaves is enriched proteins, carbohydrates and dissolved solutes. Seedlings inoculated with wild type or the *Δrtx2/rtx2+* complemented strain had an increase in total protein concentration (600 µg/mL and 617 µg/mL, respectfully) compared to seedlings inoculated with *Δrtx2* or the 1x PBS-T negative control (350 µg/mL and 170 µg/mL, respectfully). The total protein concentration was increased in seedlings inoculated with wild type or *Δrtx2/rtx2+* by a 1.7-fold increase compared to seedlings inoculated with *Δrtx2.* The total protein concentration was measured by a Bradford assay of the extracted apoplastic fluid (Figure 1. 3).

Seedlings inoculated with wild type or the *Δrtx2/rtx2+* complemented strain had increased dissolved solutes (Ψ_w) (-2.25 and -1.75, respectfully) in the extracted apoplastic fluid as compared to the seedlings inoculated with *Δrtx2* or the 1x PBS-T negative control (-0.40 and -0.50, respectfully). The solute concentration was increased in seedlings inoculated with wild type or *Δrtx2/rtx2+* by a 5.6 and 4.4-fold increase (respectfully) compared to seedlings inoculated with *Δrtx2* (Figure 1. 4). The differences in dissolved solutes (Ψ_w) were statistically significant based on a Student's *t*-test using $p \leq 0.05$. We reason that this increase in dissolved solutes indicates plant cellular leakage.

Total carbohydrates present within the apoplastic fluid were measured two ways. First, the carbohydrates present were measured through a phenol-sulfuric acid assay (Albalasmeh *et al.,* 2013). The apoplastic fluid extracted from seedlings inoculated with wild type (525 µg/mL) or the *Δrtx2/rtx2+* complemented strain, (275 µg/mL) had

increased total carbohydrates compared to the seedlings inoculated with *Δrtx2* (25 μg/mL) or the 1x PBS-T negative control (25 μg/mL) (Figure 1. 5A). Carbohydrate content was also quantified using an anthrone-sulfuric acid assay that measures neutral sugars (Leyva *et al.,* 2008). The overall concentration of carbohydrates present in the apoplastic fluid using the anthrone-sulfuric acid assay followed the same trends observed for the phenol-sulfuric acid assay. The apoplastic fluid extracted from seedlings inoculated with wild type (5, 010 μg/mL) or *Δrtx2/rtx2+*(3, 000 μg/mL) had increased total carbohydrates compared to the seedlings inoculated with *Δrtx2* (250 μg/mL) or the 1x PBS-T negative control (800 μg/mL) (Figure 1. 5B). The total carbohydrate concentration, regardless of the form of measurement, was increased in seedlings inoculated with wild type or *Δrtx2/rtx2+* by 20-fold when measured by the phenol-sulfuric acid assay and 11-fold when measured by the anthrone-sulfuric acid assay compared to seedlings inoculated with *Δrtx2* (Figure 1. 5). The carbohydrate concentrations were statistically significant for the phenol-sulfuric acid assay based on a Mann-Whitney test ($p \leq 0.05$). The carbohydrate concentrations were also statistically significant for the anthrone-sulfuric acid assay based on a Student's *t*-test using $p \leq 0.05$. The phenol-sulfuric acid assay data was not evenly distributed. The appropriate statistical analysis for this data was the Mann-Whitney test. However, the dataset for the anthrone-sulfuric acid assay was evenly distributed and Student's *t*-test was used for data analysis.

Collectively, the results from the extracted apoplastic fluid support our initial hypothesis RTX2 mediates plant cell lysis that releases nutrients into the leaf apoplastic space where *P. stewartii* resides.

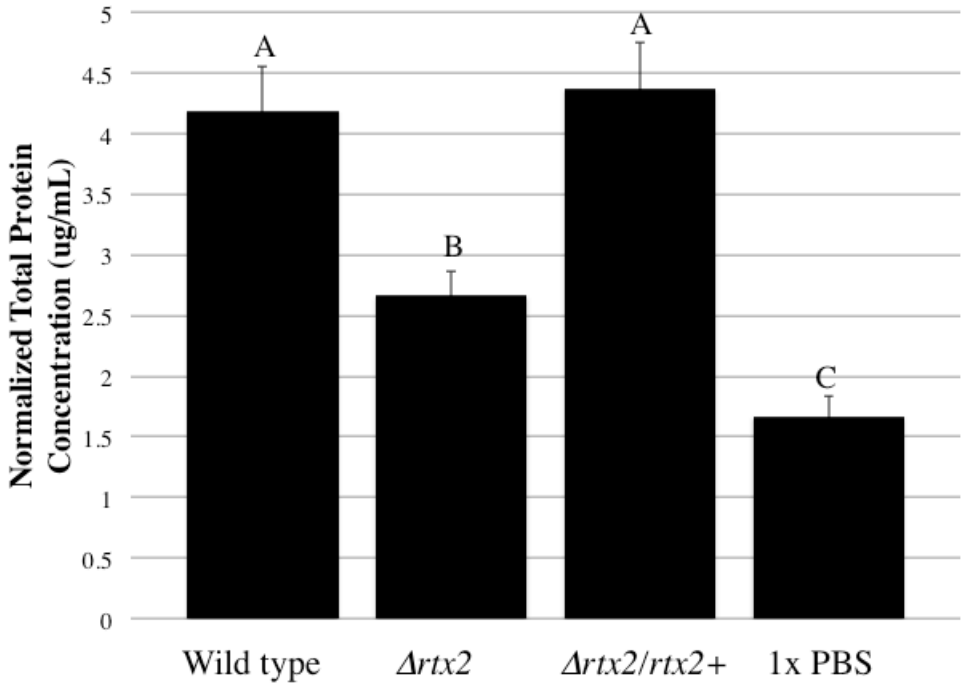


Figure 1. 3. Total protein content in apoplastic fluid extracted from whorl-inoculated sweet corn seedlings.
The Bradford assay (Bio-Rad, Hercules, CA) was used to determine total protein concentration in the extracted apoplastic fluid from seedlings (var. Jubilee) inoculated with wild type *P. stewartii* (DC283), *Δrtx2*, *Δrtx2/rtx2+*, or 1x PBS following the whorl inoculation method by (Ham *et al.,* 2006). Seedlings inoculated with wild type or *Δrtx2/rtx2+* had elevated levels of normalized total protein concentration (4.18 and 4.367, respectfully) compared to *Δrtx2* or 1x PBS (2.66 and 1.66, respectfully). Samples were normalized to an A-B scale (y=1+(x-A)(b-a)/(B-A), where A is the minimum value of the samples, B is the maximum of the samples, a is 1, b is 10, and x is the data point). Error bars represent the standard error from the mean. . Letters (A, B, and C) indicate significant differences in the total protein concentration between *P. stewartii* strains. The differences were statistically significant based on a Mann-Whitney test at the 0.05 level.

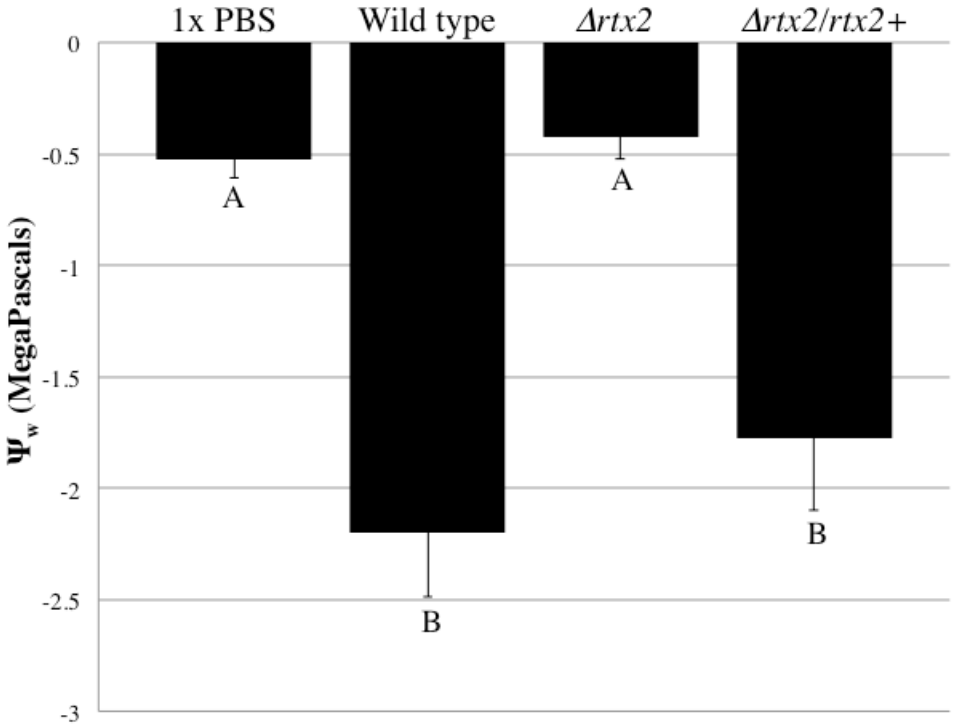
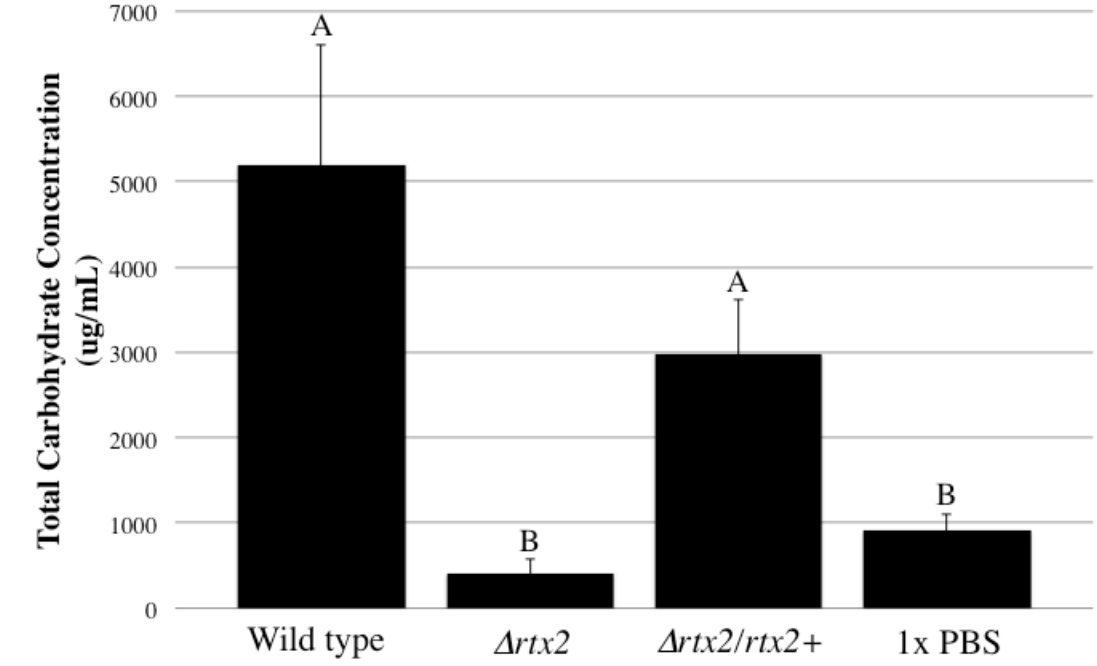


Figure 1. 4. Water potential (Ψ_w) of apoplastic fluid extracted from whorl-inoculated sweet corn seedlings.
Water potential was used to determine the solute concentration in the extracted apoplastic fluid from seedlings (var. Jubilee) inoculated with wild type *P. stewartii* (DC283), *Δrtx2*, *Δrtx2/rtx2+*, or 1x PBS following the whorl inoculation method by (Ham *et al.,* 2006). Seedlings inoculated with wild type or *Δrtx2/rtx2+* (-2.25 and -1.75, respectfully) had elevated levels of dissolved solutes compared to *Δrtx2* or the 1x PBS-T (-0.40 and -0.50, respectfully). The dissolved solute concentration was increased in seedlings inoculated with wild type by a 5.6 fold increase compared to seedlings inoculated with *Δrtx2*. Error bars represent the standard error from the mean. Letters (A and B) indicate significant differences in the water potential (Ψ_w) between *P. stewartii* strains. The differences were statistically significant based on a Student's *t*-test using $p \leq 0.05$.

A. Carbohydrate content in apoplastic fluid extracted from whorl-inoculated sweet corn seedlings as measured by the phenol-sulfuric acid assay.



B. Carbohydrate content in apoplastic fluid extracted from whorl-inoculated sweet corn seedlings as measured by the anthrone-sulfuric acid assay to measure.

