ELUCIDATION OF THE MOLECULAR HOST-PATHOGEN INTERACTIONS THAT INFLUENCE SEED-TO-SEEDLING TRANSMISSION OF ACIDOVARAX CITRULLI

by

KAMEKA LATOYA JOHNSON

(Under the Direction of Ronald Walcott)

ABSTRACT

Bacterial fruit blotch (BFB) caused by the seedborne bacterium, Acidovorax citrulli is a serious economic threat to cucurbit production worldwide. Bacterial fruit blotch occurs sporadically, but it can cause up to 100% loss under ideal field conditions. Gaining a better understanding of the biology of BFB could potentially lead to more effective management strategies. Type III secretion (T3S), type II secretion (T2S) and quorum sensing (QS) are involved in bacterial pathogenicity. The role of these systems in A. citrulli pathogenicity and, more importantly, seed colonization and BFB seedling transmission was investigated using the sequenced strain AAC00-1. T3S is important for pathogenesis and facilitates the translocation of virulence proteins directly into the plant host cell. A type three secretion system (T3SS) mutant of A. citrulli was non-pathogenic on watermelon seedlings but still grew at wild type levels on seed. When used as a biocontrol blossom protectant the T3SS mutant reduced seed infestation by A. citrulli. Additionally when applied as a seed treatment to naturally infested seed, the nonpathogenic strain reduced seedling transmission by 37% in the greenhouse. In this study, we found that A. citrulli has a functional type two secretion system (T2SS) which is important for colonization of seed and seed-to-seedling transmission of BFB. An A. citrulli type two secreted
endoglucanase was also found to be important for seed colonization, but xylanase and pectate lyase were not required. *A. citrulli* encodes the quorum sensing homologs, *accI* (acyl homoserine lactone synthase) and *accR* (transcriptional regulator). Unlike the *aacR* mutant, the *aacI* mutant of AAC00-1 was reduced in virulence and in its ability to be transmitted from seed to seedlings. Interestingly both mutants colonized watermelon seed at wild-type rates.

**KEYWORDS:** *Acidovorax avenae* subsp. *citrulli*, β-1, 4- endoglucanase, LuxR, LuxI, *hrcC, gspG*, watermelon fruit blotch
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DEDICATION

Thanks you to all the graduate students that helped me throughout my time here and my fellow Jamaicans who helped to make Athens feel like home. To Dr. and Mrs. Mims, thank you for helping me get settled in this country. To my family, you were my inspiration, thank you. To my mother, thank you for your advice and encouragement and thank you for the poem, labor for learning. It kept me going. To my partner in crime, Dan, thank you for everything.
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The family *Cucurbitaceae* includes various crops that are used as a food source in regions around the world. These plants are widely distributed in the tropics and warm temperate regions of Asia, Africa and the Americas (Nayar and More, 1998). Cucurbits are mostly climbers, rarely woody and are prolific seed producers (Robinson and Decker-Walters, 1997). The term cucurbit was used to describe the cultivated members of the Cucurbitaceae. Major cultivated cucurbit crops are cucumber (*Cucumis sativa*), melon (*C. melo cantalupensis*), honeydew (*C. melo inodurus*) and watermelon (*Citrullus lanatus*). Other economically important crops include pumpkin (*Cucurbita pepo*), squash (*C. maxima*), and ornamental gourds (*C. moschata*). In addition to these major crops, more than 20 minor crops are produced for food in regions around the globe.

Watermelon originated in the Kalahari desert in Africa where the watermelon fruit was thought to be a source of water for travelers (Nayar and More, 1998; Robinson and Decker-Walters, 1997). The watermelon fruit is approximately 92% water and a significant source of vitamins A and C, fiber, and potassium (Nayar and More, 1998). The earliest record of a watermelon fruit harvest was in Egypt approximately 4000 years ago as documented in art and hieroglyphs of ancient Egypt (Robinson and Decker-Walters, 1997). The plant can now be found in the Mediterranean, Asia, Europe, and the new world where it was introduced in the early
Watermelon was first cultivated in the US in 1629 in Massachusetts and currently it is grown in 44 states (http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1183). In 2009, the United States was the fourth largest producer of watermelon (3,682 million pounds). China was the number one producer at 150,576 million pounds (http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1183). Florida, Georgia, Texas, and California were the highest watermelon producers, planting 25,800, 23,000, 20,900 and 12,400 acres, respectively. The fresh market watermelon yield for Georgia was worth $67.6 million USD and $460.7 million USD for the US (http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1183).

Vegetable seed is also a high value commodity that is traded internationally. In 2008 the world seed market was valued at $36.5 billion USD, of which, approximately 66% is accounted for by field crop seed and 34% is vegetable and horticultural seed (http://www.worldseed.org/isf/seed_statistics.html). The United States has the largest domestic seed market (~ $8.5 billion USD) and was closely followed by China at $4.0 billion USD in 2008. The United States is the largest importer and exporter of vegetable seed with an estimated $458 and $280 million USD in exports and imports, respectively, in 2008.

Watermelon fruit and seed production can be negatively impacted by bacterial fruit blotch (BFB) a disease caused by the Gram negative bacterium *Acidovorax citrulli* (Schaad *et. al.*, 1978; Schaad *et. al.*, 2008; Willems *et. al.*, 1992). Even though it is widely accepted that seed represent the most significant source of primary inoculum, BFB management in seed and fruit production fields is difficult, where 50 to 100% yield loss can occur under ideal field
conditions. Improved management of BFB will therefore require a better understanding of *A. citrulli* biology, specifically, the host-pathogen interactions that influence seed-to-seedling transmission of the bacterium.

**ACIDOVORAX CITRULLI**

*Acidovorax citrulli* was first recovered from watermelon seedlings at the Georgia Agricultural Experiment Station in Griffin, GA in 1965 (Webb and Goth, 1965). The seedlings were grown from seed that were thought to have originated from Turkey. Numerous seedlings were observed to have necrosis on cotyledons and true leaves and these symptoms often resulted in premature seedling death. Symptoms included small irregular shaped, water-soaked spots on leaves that eventually coalesced to form larger blighted areas.

Based on morphological and physiological characteristics, the pathogen was classified as a member of the *Pseudomonadaceae* family (Schaad *et. al.*, 1978). The bacterium was thought to be most similar to *Pseudomonas pseudoalcaligenes* because of its restricted ability to utilize sugars, and monotrichous flagellar arrangement (Schaad *et. al.*, 1978). Eventually, the pathogen was taxonomically placed as a subspecies of *P. pseudoalcaligenes* (*P. pseudoalcaligenes* subsp. *citrulli*) based on its unique ability to infect watermelon seedlings. However, *P. pseudoalcaligenes* strains failed to react with an antibody raised against the watermelon pathogen, indicating a dissimilarity (Schaad *et. al.*, 1978). Subsequently, DNA-rRNA hybridization showed that *P. pseudoalcaligenes* subsp. *citrulli* belonged to the *Acidovorax* rRNA complex in the rRNA superfamily III, and the bacterium was transferred to the *Acidovorax* genus and renamed *A. avenae* subsp. *citrulli* (Willems *et. al.*, 1992). Most recently Schaad *et. al.* (2008) showed that the bacterium had low similarity (less than 50%) based on DNA/DNA hybridization.
to other _A. avenae_ subspecies and recommended that the pathogen be elevated to species level and renamed _A. citrulli_. I agree with these findings and will refer to the pathogen as _A. citrulli_ throughout this dissertation.

_Acidovorax citrulli_ produces non-fluorescent (on King’s B medium), round, smooth, and slightly convex colonies capable of growing at 41°C but not 4°C (Schaad et. al., 1978). The gram-negative aerobic rod-shaped bacterium is approximately 0.5 – 1.7 μm in length and bears a single polar flagellum (Schaad et. al., 1978). The bacterium has a GC content of 67.2 - 68.53% and is catalase, oxidase, lipase and urease positive, and arginine dihydrolase negative (Somodi et. al., 1991). _A. citrulli_ can use citrate, ethanol, fructose, lactate, β-alanine, α-aminopentanoate, ethanolamine, glutarate, L-leucine, levinulate, n-propanol, succinate, D-xylose and D-serine as sole carbon sources (Saddler, 1994; Schaad et. al., 1978). On the other hand, carbon sources such as mannose, ribose, salicin, sorbitol, adonitol, benzoate, cellobiose, dextrin, dulcitol, erythitol, inositol, inulin, lactose, maltose, melezitose, raffinose, rhamose, sucrose, D-tartate and trehalose were utilized by the strains tested (Schaad et. al., 1978).