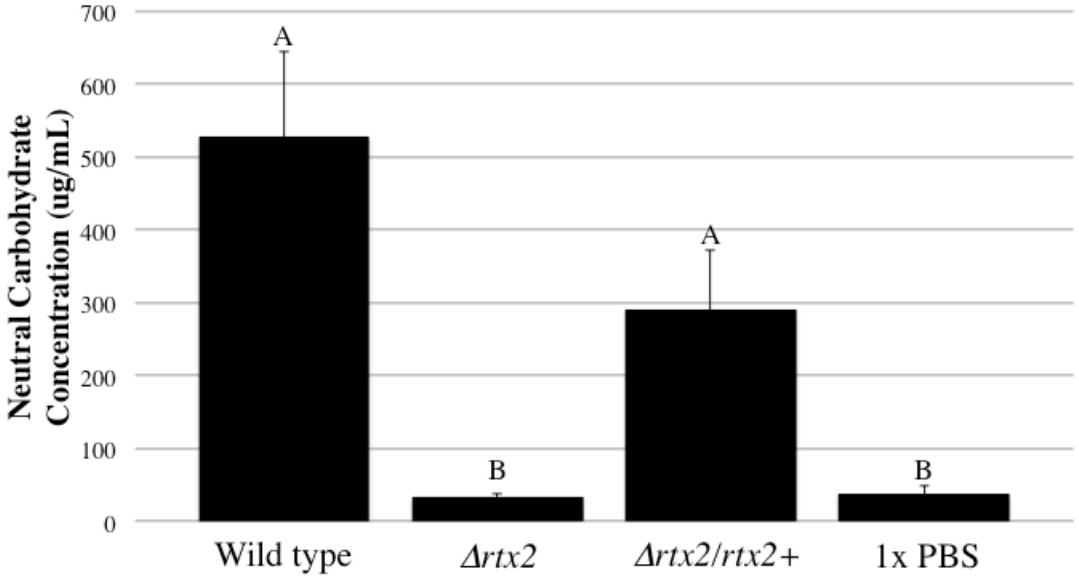


Figure 1. 5. Carbohydrate content in extracted apoplastic fluid from whorl-inoculated sweet corn seedlings.

Both the phenol-sulfuric acid (A) and anthrone-sulfuric acid (B) assays were used to determine total carbohydrate concentration in the extracted apoplastic fluid from seedlings (var. Jubilee) inoculated with wild type *P. stewartii* (DC283), *Δrtx2*, *Δrtx2/rtx2+*, or 1x PBS following the whorl inoculation method by (Ham *et al.,* 2006). Seedlings inoculated with wild type (525 µg/mL) or *Δrtx2/rtx2+* (275 µg/mL) had elevated levels of carbohydrate content compared to *Δrtx2* (25 µg/mL) or the 1x PBS-T negative control (25 µg/mL) as quantified by the phenol-sulfuric acid assay (5A). The apoplastic fluid extracted from seedlings inoculated with wild type (5, 010 µg/mL) or *Δrtx2/rtx2+*(3, 000 µg/mL) had increased carbohydrates compared to the seedlings inoculated with *Δrtx2* (250 µg/mL) or the 1x PBS-T negative control (800 µg/mL) (Figure 5B). Letters (A and B) indicate significant differences in the carbohydrate content found in the leaf apoplastic fluid isolated from plants inoculated with either WT, *Δrtx2, Δrtx2/rtx2+* or 1X PBS-T. Error bars represent the standard error from the mean. The differences were statistically significant based on a Mann-Whitney test at the 0.05 level and Student's *t*-test using $p \leq 0.05$, respectively.

DISSCUSSION

The goal of this study was to characterize the role of the RTX2 protein in the *P. stewartii* pathosystem. It is hypothesized *rtx2* encodes a cytolytic protein (Roper and von Bodman, in preparation). We hypothesized that the bacteria lyse host cells to release nutrients for the bacteria to utilize in the nutrient-poor environment of the xylem and that this leakage of cellular content from the lysed host cells results in the disease phenotype of water-soaked (WS) lesions (Roper and von Bodman, in preparation). When imaged by SEM, the WS lesions in plants inoculated with WT contain cells that are collapsed likely due to cell lysis, (Figures 1. 1A and 1. 1B). Leaf cells in plants inoculated with *Δrtx2* are turgid, maintain their original rectangular morphology, and are indistinguishable from cells inoculated with 1x PBS control. Additionally, corn leaves harvested from seedlings infected with wild type and *Δrtx2/rtx2+* prominently stained with trypan blue, indicating that the plant cell membranes are structurally compromised in the WS lesions. We hypothesize that this observed collapse and cell death is due to lysis of plant cells caused by the lytic activity of RTX2 (Figures 1. 2A and 1. 2B). In support of this hypothesis, the leaf apoplastic fluid of corn seedlings infected with wild type and *Δrtx2/rtx2+ P. stewartii* have an increase in protein, dissolved solutes, and carbohydrates (Figures 1. 3, 1. 4, 1. 5A, and 1. 5B, respectfully). We reason that this occurs due to leakage of plant cell contents due to the lytic action of *P. stewartii*.

Future studies to characterize the RTX2 protein include the recombinant expression and purification of the RTX2 protein. The recombinant protein could then be applied to maize protoplasts, which could be visualized for lysis by microscopy. In

addition, it would be valuable to incorporate *rtx2* into a constitutively expressed plasmid and transform the construct into a non-WS lesion forming bacterium, such as *E. coli*. This bacterium could then be inoculated into sweet corn and evaluated for the ability to incite WS lesions to determine if RTX2 is specifically responsible for lesion formation.

It is still unknown where RTX2 localizes within the bacterial cell. Structural comparisons of the protein domains suggest that it is localized to the cell envelope. I made attempts to observe the localization of RTX2 within the bacterial cell but was unsuccessful. In the future, localization of RTX2 will be further explored.

MATERIALS AND METHODS

Strains and Growth Conditions. The *Pantoea stewartii* subsp. stewartii (DC283) and *Escherichia coli* strains were grown on solid Nutrient Agar (NA) (Difco Laboratories, Detroit, MI) or in liquid culture (Luria-Bertani (LB) medium (Difco, Laboratories, Detroit, MI) at 28ºC and 37ºC, respectfully. The antibiotics nalidixic acid, kanamycin (at a concentration of 30 μg/mL,) gentamycin (2.5 μg/mL), or chloramphenicol (35 μg/mL) were added to the medium when appropriate for the *P. stewartii* strains. Kanamycin (30 μg/mL), ampicillin, streptomycin (at a concentration of 100 μg/mL), or chloramphenicol (35 μg/mL) were added to the medium when appropriate for the *E. coli* strains. Clones were created utilizing *E. coli* DH10β and S17-1λ was the donor utilized for conjugal transfer to carry the RK-2 based plasmid constructs into *P. stewartii* subsp. stewartii (DC283). All bacterial strains and plasmids used in this study are listed in Table 1.

Standard DNA Manipulations. The Dneasy DNA extraction kit (Qiagen, Valencia, CA) was used to extract genomic DNA per manufacturer's instructions. The Zyppy Plasmid Miniprep kit (Zymo Research Corporation, Irvine, CA) was used to extract the plasmids per manufacturer's instructions. Herculase II Fusion DNA polymerase (Agilent Technologies, Santa Clara, CA) was used to amplify DNA fragments and synthetic oligonucleotide primers were ordered from Integrated DNA Technologies (IDT) (Coralville, IN). Restriction enzymes were purchased from New England Biolabs (NEB) (Ipswich, MA).

Mutant construction. The *P. stewartii Δrtx2* strain used in this study was created in the wild type (DC283) background through Gateway (GW) technology (Life Technologies, Inc., Carlsbad, CA). The GW system has been described by (Choi & Schweizer, 2005). The *Δrtx2* mutant strain was created previously by (Roper and von Bodman, in preparation). The *rtx2* mutant strain was complemented previously by (Roper and von Bodman, in preparation). All of the primer sets and sequences used in this study are listed in Table 2.

Scanning Electron Microscope (SEM) micrographs of water-soaked lesions. Whorl inoculations were performed on seven-day old sweet corn seedlings (var. Jubilee) grown in a growth chamber with 12-hour light/dark cycles and 80% humidity. Seedlings were inoculated with 100 μL of a bacterial suspension contained approximately $1x10^7$ cfu/mL in 1x PBS buffer containing 0.2% Tween20 (1x PBS-T). The bacterial suspension was pipetted into the whorl of the seedling. Four days post-inoculation, the oldest leaf was harvested and prepared to be imaged. Leaf samples for top-down imaging were placed on double-sided tape adhered to the pedestal of the SEM. Leaf samples for cross-section imaging were cut laterally and placed standing vertically on double-stick tape adhered to the pedestal of the SEM. Leaf samples were imaged using the Hitachi Tabletop scanning electron microscope (SEM). Leaf samples were either imaged (1) top-down or as (2) cross-sections.

Visualization of cellular lysis in water-soaked lesions. Whorl inoculations were performed on seven-day old sweet corn seedlings (var. Jubilee) were inoculated as described above. Four days post inoculation, the oldest leaf was harvested and prepared to be stained following the trypan blue method described below.

Following a trypan blue staining method previously described by (Heese *et al*, 2007), leaves were submerged in trypan blue staining solution (6 vol of ethanol, 1 vol of water, 1 vol of lactic acid, 1 vol of glycerol, 1 vol of phenol, 0.067% wt/vol trypan blue) in 15 mL falcon tubes and heated in a boiling water bath for 10 minutes. After 30 minutes of cooling at room temperature, the trypan blue staining solution was removed and the leaf samples were rinsed three times with sterile water. Leaves were destained in Chloral hydrate (2.5 g/ml) for 24 hours on a rotary shaker. For microscopy, the chloral hydrate was replaced with 60% glycerol to prevent the leaf samples from drying out during visualization. Leaves were imaged under 20x magnification, using DM4000, Leica Microsystems CMS (GmbH, Wetzlar, Germany) microscope equipped with LAS (Leica application suite) v.4.2, Leica Microsystems CMS software (GmbH, Wetzlar, Germany).

Quantification of total protein, solutes, and carbohydrates in sweet corn leaf apoplastic fluid. Whorl inoculations were performed on seven-day old sweet corn seedlings (var. Jubilee) were inoculated as described above. A single leaf was harvested from each inoculated plant four days post-inoculation. Leaf tissue was individually weighed, followed by surface sterilization using the following procedure; leaves were

soaked in 70% ethanol followed by 10% bleach and rinsed twice in sterile diH_2O (30 seconds per solution). The apoplastic fluid was then extracted from the leaf by centrifugation (modified protocol from Joosten, 2012). Briefly, the leaf was rolled gently and inserted into the top portion of a P1000 filter tip (USA Scientific) (the bottom portion had been removed) and placed in a microcentrifuge tube. Samples were centrifuged at 8, 000 rpm for 1-2 minutes to collect apoplastic fluid. The leaf was removed from the tube and the apoplastic fluid was filtered using Ultrafree- MC-VV centrifugal filters 0.1 μm (EMD Millipore, Darmstadt, Germany) to remove whole bacterial cells.

Total protein concentration, dissolved solutes, and neutral sugar carbohydrate content was quantified in the apoplastic fluid collected from healthy and infected leaves. The total protein concentration was determined by using the Quick start Bradford assay (Bio-Rad, Hercules, CA). Protein content was quantified in 20 μL of filtered apoplastic fluid using a BSA standard curve. This value was then converted to μg/ml of apoplastic fluid and normalized by an A-B scale. The A-B normalization equation used was (y=1+(x-A)(b-a)/(B-A), where A is the minimum value of the samples, B is the maximum of the samples, a is 1, b is 10, and x is the data point.

Water potential of the filtered apoplastic fluid was quantified using a Psypro-water potential data-logger fitted with the PCT-55/PST-55 soil hygrometer/psychrometer (Eli Tech Group WESCOR) probe. A small paper filter was placed in the collection chamber and 10 μL of the apoplstic fluid was applied to the paper filter. The probe was then placed in the chamber and water potential values were collected every hour for

twenty hours. The water potential values were calculated based on a sodium chloride standard curve.

Carbohydrate content was determined through two methods: (1) phenol-sulfuric acid assay (Albalasmeh *et al.,* 2013; Matsuko *et al.,* 2005) to measure total carbohydrates and (2) anthrone-sulfuric acid assay (Leyva *et al.,* 2008) to measure neutral sugars. Briefly, the phenol-sulfuric acid assay was completed using 1:100 diluted filtered apoplstic fluid. This solution was then added to 500 µL of 5% water-saturated phenol and 2.5 mL sulfuric acid. Samples were vortexed briefly and cooled at room temperature for 15 mins. Optical density (OD_{488} nm) was taken to measure the colorimetric change. This value was then used to calculate the total carbohydrate concentration was calculated using a glucose standard curve. The anthrone-sulfuric acid assay was performed using 1:100 diluted filtered apoplastic fluid. This solution was then added to 1 mL of chilled sulfuric acid and 2 mL of chilled anthrone solution. Samples were boiled for 15 mins and cooled at room temperature for 15 mins. Optical density (OD_{488} nm) was taken to measure the colorimetric change. This value was then used to calculate the total carbohydrate concentration was calculated from a glucose standard curve.