**Epidemiology**

Bacterial fruit blotch was first observed in commercial watermelon fields in 1989, where the pathogen caused up to 100% fruit yield losses (Somodi et. al., 1991). It can also cause 5-50% loss in neighboring fields depending on environmental conditions (Latin and Hopkins, 1995). The bacterium is seedborne and early symptoms including water-soaked lesions on the undersides of cotyledons and along hypocotyls can develop on seedlings 7 – 10 days after planting (Latin and Hopkins, 1995). Leaf lesions are dark brown and can appear water-soaked on the undersides of leaves. On the fruit, dark olive-green, irregularly-shaped water-soaked lesions
can develop on the upper surfaces (Latin and Hopkins, 1995). These lesions can result in the rupture of the rind and a sticky effervescent ooze is frequently observed (Latin and Hopkins, 1995). Secondary colonization by saprophytes can lead to fruit rot.

The BFB disease cycle begins with infested seed that represent the most significant source of primary inoculum. Seedlings are often used to plant watermelon fields and transplant house conditions, including high relative humidity, high temperature, high plant populations and overhead irrigation, favor pathogen multiplication and spread (Latin et. al., 1995). Overhead irrigation leads to secondary inoculum spread by splash dispersal, which increases the number of infected seedlings in transplant production facilities (Latin and Hopkins, 1995). *A. citrulli* can also overwinter in the field on seed from infected fruits. As seedlings emerge from these seed they can develop lesions that serve as a sources of inoculum and initiate secondary disease cycles in the field (Hopkins, 1993). The ability of the pathogen to overwinter on fruit debris and grow epiphytically on non-host plants may explain some recurring BFB outbreaks, but in most cases outbreaks are initiated from infested commercial seed sources (Latin and Hopkins, 1995).

**Geographic distribution and host range**

Since the first BFB outbreaks, the disease has been reported in cucurbit producing regions around the world including the Americas (Brazil, Costa Rica, Nicaragua, and the United States), Asia (Taiwan, Japan, China), the middle east (Israel and Turkey) and Australia (Burdman et. al., 2005; Cai et. al., 2005; Deng et. al., 2010; Macagnan et. al., 2003; Martin and Horlock, 2002; Mirik et. al., 2006; Munoz and Monterroso, 2002; Shirakawa et. al., 2000; Somodi et. al., 1991). Generally, BFB outbreaks can occur wherever watermelons are produced in hot humid environments. In the United States, BFB as been reported in Florida, Georgia,
South Carolina, North Carolina, Illinois, Missouri, Iowa, Oregon, Oklahoma, Texas and Delaware (Babadoost and Pataky, 2002; Black et al., 1994; Evans and Mulrooney, 1991; Hamm et al., 1997; Jacobs et al., 1992; Langston et al., 1999b; Latin and Rane, 1990; Somodi et al., 1991). Additionally, BFB outbreaks have occurred on other curcurbit hosts including *Cucumis melo reticulates* (rockmelon), *Cucumis melo* var. *inodorus* (honeydew), *Cucurbita pepo* (pumpkin), *Citrullus lanatus* var. *citroides* (citronmelon), *Cucumis myriocarpus* (prickly paddy melon) and *Cucumis sativus* (cucumber) (Isakeit et al., 1997; Isakeit et al., 1998; Langston et al., 1999a; Martin and O'Brien, 1999). *A. citrulli*, identified using fatty acid profiles, has been reported on tomato seed imported to Israel (Assouline et al., 1997). When inoculated onto fruits of tomato, bell pepper and eggplant, lesions were observed (Nascimento et al., 2004).

**Genetic diversity of Acidovorax citrulli**

In the past decade, increases in frequency of BFB outbreaks and expansion of the pathogen’s host range suggested changes in *A. citrulli* population structure. Diversity within an Australian *A. citrulli* population was first shown by O'Brien and Martin in 1999 using sole substrate utilization profiles and pathogenicity assays (O'Brien and Martin, 1999). The diversity of *A. citrulli* was further assessed using strains from the USA, Brazil, Australia, Thailand, Israel and China and from different cucurbit hosts including watermelon, cantaloupe and pumpkin using fatty acid analysis and DNA fingerprinting (Walcott et al., 2004). DNA fingerprint analysis by restriction endonucelase digestion and pulse field gel electrophoresis revealed the existence of at least 28 unique haplotypes designated A-Z, E2, and E3 (Burdman et al., 2005; Walcott et al., 2004). These haplotypes clustered into two groups designated I and II that could also be delineated by fatty acid methyl ester composition, sole carbon source utilization profiles,
pathogenicity on different cucurbit hosts and DNA fingerprinting using REP-PCR (Walcott et al., 2004). Approximately 93% of group II strains were isolated from watermelon, compared to only 18% of group I. Additionally the group II pathogens were reported to be more aggressive on watermelon than group I, while group I strains were more aggressive on cantaloupe and squash. Ninety six percent of the group II strains were sensitive to copper and capable of utilizing L-leucine while only 17% of group I strains were copper sensitive (Walcott et al., 2004).