Cellular localization of RTX2. Attempts were made to determine the localization of RTX2 within the bacterial cell using two methods: (1) V5 tagging the RTX2 protein and (2) enhanced green fluorescent protein (EGFP) expressed on the C-terminus of the RTX2 protein. Briefly, the *rtx2* gene was PCR amplified from the TOPOXL-*rtx2* plasmid

(pMCR23) using attB1:rtx2b fwd/ attB2:rtx2b rev primers. Once the *rtx2* gene was confirmed by restriction enzyme digest with EcorI and conventional PCR using rtx2bfwd/rtx2brev primers. The *rtx2* gene was cloned into pDONR221 to create the entry vector pDONR221::*rtx2* using Gateway (GW) technology (Life Technologies, Inc., Carlsbad, CA) as described by (Choi & Schweizer, 2005), creating plasmid pKW013. The *rtx2* gene was subcloned into the GW compatible destination vector, pRH006 (EMBL accession #AJ606312, BCCM accession number LMBP 5417). The plasmid pRH006 (Hallez *et al.,* 2007) places a C-terminal EGFP tag and expression of the *rtx2-gfp* is driven by the *lacZ* promoter. Cloning of pDONR221::*rtx2 w*ith the destination vector, pRH006 was not successful. However, if it had been successful, the pRH006::*rtx2* plasmid would then be electroporated into the *P. stewartii Δrtx2* mutant. The bacterial cells would then be imaged using fluorescent microscopy with excitation 475-495 nm and emission 520-560 nm to visualize localization of the fluorescence. Bacterial cells would be induced by IPTG to increase the expression levels of the RTX2-EGFP protein under the control of the *lacZ* promoter.

I also attempted to create a RTX2-V5 fusion protein that could be used to immunologically determine the localization of RTX2 within the bacterial cell. I tried to subclone *rtx2* from the entry vector pDONR221::*rtx2* into the destination vector, pDEST42 (Life Technologies, Inc., Carlsbad, CA) which is GW compatible and places *rtx2* under the control of the bacteriophage T7 promoter. This cloning was not successful. Ideally, pDEST42::*rtx2* plasmid would be transformed into *E. coli* BL21 and gene expression would be induced with IPTG. The V5 tag would have been located using

the anti-V5 FITC fluorescent antibody (Novex by Life Technologies, Inc., Carlsbad, CA). The localization would have been observed under fluorescent microscopy using the FITC filter (excitation and emission spectrum peak wavelengths 495 nm and 519 nm, respectively).

Table 1. 1. Bacterial Strains and Plasmids

Strain or Plasmid	Relevant genotypes	Source or Reference
Pantoea stewartii* subsp. *stewartii		
DC283	Wild type, Nal^r	(Coplin et al., 1986)
Δrtx2 (CR64)	Nal^r, knockout in *rtx2*	Roper *et al.,* unpublished data
Δrtx2/rtx2+ (CR54)	Nal^r, Ap^r, *rtx2* complement	Roper *et al.,* unpublished data
Escherichia coli		
DH10B	F– endA1 *recA1 galE15 galK16* n*upG rpsL lacX74 80lac Z M15 araD139* (ara,leu)7697 *mcrA* (*mrr-hsdR MS-mcrBC*) –	Invitrogen
S17-1pir+	RP4, Mob+, Sm^r	(Simon et al., 1983)
EC100pir+	F *mcrA (mrr-hsdRMS-mcrBC), 80dlacZ, M15 lacX74, recA1, endA1araD139,(ara, leu)7697galU galK – rpsL nupG pir*+(DHFR)	Epicentre
Plasmids		
pUC18R6K-mini-Tn7-cat	Cm^r, Ap^r	(Choi et al., 2005)
pAUC40	Suicide vector, Gateway *attR,* Cm^r, Sm^r	(Carlier et al., 2009)
pFLP2	*sacB+,* FLP recombinase, Ap^r	(Kaniga et al., 1991)
pKD4	Km^r, source of kan cassette	(Datsenko & Wanner, 2000)
pSP856	Gm^r, source of gent cassette	(Choi et al., 2005)
pCR8/GW/TOPO	Cloning vector, Sp^r	Invitrogen
pDONR221	Cloning vector, Km^r, Ze^r	Invitrogen
pBBR1MCS-4	Broad host range vector, Ap^r	(Kovach et al., 1995)
pMCR29	r*tx2* cloned into pBBR1, Ap^r	Unpublished manuscript
pMCR31	*rtx2*::kan cloned into pDONR/Zeo, Ze^r	Unpublished manuscript
pMCR32	*rtx2*::kan cloned into pAUC40, Ap^r	Unpublished manuscript
pKW013	r*tx2* cloned into pDONR221, Km^r	This study

Nal^r, nalidixic acid; Ap^r, ampicillin; Km^r, kanamycin; Gm^r, gentamycin; Sm^r, streptomycin; Sp^r, spectinomycin; Cm^r chloramphenicol; Ze^r, zeocin resistance.

Table 1. 2. Primer Sequences

Primer	Sequence	Source or Reference
attB1:rtx2b fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGGGCGGC GATAACACGCTAAGCGTCAG	This study
attB2:rtx2b rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATACCTGATT TATCGCGCAACGTTGCGCGC	This study
rtx2bfwd	GTGGGCGGCGATAACACGCTAAGCGTCAG	This study
rtx2brev	ATACCTGATTTATCGCGCAACGTTGCGCGC	This study

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Chapter 2: Characterization of an Alpha-2-Macroglobulin in *Pantoea stewartii* subsp. *stewartii*.

ABSTRACT

Pantoea stewartii is a bacterial plant pathogen that causes Stewart's Wilt of sweet corn. *P. stewartii* is transmitted by the corn flea beetle, which creates wounds in the plant and allows the bacterium to enter the xylem and leaf apoplast resulting in characteristic water-soaked lesions and wilting. The *P. stewartii* genome has been fully sequenced and two putative alpha-2-macroglobulin (α_2M) genes have been identified. The α_2M protein is a known serine protease inhibitor in eukaryotic organisms that binds non-self proteases and degrades them. In prokaryotes, the α_2M gene can be found juxtaposed next to a gene that encodes a penicillin-binding protein (PBP) associated with cell wall repair. It has been suggested that the α_2M protein protects the bacteria from non-self proteases and the PBP repairs damaged peptidoglycan that may result from a damage cell envelope. Proposed functions of the α_2M protein include subverting host proteases. The α_2M protein may also function in defense against other bacterial proteases present in multicellular microbial communities. The goal of this study was to characterize the role of α_2MI during the host-pathogen interaction between *P. stewartii* and sweet corn.

INTRODUCTION

Pantoea stewartii subsp. stewartii DC283 (syn. *Erwinia stewartii*) is a serious pathogen of sweet corn and the causal agent of Stewart's wilt (Bradbury, 1967). This Gram-negative bacterium is transmitted by *Chaetocenema pulicaria* (Melsheimer), commonly known as the corn flea beetle (Braun, 1982). *P. stewartii* causes disease in sweet corn by colonizing the leaf apoplast and xylem tissues. Successful colonization and infection leads to the development of disease symptoms, including water-soaked (WS) lesions and wilting (Pataky & Ikin, 2003; Ham *et al.,* 2006; Roper, 2011). Thus far, several types of virulence factors have been identified in the *P. stewartii* system including (1) a type III secretion systems (T3SS), (2) stewartan exopolysaccharide (EPS), (3) an endoglucanase enzyme, and (4) a siderophore-dependent iron uptake pathway (von Bodman & Farrand, 1995; Coplin *et al.,* 1992; Dolph *et al.*, 1988; Roper, 2011; Merighi, *et al.,* 2003; Carlier *et al*., 2009; Majdalani *et al.*, 2005; Coplin *et al.,* 1996; Mohammadi *et al.*, 2012; Burbank *et al.,* 2014).

In eukaryotic systems, alpha-2-macroglobulin (α_2M) is part of the innate immune system and acts as a protease inhibitor that aids in defense against pathogens (Starkey & Barrett, 1973; Armstrong & Quigley, 1999). α_2M inhibits the activity of non-self proteases, although the discrimination of self versus non-self is not fully understood (Budd *et al*., 2004). α_2M contains a 'bait' that attracts proteases with high affinity. When a protease binds to the bait, proteolytic cleavage by the protease induces an irreversible conformational change of α_2M (Starkey & Barrett, 1973; Armstrong & Quigley, 1999) and a previously internalized thioester bond is exposed, which mediates attachment to

carbohydrates found on biological surfaces (Law & Dodds, 1997). The conformational change causes α_2M to sequester the protease within the α_2M protein complex, ultimately resulting in degradation of the protease (Budd *et al.,* 2004).

The most well-documented α_2M protein is found within the plasma of the horseshoe crab, *Limulus polyphemus,* and aids in blood clotting, as part of the innate immune system (Armstrong & Quigley, 1999; Budd *et al.,* 2004, Chaikeeratisak *et al.,* 2012). The *L. polyphemus* α_2M protein forms a tetrameric complex and functions to inhibit proteases of various parasites and pathogens from spreading systemically within the host (Armstrong & Quigley, 1999; Budd *et al.,* 2004). Many insects, such as mosquitos, flies, and hard ticks also possess the α_2M protein complex (Christophides *et al.,* 2002; Blandin & Levashina, 2004, Chaikeeratisak *et al.,* 2012). These insects have multiple α_2M homologs, termed thioester-containing proteins (TEPs) because of the thioester motif. Similar to the α_2M protein complexes in the horseshoe crab, TEPs are hypothesized to aid in the insect host defense through phagocytosis of the pathogenic proteases when under pathogenic attack (Christophides *et al.,* 2002; Blandin & Levashina, 2004, Chaikeeratisak *et al.,* 2012). The first TEP was identified in mosquitoes (TEP1) and was characterized to target and bind to pathogens such as bacteria and protozoan parasites. The pathogen is then either phagocytized or destroyed via degradation mediated by TEP1 (Levashina *et al.,* 2001; Blandin *et al.,* 2004; Blandin & Levashina, 2004; Budd *et al.,* 2004).

A study conducted by Chaikeeratisak *et* al (2012), investigated the α_2M protein found in the host *Pacifastacus monodon*, commonly known as the Asian tiger shrimp,

against proteases released from the bacterial pathogen *Vibrio harveyi. V. harveyi* is a Gram-negative, motile, bioluminescent, rod-shaped bacterium that causes disease within the shrimp host resulting in localized tissue death and ultimately systemic infection (Chaikeeratisak *et* al., 2012). Chaikeeratisak *et* al (2012) identified that the Asian tiger shrimp α_2M protein is a broad range protease inhibitor active against bacterial proteases, specifically elastase and aminopeptidases.

Recent advances in computational biology have made it possible to accurately predict putative gene function using comparative genomics. In a previous study, BLAST searches by Budd, *et al* (2004) revealed numerous bacterial organisms containing genes homologous to metazoan α_2M. It was previously thought that only invertebrates and vertebrates have α_2M-like protein complexes. However, a closer examination of bacterial genomes revealed putative α_2M genes. It is hypothesized that bacterial systems obtained α_2M through horizontal gene transfer from the hosts they invade, as observed in the human pathogens *Escherichia coli* and *Pseudomonas aeruginosa* (Budd *et al*., 2004, Robert-Gethon *et al.,* 2013). Both of these bacterial pathogens possess α_2M-like proteins, (ECAM and MagD, respectfully) which are capable of binding to human neutrophil elastase, a serine proteinase secreted by neutrophils and macrophages during inflammation to destroy pathogenic bacteria (Robert-Gethon *et al.,* 2013). It is hypothesized that once *P. aeruginosa* establishes a systemic infection, the initial "aggressive" virulence factor, the type three-secretion system, is no longer needed to protect the bacteria from initial host detection (Robert-Gethon *et al.,* 2013). However, in order to increase bacterial survivability, MagD would be necessary to act as a protease

inhibitor to protect the bacteria from the host neutrophil elastases (Robert-Gethon *et al.,* 2013). Other examples of bacterial protease inhibitors include ecotin and Ivy, which protect against neutrophils and host lysozymes, but it is likely that other protease inhibitors exists within bacterial systems that have not yet been identified (Robert-Gethon *et al.,* 2013).

In numerous bacteria including those in the *Enterobacteriaceae* family, α_2M and *pbp,* encoding a penicillin-binding protein (PBP), are commonly found to be in close proximity to one another within the genome. The two genes typically co-occur and are often adjacent to one another in the same operon as predicted by Budd *et al*., (2004). The PBP is a cell wall repair protein found in Gram-negative bacteria that functions to repair the peptidoglycans that comprises the cell wall by catalyzing transglycosylation and transpeptidation processes (Tipper & Strominger, 1965; Sauvage *et al.,* 2008).

This co-transcription of α_2M and *pbp* suggests they work together (Budd *et al*., 2004). The α_2M protein is hypothesized to protect the bacteria from non-self proteases and the PBP repairs the damaged peptidoglycan that results from perturbations in the cell envelope (Neves *et al.,* 2012).

There are several proposed functions for the α_2M protein complex in bacterial pathogens, including subverting host proteases, facilitating bacterial colonization and defense against host and other bacterial proteases present in multicellular microbial communities (Budd *et al.,* 2004; Doan *et al.,* 2008, Neves *et al.,* 2012). The *P. stewartii* genome has been fully sequenced and two putative α_2M genes as well as a putative *pbpC*-encoding gene have been identified. It is possible that *P. stewartii* obtained α_2M through

horizontal gene transfer from either the insect or the plant host as suggested by Budd *et al.,* (2004). Both hosts utilize serine protease inhibitors as part of the innate immune system, however it has yet to be determined if they possess an α_2M homolog.

A *P. stewartii* mutant defective in *α_2MI* was created and assessed for virulence, host colonization and attachment to surfaces. In addition, quantitative RT-PCR was performed to analyze gene expression for *α2MI, α2MII,* and *pbpC* to evaluate the impact to *α2MI, α2MII,* and *pbpC* transcription in several *P. stewartii* strains that are compromised in the production of the cell surface macromolecule, exopolysaccharide. The goal of this study was to characterize the role of α_2MI in host-pathogen interactions between *P. stewartii* and sweet corn and to better understand bacterial α_2M and its contribution to pathogenicity.

RESULTS

***Δα₂MI* mutant exhibits increased surface attachment.** A screen of *P. stewartii* mutants created through random insertion of the MARxT7 transposon in the *ΔwceO/ΔoxyR* genetic background was used to identify several mutants with differences in surface attachment (Burbank, Nanthavong, and Roper, unpublished data). The *ΔoxyR* mutant hyper-attaches, which suggests that OxyR could be a negative regulator of adhesion (Burbank and Roper, unpublished data). The goal of the original mutant bank screen performed by Maxine Nanthavong, (a student in the National Science Foundation-Research Experience for Undergraduates program), was to look for suppressors of the hyper-adhesion phenotype in the *ΔoxyR* mutant. EPS can interfere with the attachment of the cells to surfaces, making it difficult to measure differences in attachment, thus, the attachment assays were performed in an EPS minus strain, *ΔwceO* (Carlier *et al*, 2007). Random mutants were screened using a crystal violet (CV) staining method (O'Toole *et al.,* 1998; Koutsoudis *et al.,* 2006) to quantify attachment. A hyper-attaching mutant was recovered and the insertion of the MARxT7 transposon was mapped to *α₂MI* (ASAP ID # ACV-0288726, GenBank Accession # EHT99557), which encodes a putative alpha-2-macroglobulin I. This mutant had adhesion values higher than the parental strains phenotypes (*ΔwceO* and *ΔwceO/ΔoxyR* backgrounds, respectively) (Figure 2. 1). These differences were statistically significant, based on a Student's *t*-test using $p \leq 0.05$.

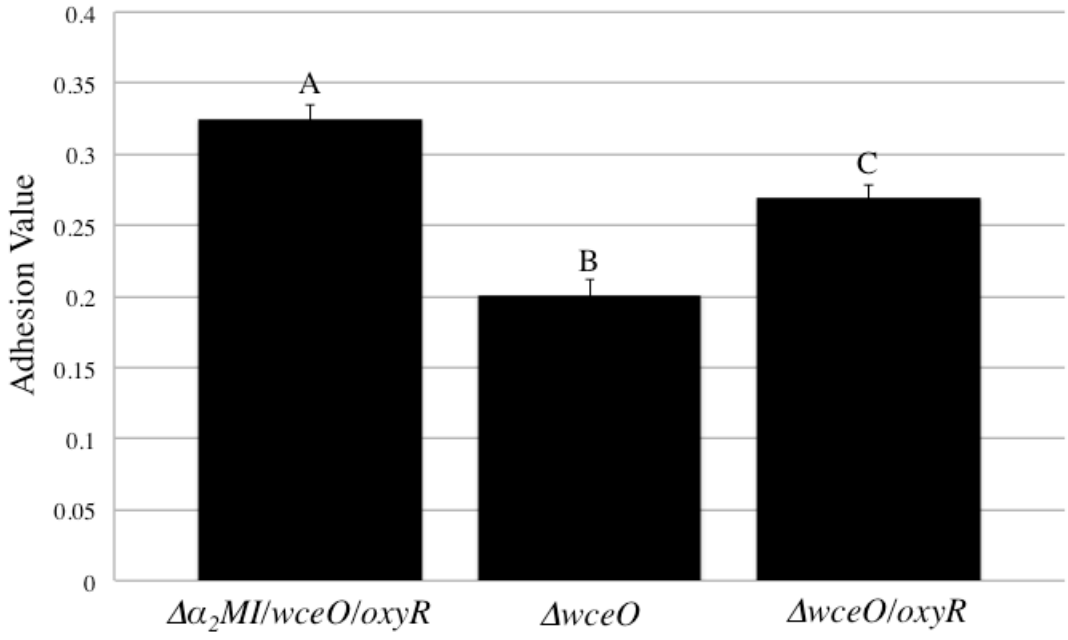


Figure 2. 1. *Δα$_2$MI/wceO/oxyR* exhibits increased surface attachment.
The insertion of the MARxT7 transposon in a hyper-attaching mutant was mapped to *α$_2$MI*. This mutant was screened for cell-surface attachment using a crystal violet (CV) assay (O'Toole *et al.,* 1998; Koutsoudis *et al.,* 2006). Optical density (OD_{600}) readings were normalized to initial cell growth. *Δα$_2$MI/wceO/oxyR* demonstrated increased adhesion compared to *ΔwceO* and *ΔwceO/oxyR*. The adhesion values were calculated as (adhesion value = (CV value-CV blank value/initial growth value-blank value)). Vertical bars represent the standard error of the mean. Letters (A, B, & C) indicate significant differences in the adhesion values. These differences were statistically significant based on a Student's *t*-test using $p \leq 0.05$.

To test the role of *α_2MI* in virulence, we constructed an *$\Delta\alpha_2$MI* mutant in the *P. stewartii* DC283 wild type background. This mutant was also tested for differences in attachment using the CV assay. The *$\Delta\alpha_2$MI* mutant also demonstrated hyper-attachment as compared to wild type *P. stewartii*, resulting in mean adhesion values higher than wild type DC283 (Figure 2. 2). These differences were statistically significant based on a Mann-Whitney test at the 0.05 level.

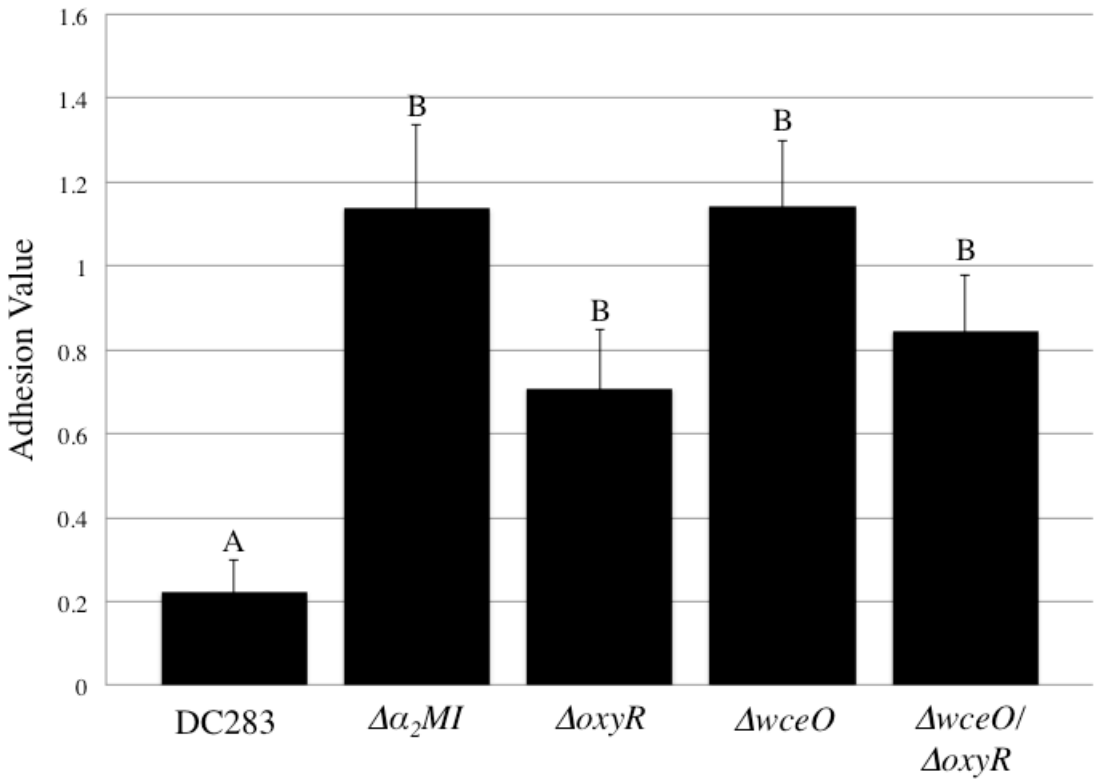


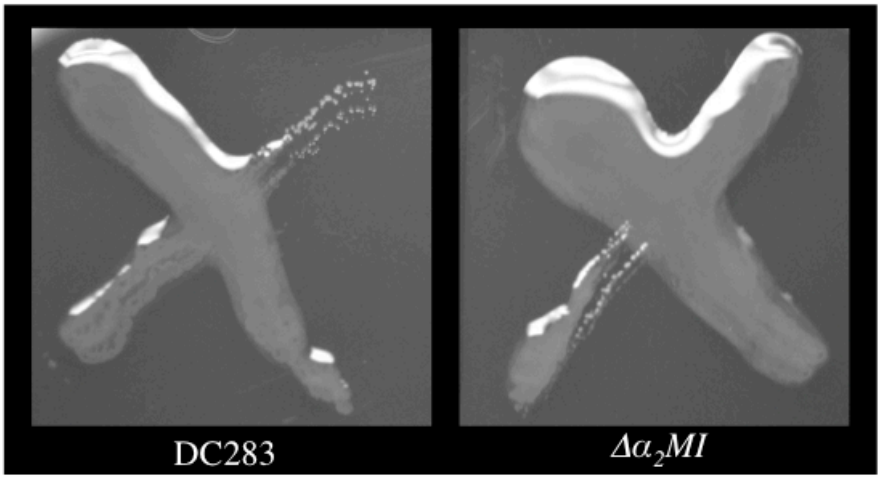
Figure 2. 2. Adhesion of *P. stewartii Δα₂MI* to polyvinyl chloride.
The *Δα₂MI* mutant strain was screened for cell-surface attachment using a crystal violet (CV) assay and compared to wild type and three mutant strains known to have increased attachment. The *Δα₂MI* mutant demonstrated a significant increase in attachment compared to the wild type. Vertical bars represent the standard error of the mean. Letters (A and B) indicate significant differences in the adhesion values. These differences were statistically significant based on a Mann-Whitney test at the 0.05 level between wild type and the mutant strains.

***P. stewartii Δα$_2$MI* produces wild type amounts of exopolysaccharide (EPS).**

Levels of stewartan exopolysaccharide (EPS) production affect surface adherence in *P. stewartii* and the ability to properly form biofilms within the plant host (Koutsoudis *et al.,* 2006). *P. stewartii* deletion mutants of *wceO* and *oxyR* produce a lower concentration of EPS (ug/mL carbohydrate) compared to wild type when evaluated with the phenol-sulfuric acid assay (Carlier *et al.,* 2007; Burbank and Roper, 2014). These mutants also demonstrate increased attachment when evaluated through a CV attachment assay (Carlier *et al.,* 2007; Burbank and Roper, 2014). When *α$_2$MI* was knocked out in a *Δwceo/ΔoxyR P. stewartii* strain during a mutant screen for increased attachment phenotypes, the triple mutant was identified to exhibit increased hyper-attachment (Burbank, Nanthavong, and Roper, unpublished data). To assess if *α$_2$MI* contributes to the hyper-attachment phenotype and EPS production, I generated a *Δα$_2$MI* mutant in a *P. stewartii* wild type background. The phenol-sulfuric acid assay was conducted to quantitate the concentration of EPS in the *Δα$_2$MI* mutant and wild type strain. Qualitatively, my results indicate there was no difference in growth or visible EPS produced (Figure 2. 3A) in *α$_2$MI* on NA plates containing 0.2% glucose. Strains were grown in liquid culture (AB minimal media or LB rich media with the addition of 0.4% glucose), and EPS was extracted from the culture supernatant and quantified using the phenol-sulfuric acid colorimetric assay described by Matsuko *et al.* (2005). Total EPS was normalized to starting cell density (OD_{600}). There was no significant difference in the amount of EPS produced by *Δα$_2$MI* in liquid culture (Figure 2. 3B) based on a Student's *t*-test using $p \leq 0.05$. This resulting information suggests that the α_2MI protein does not

affect stewartan EPS production. However, α_2MI may be contributing to bacterial adhesion by functioning as an adhesion protein or promoting factors that aid in attachment to surfaces.

A.


DC283
$\Delta\alpha_2MI$

B.

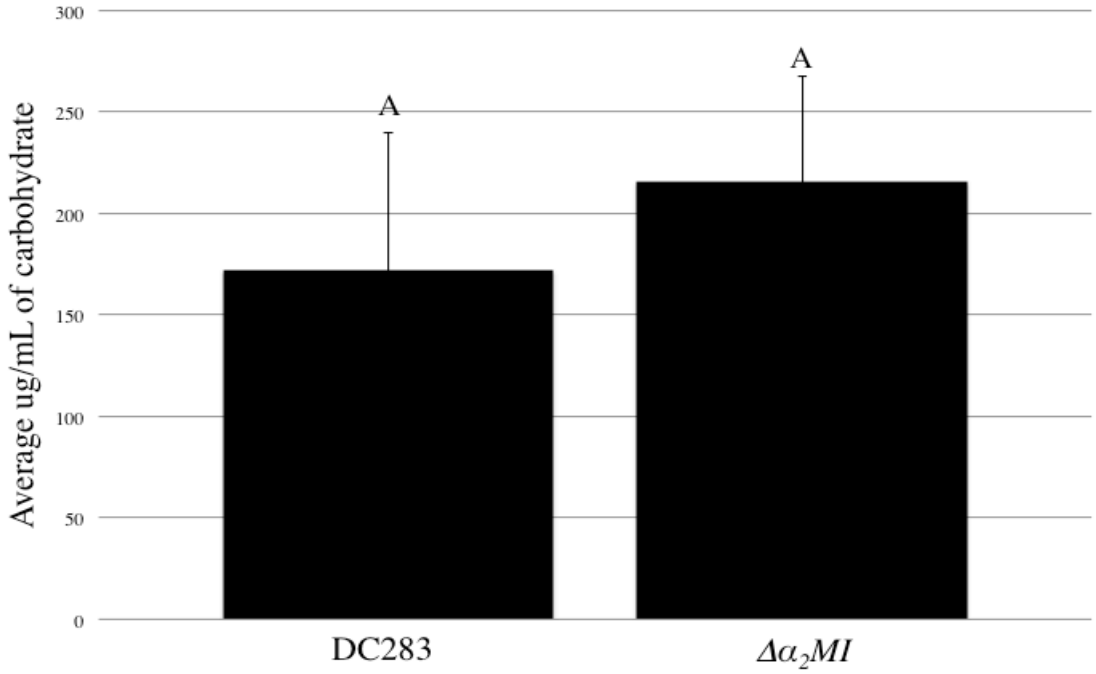

300
250
200
150
100
50
0
Average ug/mL of carbohydrate
A
A
DC283
$\Delta\alpha_2MI$

Figure 2. 3. Stewartan exopolysaccharide production of wild type and *Δα₂MI*. A) Qualitative analysis on NA+ 0.2% glucose plates indicated no change in EPS production in the *Δα₂MI* mutant. B) Quantitative analysis of EPS measured by the phenol-sulfuric acid colorimetric assay indicated there was no significant difference in the amount of EPS produced by *Δα₂MI* in liquid culture based on a Student's *t*-test using $p \leq 0.05$. Letter (A) indicates no significant differences in the EPS production measured by the phenol-sulfuric acid assay.

Wild type and *Δα$_2$MI P. stewartii* grow at the same rate in LB media.

I generated a mutation in *α$_2$MI* in the wild type background to assess effects on attachment. However, loss of function mutations often affect bacterial growth and development. A growth curve analysis was performed in order to determine if wild type and *Δα$_2$MI* demonstrate differences in the rate of growth. Fresh single colonies of wild type DC283 and *Δα$_2$MI* were inoculated in 2 mL LB medium, and incubated at 28° C with shaking at 180 rpm for 24 hours. Bacterial cultures were sub-cultured 1:10 in fresh LB and allowed to grow with shaking at 28° C for 25 hours. An optical density (OD_{600}) reading was taken every 30 minutes for the first 6.5 hours and the final reading after 25 hours (Figure 2. 4). There was no statistically significant difference in the rate of growth between the wild type and the *Δα$_2$MI P. stewartii* strain using the Student's *t*-test using $p \leq 0.05$. Therefore, the mutation in the *α$_2$MI* gene does not affect growth rate.

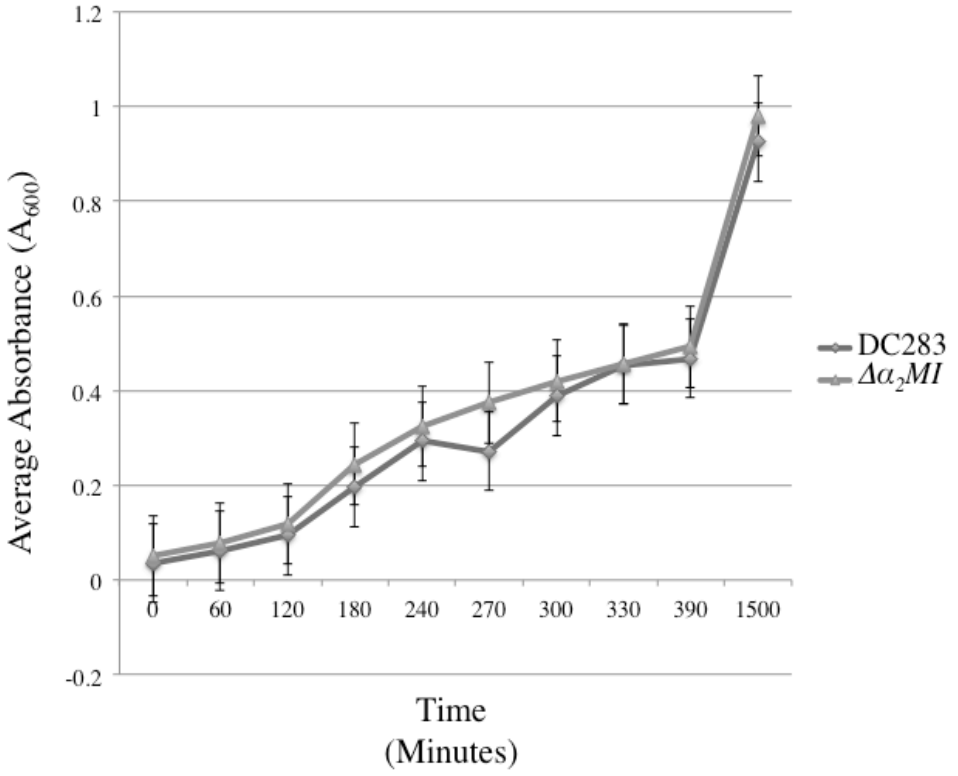


Figure 2. 4. *Δα₂MI* has the same growth rate as wild type *P. stewartii*. Colonies were grown in LB medium for 25 hours and the OD_{600} was measured every 30 minutes. The vertical error bars represent the standard error of the mean. There were 4 replicates per biological strain. There is no statistically significant difference between wild type and *Δα₂MI* (Student's *t*-test using $p \leq 0.05$).

The *α₂MI* mutant is compromised in WS lesion development, but not host colonization. Virulence was assessed by measuring water-soaked (WS) lesion length and by monitoring overall disease progression. The length of the WS lesions seven days post-inoculation on the plants inoculated with *Δα₂MI* were significantly shorter than the plants inoculated with the wild type (DC283) based on a Mann-Whitney test at the 0.05 level (Figure 2. 5). Disease progression was assessed daily for seven days by a general Stewart's wilt disease rating system between 0 and 4 where 0= no symptoms, 1= a few lesions but no bacterial biofilm ooze, 2= many lesions and bacterial biofilm ooze, 3= severe lesions and bacterial biofilm ooze, and 4 = leaf necrosis and cellular collapse (Figure 2.6). The disease progress in both the wild type and the *Δα₂MI* were not statistically different, based on a Mann-Whitney test at the 0.05 level. The colonization of the apoplast was not statistically significant between *Δα₂MI* and wild type (Figure 2. 7), suggesting that the mutation of *α₂MI* does not affect the initial onset of the water-soaked lesion phase of infection or apoplast colonization. The negative control for the whorl inoculations were plants inoculated with 1x PBS-T, which did not develop disease symptoms. These differences were not statistically significant, based on a Mann-Whitney test at the 0.05 level.

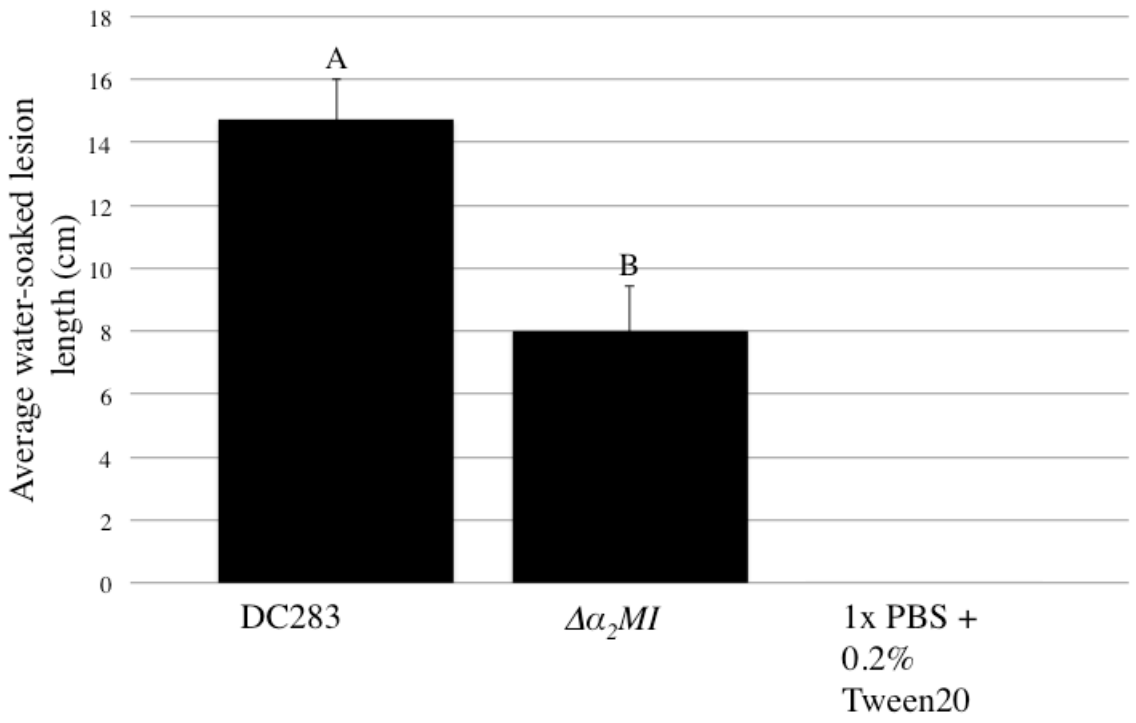


Figure 2. 5. Water-soaked lesion length of wild type and *Δα₂MI*. Whorl inoculations were performed on seven-day old seedlings of a susceptible sweet corn variety (var. Jubilee). Seedlings were inoculated with a bacterial suspension containing $1x10^7$ cells/mL re-suspended in 1x PBS buffer containing 0.2% Tween20 (1x PBS-T). The inoculum was placed into the whorl of the seedling. Following seven days post-inoculation, the water-soaked lesions were measured on the oldest leaf. The vertical error bars represent the standard error of the mean. Letters (A and B) indicate significant differences in the WS lesion length. These values are statistically significant based on a Mann-Whitney test at the 0.05 level.

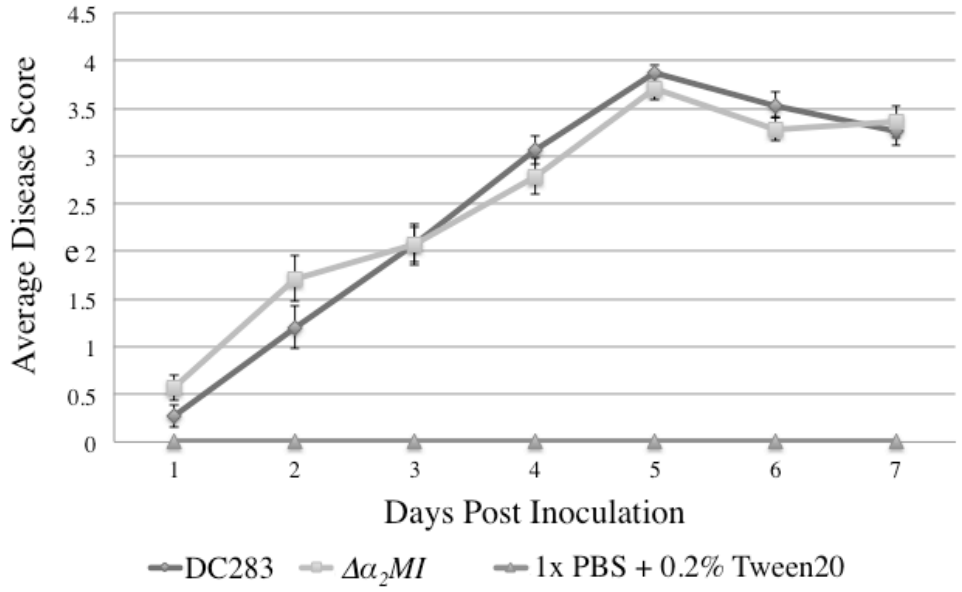


Figure 2. 6. Overall disease development in sweet corn plants. Whorl inoculations were performed on seven-day old seedlings of a susceptible sweet corn variety (var. Jubilee). Seedlings were inoculated with a bacterial suspension containing $1x10^7$ cells/mL re-suspended in 1x PBS buffer containing 0.2% Tween20 (1x PBS-T). The inoculum was placed into the whorl of the seedling. Disease ratings were performed daily based on the rating system mentioned above. The vertical error bars represent the standard error of the mean. These differences were not statistically significant based on a Mann-Whitney test at the 0.05 level.

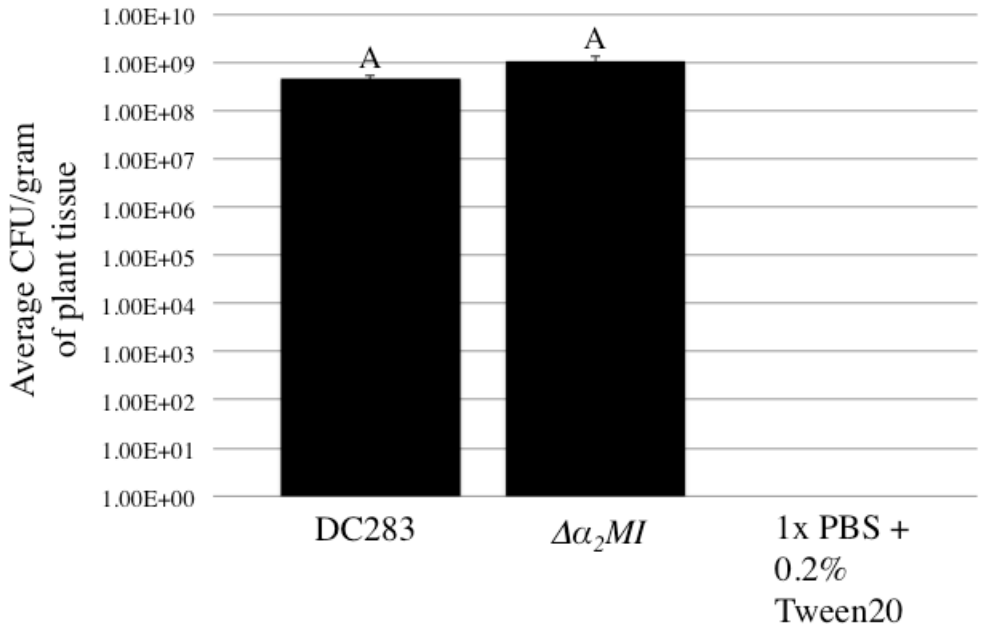


Figure 2. 7. *In planta* colonization of wild type and *Δα$_2$MI*. Whorl inoculations were performed on seven-day old seedlings of a susceptible sweet corn variety (var. Jubilee). Seedlings were inoculated with a bacterial suspension containing 1x10^7 cells/mL re-suspended in 1x PBS buffer containing 0.2% Tween20 (1x PBS-T). The inoculum was placed into the whorl of the seedling. The bacteria were isolated from the infected seedlings and quantified. Seedlings inoculated with wild type (DC283) served as the positive control and seedlings inoculated with 1x PBS-T buffer alone served as the negative control. Vertical error bars represent the standard error of the mean. Letter (A) indicates no significant differences in the colonization abilities of either strain. These differences were not statistically significant based on a Mann-Whitney test at the 0.05 level.

Virulence of *Δα₂MI* was also tested using the scratch-inoculation technique, which creates wounds mimicking the natural infection process by the corn flea beetle and allows the bacteria to enter the xylem as well as the leaf apoplast. This inoculation method allows assessment of both the water-soaked lesion and wilting phases of the disease. Seven days post-inoculation, similar levels of wilting were observed in both wild type and *Δα₂MI*-inoculated plants, suggesting that the wilting phase of infection is not affected by a mutation in *α₂MI* (Figure 2. 8). Following isolation of bacterial populations, colonization of the *Δα₂MI* mutant was the same as wild type *P. stewartii* (Figure 2. 9). The negative control for the scratch inoculations were plants inoculated with 1x PBS-T, which did not exhibit disease symptoms. These differences were not statistically significant based on a Mann-Whitney test at the 0.05 level.

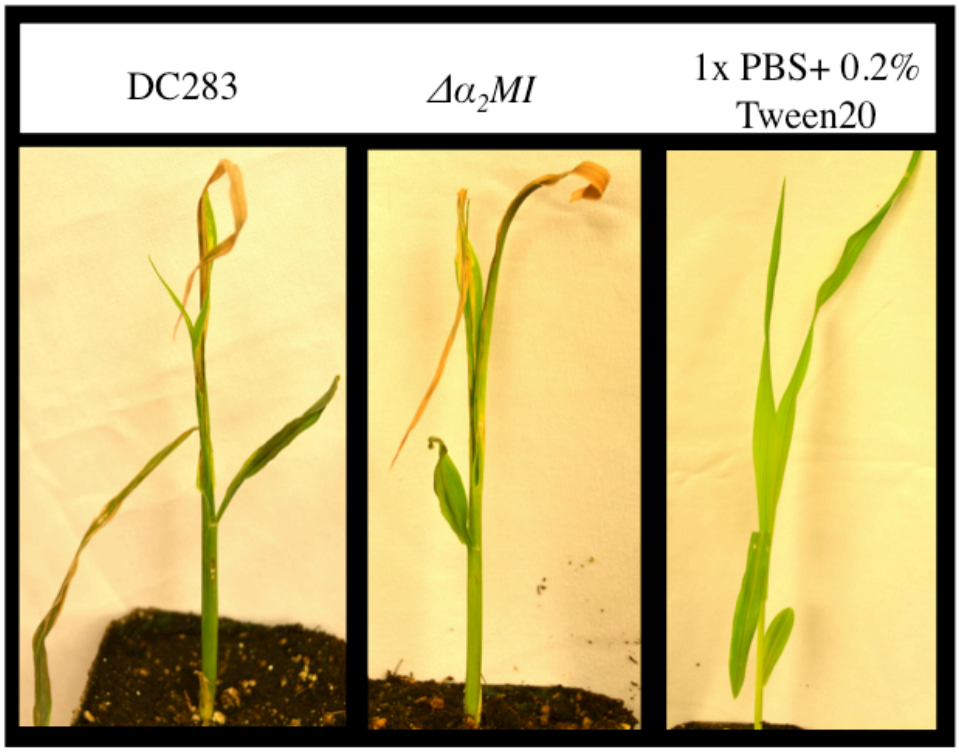


Figure 2. 8. Development of Stewart's wilt in scratch inoculated seedlings. Five µl of inoculum containing $5x10^7$ cells/mL in sterile 1x PBS-T was placed over a wound, created by scratching the stem of a seven-day old seedling with a sterile 20-gauge needle. Plants were observed for development of wilting symptoms for seven days post-inoculation. Seedlings inoculated with wild type (DC283) served as the positive control and seedlings inoculated with 1x PBS-T buffer alone served as the negative control.

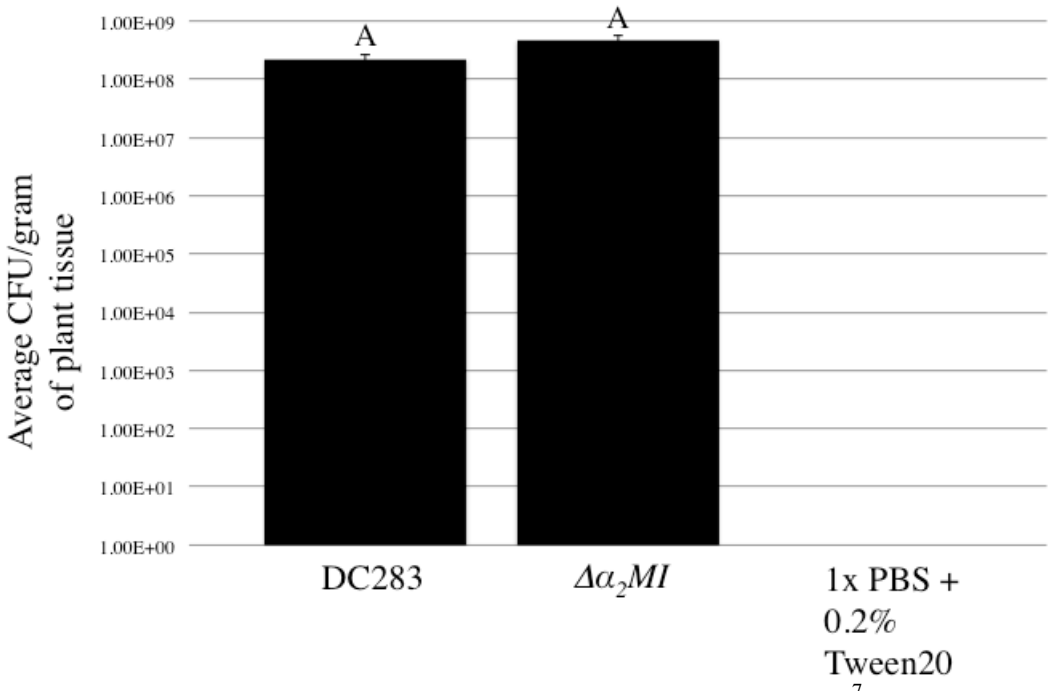


Figure 2. 9. Host colonization. Five µl of inoculum containing 5x10^{7} cells/mL in sterile 1x PBS-T was placed over a wound created by scratching the stem of a seven-day old seedling with a sterile 20-gauge needle. Plants were observed for development of wilting symptoms after seven days post-inoculation and bacteria were isolated from the infected seedlings and quantified. Seedlings inoculated with wild type (DC283) served as the positive control and seedlings inoculated with 1x PBS-T buffer alone served as the negative control. Vertical error bars represent the standard error of the mean. Letter (A) indicates no significant differences in the colonization abilities of either strain. These differences were not statistically significant based on a Mann-Whitney test at the 0.05 level.

Gene expression analysis in the *α₂MI* mutant. RNA was isolated from *P. stewartii* wild type DC283, *Δα₂MI, ΔoxyR, ΔwceO,* and *ΔwceO*/*ΔoxyR* for gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR). In the wild type background, *oxyR* and *wceO* have specific functions that contribute to host colonization and virulence of *P. stewartii.* OxyR is a redox-sensing transcription regulator that that belongs to the regulatory hierarchy that controls exopolysaccharide (EPS) production and the wilting phase of Stewart's wilt disease (Burbank & Roper*,* 2014). WceO is necessary for stewartan EPS synthesis and contributes to virulence (Carlier *et al*, 2007).

Interestingly, mutations in *Δα₂MI, ΔoxyR,* and *ΔwceO* in the wild type background have a hyper-attaching phenotype when compared to the wild type using the crystal violet assay (O'Toole *et al.,* 1998; Koutsoudis *et al.,* 2006). However, unlike *ΔoxyR* and *ΔwceO*, *Δα₂MI* produces wild type levels of EPS.

To take a closer look at the gene expression level, three genes (*α₂MI, α₂MII,* and *pbpC*) ((ASAP ID # ACV-0288726, GenBank Accession # EHT99557), (ASAP ID # ACV-0288724, GenBank Accession # EHT99559), and (ASAP ID # ACV-0288727, GenBank Accession # EHT99556), respectively) were monitored for relative gene expression in varying genetic backgrounds. These genes were selected because of the initial identification of *α₂MI* as a possible candidate responsible for adherence to surfaces (Burbank, Nanthavong, and Roper, unpublished data). Interestingly, *P. stewartii* has two hypothetical α-2-macroglobulin genes in close proximity within the genome, as well as an additional gene that is hypothesized to encode a penicillin binding protein (PBP).

In this study, analyses of gene expression of both α-2-macroglobulin genes were performed (*α₂MI* and *α₂MII*). In many bacterial systems, such as *E. coli* and *Anabaena*, *α₂M* is part of a conserved gene set with *pbp*. The *α₂M* and *pbp* genes are typically located in the same operon and are thought to be co-transcribed (Budd *et al.,* 2004). These two proteins are hypothesized to have a physical interaction with one another and are proposed to work together to evade host proteases (Budd *et al.,* 2004; Dandekar *et al.,* 1998; Doan & Gettins, 2008). PbpC has been documented as a key player in peptidoglycan biosynthesis for use in the bacterial cell wall in Gram-negative bacteria (Evans *et al.*, 2013).

qRT-PCR analysis was performed to compare the relative gene expression of *α₂MI, α₂MII,* and *pbpC* in *P. stewartii*. The housekeeping genes, *ffh* (GenBank Accession #EHT99250) and *proC* (GenBank Accession #EHU02233) (Takle *et al.,* 2007; Burbank, dissertation 2014), were used as reference genes. *α₂MI, α₂MII,* and *pbpC* transcript expression levels were normalized to *ffh* and *proC*. We determined that expression of *pbpC* in the *Δα₂MI* mutant background was 44-fold up-regulated as compared to the wild type, *ΔoxyR,* and *ΔwceO* strains (Figure 2. 10). *pbpC* gene expression was also up regulated 8-fold in the *ΔwceO/ΔoxyR* mutant background. Interestingly, in the *ΔwceO /ΔoxyR* mutant background, *α₂MII* gene expression was also up-regulated 18-fold. Gene expression of *α₂MI* did not significantly change in any of the strains under examination. The observed increase in expression level of *pbpC* in the *Δα₂MI* mutant suggests that *α₂MI* and *pbpC* could be functioning together. Similar to other bacterial systems, *α₂MI* and *pbpC* in *P. stewartii* are in close proximity to one another within the genome and

could be part of the same operon under the same response regulator. It is plausible that the increased production of PBPC is a means to compensate for the loss of α_2*MI*.

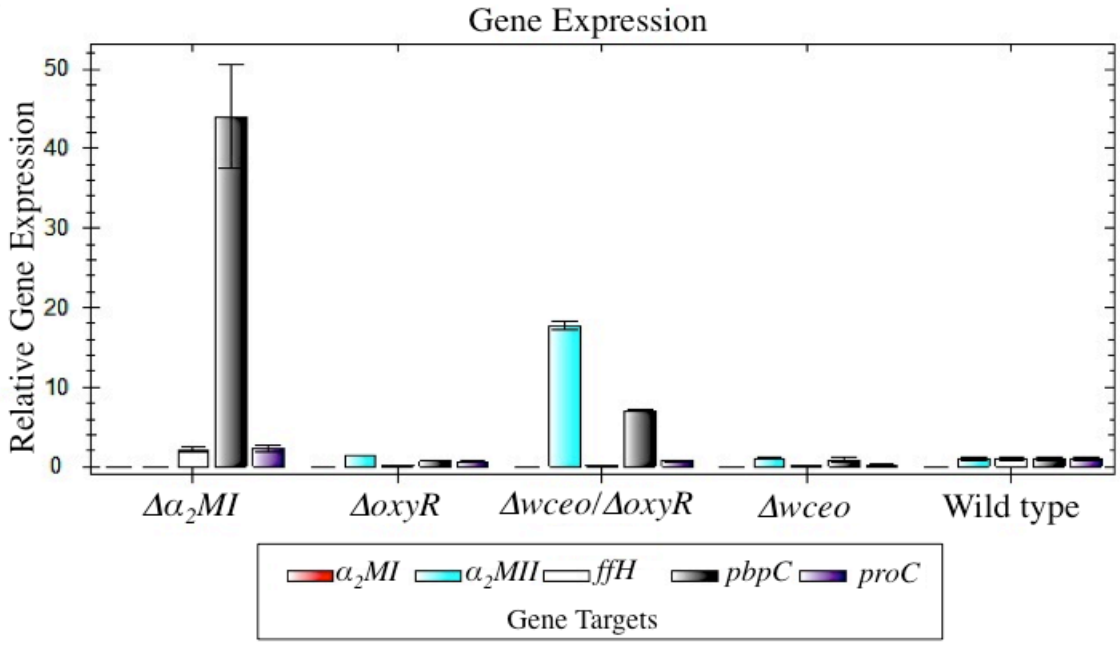


Figure 2. 10. Gene expression of *α₂MI, α₂MII,* and *pbpC* genes in wild type and mutant *P. stewartii*. Wild type *P. stewartii* and four mutated strains (*Δα₂MI, ΔoxyR, ΔwceO,* and *ΔwceO*/*ΔoxyR*) were analyzed by qRT-PCR for overall expression of three target genes (*α₂MI, α₂MII,* and *pbpC*). Gene expression of the target genes was normalized to two housekeeping genes (*ffh* and *proC*). Error bars represent the standard deviation of the mean.

DISSCUSSION

The goal of this study was to characterize the functional role of bacterial α_2MI in the host-pathogen interaction between *P. stewartii* and sweet corn. Interestingly, the WS lesions that developed in seedlings inoculated with *Δα₂MI* were significantly shorter in length when compared to the wild type (Figure 2. 5). Based on the increased adherence phenotype we observed *in vitro,* we postulate that the *Δα₂MI* strain could be restricted in movement within the leaf apoplast, which could lead to truncated lesion development.

My experimental results demonstrated that *Δα₂MI* colonizes the corn seedling at a similar rate as wild type, regardless of the inoculation method (Figures 2. 7 and 2. 9). This suggests *α₂MI* does not affect bacterial host colonization (Figures 2. 7 and 2. 9), but possibly reduces bacterial virulence, as observed by the truncated WS lesion length (Figure 2. 5). Infecting bacteria colonies can be restricted to a small region within the plant apoplast due to reduced bacterial virulence or increased susceptibility to host defense mechanisms. These bacterial colonies will not be able to spread from the site of infection to other regions of the apoplastic space within the leaf. To test whether *α₂MI* affects bacterial dispersion within the host apoplastic space, the bacterial concentration will be quantitated in leaf tissue at discrete distances from the point of inoculation. I hypothesize that *α₂MI* contributes to *P. stewartii* dispersion within the leaf and virulence, thus I expect strains of *Δα₂MI* will be less virulent and colonization of the plant host will be localized at the site of inoculation. The cell density of the *Δα₂MI* strain will be lower in leaf tissue further away from the site of inoculation. The WS lesion lengths should be recovered once *Δα₂MI* is complemented. Complementation will occur through

chromosomal insertion of *α₂MI* into the *glmS* locus, which is a single, conserved chromosomal location previously demonstrated in various bacterial species by (Choi *et al.,* 2005).

Expression of *α₂MI, α₂MII,* and *pbpC* was assessed by quantitative RT-PCR (qRT-PCR) in five distinct genetic backgrounds of *P. stewartii*: wild type *Δα₂MI, ΔoxyR, ΔwceO,* and *ΔwceO*/*ΔoxyR.* These mutants demonstrate an increased attachment phenotype and qRT-PCR was utilized to correlate transcriptional changes of *α₂MI, α₂MII,* and *pbpC* impacted by the loss of *α₂MI, oxyR, wceO,* and *wceO*/*oxyR*. Bacteria with a null *α₂MI* gene are likely to be more susceptible to plant host protease attack and could experience greater damage to their cell wall due to degradation of proteins in the cell envelope. As a result of increase cell wall damage the bacterium likely up-regulates *pbpC* to enhance cell wall maintenance and repairs. My results determined through qRT-PCR that the expression of *pbpC* in the *Δα₂MI* mutant background was significantly up regulated, specifically a 44-fold increase when compared to the wild type (Figure 2. 10). *pbpC* gene expression was up regulated 8-fold in the *ΔwceO /ΔoxyR* mutant background. Interestingly, in the *ΔwceO /ΔoxyR* mutant background, *α₂MII* gene expression was also up regulated 18-fold. No significant gene expression changes were observed for *α₂MI* in any of the mutant strains compared to the wild type (Figure 2. 10). This observation supports my initial hypothesis that α_2MI and PBP may co-function to defend against non-self proteases and reinforce the bacterial cell wall. This allows the bacterium to resist host proteases. Overall, my experiments demonstrated that *α₂MI* contributes to bacterial virulence in leaf blight phase of development and bacterial adhesion.

To continue this study, mutants in *α_2MII* and *pbpC* will be constructed in *P. stewartii*. This would allow us to assess the contributions of these proteins to bacterial attachment and protection against non-self proteases encountered in the plant.

To determine that α_2MI or α_2MII are indeed protease inhibitors would require recombinant expression and purification of these proteins. These proteins would be compared to serine-proteases, such as trypsin, and assessed for inhibitory activity. Protease degradation by α_2MI or α_2MII would be compared to a known inhibitor of trypsin, such as serum alpha-1 trypsin through a trypsin activity assay.

It is hypothesized that the proteins α_2MI and PBPC are physically interacting with one another but it has not been determined in a bacterial system (Budd *et al.,* 2004). The yeast-two-hybrid assay would be used to determine whether the two proteins physically interact, with the goal of further determining the functional relationship between α_2MI and PBPC.

MATERIALS AND METHODS

Strains and Growth Conditions. The *Pantoea stewartii* subsp. stewartii (DC283) and *Escherichia coli* constructs were grown on solid Nutrient Agar (NA) (Difco Laboratories, Detroit, MI) or in liquid culture (Luria-Bertani (LB) medium (Difco, Laboratories, Detroit, MI) or AB minimal media at 28ºC and 37ºC, respectfully. The antibiotics nalidixic acid or kanamycin was added to the medium when appropriate at a concentration of 30 μg/mL for the *P. stewartii* constructs. Kanamycin (30 μg/mL), ampicillin, streptomycin, spectinomycin (at a concentration of 100 μg/mL), or chloramphenicol (35 μg/mL) was added to the medium when appropriate for the *E. coli* constructs. Clones were created utilizing *E. coli* DH10β and S17-1λ was the donor utilized for conjugal transfer to carry the RK-2 based plasmid constructs into *P. stewartii* subsp. stewartii (DC283). All bacterial strains and plasmids used in this study can be found in Table 1.

Standard DNA Manipulations. The Dneasy DNA extraction kit (Qiagen, Valencia, CA) was used to extract genomic DNA per manufacture instructions. The Zyppy Plasmid Miniprep kit (Zymo Research Corporation, Irvine, CA) was used to extract the plasmids per manufacturer's instructions. Takara *Ex-Taq* DNA polymerase (Takara Bio USA, Madison, WI) was used to amplify DNA fragments and synthetic oligonucleotide primers were ordered from Integrated DNA Technologies (IDT) (Coralville, IN). Restriction enzymes were purchased from New England Biolabs (NEB) (Ipswich, MA).

Mutant construction. The *P. stewartii* mutants in this study were created in the wild type (DC283) background through Gateway (GW) technology (Life Technologies, Inc., Carlsbad, CA). The GW system has been described by (Choi & Schweizer, 2005).

The *Δα$_2$MI* mutant was created in the wild type genetic background using the primer set amg LF fwd/ amg RF rev to amplify the 200 bp upstream and downstream of the *α$_2$MI* gene (ASAP ID # ACV-0288726) (GenBank Accession # EHT99557), with the addition of BamHI and NdeI sequences to the primers, respectfully. Primer set pKD4fwdBamHI/ pKD4revNdeI amplified the kanamycin resistance cassette derived from plasmid pKD4 (Datsenko & Wanner, 2000), with the addition of BamHI and NdeI sequences to the primers, respectfully. The resulting PCR products were individually cloned into vector pCR8/GW/TOPO (Invitrogen), followed by enzyme digestion by BamHI and NdeI to excise the *α$_2$MI* gene from the pCR8/GW/TOPO vector and the kanamycin cassette from the pCR8/GW/TOPO vector. The kanamycin cassette was inserted into the backbone of the pCR8/GW/TOPO: *α$_2$MI* plasmid, through ligation (T4 DNA Ligase, NEB, Ipswich, MA) per manufacturer's instructions. This resulted in

Δα$_2$MI (pCR8/GW/TOPO::*kan*), also known as pKW005. This plasmid was recombined with LR clonase II enzyme mix per manufacturer's instructions with a GW suicide vector, pAUC40 (Carlier *et al.,* 2009) to create plasmid pKW006. The mutagenesis construct was transformed into the *E. coli* strain S17-1λ for conjugation with wild type *P. stewartii*. The resulting transformants were then screened for sucrose sensitivity to verify that the suicide vector pKNG101 was successfully removed. Sucrose resistant colonies were then selected for Kan^R and Strep^S for a second verification that the

pKNG101 vector was excised. The double crossover event was confirmed through two PCR reactions: (1) primers specific to amplify the flanking regions of the *α₂MI* gene and (2) primers specific to the flanking regions of the Kan^R marker of the mutant construct. The PCR products were sequenced and then entered into NCBI BLAST to confirm the mutation.

The kanamycin cassette was removed from the *P. stewartii* subsp. stewartii DC283 chromosome by Flp recombinase, which is carried on the plasmid pFLP2 (Hoang *et al.,* 1998). This resulted in an unmarked strain for the *α₂MI* mutant. The removal of the kanamycin cassette resulted in an 85 bp *frt* scar to create KW009 (*Δα₂MI*). The pFLP2 plasmid was cured through sucrose selection.

P. stewartii Δα₂MII and *ΔpbpC* mutants are also in the process of being made. In short, the *Δα2MII* and *ΔpbpC* mutants were created using primer sets 500bp A2M2fwd/ 500bp A2M2rev amplified 500 bp up and downstream of *α2MII* (ASAP ID # ACV-0288724) (GenBank Accession # EHT99559) and pKD4 kan::A2M2 fwd.2/ pKD4 kan::A2M2 rev.2 with the addition of SacI and AgeI sequences to the end of both primer sets. Primer sets 500bp pbpCfwd/ 500bp pbpCrev amplified 500 bp up and downstream of *pbpC* (ASAP ID # ACV-0288727) (GenBank Accession # EHT99556) and pKD4 kan::pbpC fwd.2/ pKD4 kan::pbpC rev.2 with the addition of AscI and AfeI restriction sites to the end of both primer sets. The kanamycin resistance cassette was derived from plasmid pKD4 (Datsenko & Wanner, 2000) for both *Δα₂MII* and *ΔpbpC*. The resulting PCR products were individually cloned into the vector pCR8/GW/TOPO (Invitrogen), followed by enzyme digestion (SacI/AgeI and AscI/AfeI, respectfully), and

ligation as described above for the *Δα₂MI* mutant. This resulted in *Δα₂MII* (pCR8/GW/TOPO: kan) and *ΔpbpC* (pCR8/GW/TOPO: kan) pKW011 and pKW012, respectively. These plasmids were recombined with pAUC40 using the LR clonase II enzyme mix per manufacturer's instructions (Carlier *et al.,* 2009) to create plasmid pKW014 (*Δα₂MII*). The *ΔpbpC* construct is not yet in the suicide vector pAUC40. The mutagenesis constructs will be transformed into *E. coli* strain S17-1λ for conjugation with wild type *P. stewartii,* followed by sucrose selection to verify that the pAUC40 backbone was successfully removed. Confirmation of the double crossover event would occur through two PCR reactions as described for the *Δα₂MI* construct.

The *Δα₂MI* mutant strain is in the process of being complemented. The *α₂MI* ORF with an additional 200 bp upstream was amplified by conventional PCR using primer set A2MIfwdXhoI Comp/A2MIrevKpn1 Comp and cloned into the XhoI and KpnI restriction sites of the pUC18R6K-mini-Tn7-cat vector system developed by (Choi *et al.,* 2005). The complementation construct was transformed into *E. coli* strain S17-1λ and conjugated into *P. stewartii* subsp. *stewartii Δα₂MI*, which did not result in any viable transformants. The pUC18R6K-mini-Tn7-cat constructs are expected to integrate into the *glmS* locus, which is a single, conserved chromosomal location previously demonstrated in various bacterial species by (Choi *et al.,* 2005). Once the insertion occurs into this specific site, DNA sequencing of the *glmS* locus region would confirm the complementation. All of the primer sequences used for this study can be found in Table 2.

***In planta* virulence assays**. Virulence was assessed for all strains using two inoculation methods. Whorl and scratch inoculation techniques were previously described by Ham *et al.*, (2006) and Herrera *et al.,* (2008), respectfully. Whorl inoculations were performed on seven-day old sweet corn seedlings (var. Jubilee) grown in a growth chamber with 12-hour light/dark cycles and 80% humidity. Seedlings were inoculated with 100 µL bacterial suspension that consisted of $1x10^7$ cells re-suspended in 1x PBS buffer containing 0.2% Tween20 (1x PBS-T). The bacterial suspension was pipetted into the whorl of the seedling. Disease progression was assessed daily by a disease rating system from 0 to 4 described by (Roper & von Bodman, in preparation). The disease ratings are described as (0) no symptoms, (1), few lesions and no bacterial ooze, (2) many lesions and some bacterial ooze, (3) severe lesions and bacterial ooze, and (4) leaf necrosis and cellular collapse. Plants inoculated through whorl inoculation develop WS lesions. Seedlings do not wilt when inoculated using this method because the bacteria are unable to gain access to the xylem, which requires wounding. The apoplast of the seedling is the only location that the bacterium can colonize in the absence of wounding, resulting in WS lesions.

The scratch inoculation method as described by Herrera *et al.,* (2008) was used to mimic the scratching feeding behavior of the corn flea beetle that creates wounds on the plant and allows bacteria to enter the xylem. Five µL of a bacterial suspension containing $5x10^7$ cells/mL in sterile 1x PBS-T was placed over a wound created by a sterile 20-gauge syringe needle. The disease ratings are described as (0) no symptoms, (1), few lesions, (2) spreading lesions and some bacterial ooze, (3) severe lesions, bacterial ooze,

and wilting, and (4) severe wilting and plant death. Plants were observed for development of wilting symptoms (score of 3^{+}) after seven days post-inoculation. Seedlings inoculated with wild type bacteria served as the positive control and seedlings inoculated with 1x PBS-T buffer alone served as negative control for both inoculation methods.

Quantification of bacterial populations *in planta*. Quantification of the bacterial populations for both whorl and scratch inoculation were performed using the same method. The oldest leaf was removed from each inoculated plant seven days post-inoculation. Leaf tissue was individually weighed and then surface sterilized using the following procedure: leaves were soaked in 70% ethanol followed by 10% bleach and rinsed twice in sterile diH_2O (30 seconds per solution). Following this, the leaf tissue was placed in a mesh grinding bag (Agdia, Inc. Elkhart, IN) with 2 mL 1x PBS and ground to a slurry. Serial dilutions of the plant slurry were made in 1x PBS and plated for viable colony counts on NA containing the appropriate antibiotics and incubated at 28°C for 48 hours. Colonies were then counted and bacterial populations were quantified as CFU per gram fresh tissue.

Surface attachment assay. Single colonies of *P. stewartii* wild type DC283, *Δα₂MI, ΔoxyR, Δwceo,* and *Δwceo/ΔoxyR* were grown overnight in 200 µL of attachment media (LB medium with no salt containing + 0.2% glucose) with the appropriate antibiotics in a 96 well PVC microtiter dish (BD Falcon). Samples were incubated statically at 28°C for 48 hours. Following incubation, attached cells were quantified using a crystal violet (CV) staining method previously described (O'Toole *et al.,* 1998; Koutsoudis *et al.,* 2006). Attached cells were normalized to initial cell density (OD_{600}). Adhesion values (AV)

were calculated as (AV= (CV value-CV blank value/initial growth value-blank value)) (Koutsoudis *et al.,* 2006).

Exopolysaccharide isolation and quantification. All strains were grown in AB minimal medium (Chilton *et al.,* 1974) with the addition of 0.4% glucose to an optical density of (OD_{600}=0.7). Glucose (0.2%) was added to the media to induce EPS production. Ethanol precipitation was used to extract EPS from the culture supernatant, modified from Bramhachari *et al.,* 2007. Bacterial cells were removed by centrifugation and EPS was precipitated out of the supernatant by adding three volumes of 95% ethanol at -80° C for 30 min. EPS was pelleted by centrifugation at 6,000 rpm for 20 min and then washed twice with cold 70% ethanol. EPS was re-suspended in 1x PBS at 37°C and quantified using the phenol-sulfuric acid colorimetric assay (Matsuko *et al.,* 2005). The initial cell density (OD_{600}) was used to normalize the total EPS.

For qualitative assessment of EPS production, fresh colonies were streaked in an "X" pattern on NA plates containing 0.2% glucose and the appropriate antibiotics. Plates were incubated at 28° C for 24 hours and then imaged.

Gene expression analysis. RNA was extracted following a modified method by Jahn *et al.*, (2008**).** Briefly, single colonies of *P. stewartii* wild type DC283, *Δα$_2$MI, ΔoxyR, Δwceo,* and *Δwceo/ΔoxyR* were grown overnight in LB medium with the appropriate antibiotics at 28°C for 24 hours. Bacterial cells were sub-cultured 1:10 in fresh LB and antibiotics. Once the bacterial cells reached an optical density (OD_{A600} = 0.5), four mL of

culture was used for the RNA extraction. Immediately after the RNA extraction, the RNA was treated twice with DNase using the TURBO DNase treatment (Ambion, Life technologies) per manufacturer's instructions. Following Dnase treatment, 500 ng of DNA-free RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Biorad Laboratories, Hercules, CA) per manufacturer's instructions. Quantitative real time PCR (qRT-PCR) was performed using iQ SYBR Green Supermix (Biorad Laboratories, Hercules, CA) with fresh cDNA to determine gene expression. SYBR Green fluorescence was detected using the CFX96 Real-time PCR instrument (BioRad Laboratories, Hercules, CA). The qRT-PCR data was analyzed using the CFX Manager software (BioRad Laboratories, Hercules, CA) and expressed as a fold gene expression change compared with untreated controls, plus or minus the standard deviation.

Primers for qRT-PCR analysis were created using the Beacon Design software (Premier Biosoft) and ordered from IDT. Conventional PCR analysis with genomic DNA was used to determine the optimal conditions for primer concentration and annealing temperature prior to use qRT-PCR. Gene expression was normalized to two housekeeping genes, *ffh* (GenBank Accession #EHT99250) and *proC* (GenBank Accession #EHU02233) (primer sets ffh fwd RT/ffh rev RT and proC RT fwd/proC RT rev, respectively) that were chosen based on previous work (Takle *et al.,* 2007; Burbank & Roper, 2014). Both *ffh* and *proC* were stably expressed at equal levels in all strains.

Table 2. 1. Bacterial Strains and Plasmids

Strain or Plasmid	Relevant genotypes	Source or Reference
Pantoea stewartii* subsp. *stewartii		
DC283	Wild type, Nal^r	(Coplin et al., 1986)
Δα2MI (KW009)	Nal^r, knockout in *α2MI*	This study
Escherichia coli		
DH10B	F– endA1 *recA1 galE15 galK16 nupG rpsL lacX74 80lac Z M15 araD139* (ara,leu)7697 *mcrA* (*mrr-hsdR MS-mcrBC*) –	Invitrogen
S17-1pir+	RP4, Mob+, Sm^r	(Simon et al., 1983)
EC100pir+	F *mcrA* (*mrr-hsdRMS-mcrBC*), *80dlacZ, M15 lacX74, recA1, endA1araD139*,(ara, leu)*7697galU galK – rpsL nupG pir*+(DHFR)	Epicentre
Plasmids		
pUC18R6K-mini-Tn7-cat	Cm^r, Ap^r	(Choi et al., 2005)
pAUC40	Suicide vector, Gateway *attR,* Cm^r, Sm^r	(Carlier et al., 2009)
pFLP2	*sacB+,* FLP recombinase, Ap^r	(Kaniga et al., 1991)
pKD4	Km^r, source of kan cassette	(Datsenko & Wanner, 2000)
pSP856	Gm^r, source of gent cassette	(Choi et al., 2005)
pCR8/GW/TOPO	Cloning vector, Sp^r	Invitrogen
pDONR221	Cloning vector, Km^r, Ze^r	Invitrogen
pBBR1MCS-4	Broad host range vector, Ap^r	(Kovach et al., 1995)
pKW002	pKD4 Kan cassette cloned into pCR8/GW/TOPO, for α_2*MI* mutant, Km^r	This study
pKW003	*α2MI* cloned into pCR8/GW/TOPO, Sp^r	This study
pKW005	*α2MI* :: kan cloned into pCR8/GW/TOPO, Km^r	This study
pKW006	*α2MI*:: kan cloned into pAUC40 Km^r	This study
pKW009	pKD4 Kan cassette cloned into pCR8/GW/TOPO, for *α2MII* mutant, Km^r	This study
pKW010	pKD4 Kan cassette cloned into pCR8/GW/TOPO, for *pbpC* mutant, Km^r	This study
pKW011	*α2MII*::kan cloned into pCR8/GW/TOPO, Km^r	This study
pKW012	pbpC::kan cloned into pCR8/GW/TOPO, Km^r	This study

Nal^r, nalidixic acid; Ap^r, ampicillin; Km^r, kanamycin; Gm^r, gentamycin; Sm^r, streptomycin; Sp^r, spectinomycin; Cm^r chloramphenicol; Ze^r, zeocin resistance.

Table 2. 2. Primer Sequences

Primer	Sequence	Source or Reference
Amg LF fwd	ATTCGTCCCCTGTTCGGCAA	This study
Amg RF rev	GCGCGATTAAGATAAAGCGTCAGG	This study
pKD4fwdBa		This study
mHI	CGCGGGATCCTAGGCTGGAGCTGCTTCGAAGTTC	
pKD4revNdeI	CGCGCATATGCATATGAATATCCTCCTTAGTTCCTATTCCG	This study
500bp		This study
A2M2fwd	GGGCGCAGAAAACAGATTGCCTTCATCA	
500bp		This study
A2M2rev	TTGCCGAACAGGGGACGAATGCCCG	
500bp		This study
pbpCfwd	TCGGCTATCCGCTGAGTCCACCTGC	
500bp		This study
pbpCrev	CCAGTGCGTTGGCTGGGTTAGTTGCA	
pKD4		This study
kan::A2M2		
fwd.2	TTAGAGCTCATGATTGAACAAGATGGATTGCACG	
pKD4		This study
kan::A2M2		
rev.2	TTAACCGGTGAAGAACTCGTCAAGAAGGCGATAG	
pKD4		This study
kan::pbpC		
fwd.2	TTAGGCGCGCCATGATTGAACAAGATGGATTGCACG	
pKD4		This study
kan::pbpC		
rev.2	TTAAGCGCTGAAGAACTCGTCAAGAAGGCGATAG	
A2MIfwdXho		This study
I Comp	GGCTATCGAGGAGGGCAGTACCAAAG	
A2MIrevKpn		This study
1 Comp	ATCTAGGTACCTTACTGTACATGCAGCTGCT	
ffh fwd RT	GTATCGCCTCACGCATCC	This study
ffh rev RT	CATCTGCTTCAGTTGCTCAAG	This study
proC RT fwd	CTCGGTCACGCCCAATGC	This study
proC RT rev	CTCACGCCTACCACGGAATG	This study
A2MI RT		This study
fwd	GTGGAAGATTGGTGTCAG	
A2MI RT rev	ATTACTCAGCGTCAGTTG	This study
A2MII RT		This study
fwd	TTGCTGCCATTGCGTATTG	
A2MII RT		This study
rev	TGAGATTGCCAGGTTGAGAG	
pbpC RT fwd	GGCGTTGCTGACCTATGAAG	This study
pbpC RT rev	ACACTACGATAGCGAAGATTCTG	This study

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