Segregation of A. citrulli into two groups was further confirmed by Burdman et al. (2005) using a small population of strains recovered in Israel and by Feng et al. (2009b) using multilocus sequence typing of strains collected from around the world.

**Seed transmission**

*A. citrulli* was suspected to be seed transmitted when 50-80% of seedlings from seed extracted from infected fruit developed symptoms (Sowell and Schaad, 1979). Contaminated seedlots are the primary source of inoculum for BFB outbreaks in fields and transplants houses, highlighting the importance of understanding the interactions between *A. citrulli* and the seed during early stages of germination. Seed transmission also occurs in other cucurbits including muskmelon, squash, honeydew, cucumber, rockmelon and pumpkin, but the rate of seed transmission varies among cucurbit species and cultivars (Hopkins and Thompson, 2002; O'Brien and Martin, 1999). Pathogens are believed to infest seed by three possible mechanisms. Seed may come into contact with infected ovary tissues during extraction; the pathogen may move systemically from the mother plant to ovules; or blossoms might be infected leading to seed infection within symptomless fruit. Inoculation of watermelon blossoms resulted in infested seed within asymptomatic fruits, which gave rise to infected seedlings. Bacterial fruit
blotch seedling transmission for seed generated in this manner ranged from 5 - 9% (Walcott et. al., 2003).

Management of bacterial fruit blotch of cucurbits

The most effective strategy for managing BFB is excluding *A. citrulli* from seed, fruit or transplant production systems. This requires the use of pathogen-free seed and has mandated production of seed in cool, dry climates that limit the multiplication and spread of the bacterium. It also requires rigorous seed health testing (Walcott et. al., 2003).

To exclude the pathogen from fruit production fields, seed testing must be performed. This involves the use of procedures such as seedling grow-out assays or immunomagnetic separation (IMS) and PCR (Walcott and Gitaitis, 2000). The seedling grow-out assay involves growth of seed under conditions suitable for BFB development. Seedlings are then visually examined for BFB symptoms. IMS-PCR uses paramagnetic beads coated with antibodies specific to the pathogen of interest to separate it from a mixed population of cells. DNA is then extracted from captured cells for use in PCR amplification (Walcott and Gitaitis, 2000). Despite rigorous seed health testing (up to 50,000 seed per lot by the seedling grow-out assay), BFB outbreaks continue to occur sporadically, suggesting that current seed health testing technology is inadequate for disease management.

Seed treatment with disinfectants have also been attempted for BFB (Hopkins et. al., 2003). Hopkins *et. al.* (2003) reported 0% BFB transmission when seed were treated with either hydrochloric acid or peroxyacetic acid, while a pathogen transmission rate of 42% was observed for untreated seed. Unfortunately, this study evaluated the efficacy of these treatments on seed with surface contamination of *A. citrulli*. The efficacy of these chemicals must be evaluated
using naturally infested seed, since *A. citrulli* can be localized under the seed coat (Dutta *et. al.*, 2008). Using acidic electrolyzed water (AEW), Feng *et. al.* (2009a) reported significant reductions in BFB seedling transmission with naturally infested watermelon seed with 0.5% infested seed. AEW should be evaluated on seedlots with higher infestation levels to determine its efficacy on a commercial scale (Feng *et. al.*, 2009a).

If BFB outbreaks in the field copper bactericides such as Kocide can be used for BFB management (Hopkins, 1995). Copper sprays should be applied weekly to manage BFB if infected plants are observed in the field, and biweekly in the absence of symptoms. Under greenhouse conditions drip or ebb and flow irrigation should be employed rather than overhead irrigation to limit bacterial spread (Hopkins, 1995).

As with most plant diseases, resistance is the optimal strategy for BFB management. Currently BFB resistance is not available in commercial watermelon or melon cultivars. The challenge with identifying BFB resistant plants is *A. citrulli*’s ability to infect the plant at any stage of growth. One thousand three hundred and forty-four *Citrullus* accessions obtained from the U.S. Plant Genetic Conservation Unit were tested for resistance to BFB and only five from Zimbabwe and Zambia were found to be resistant to BFB (Hopkins and Thompson, 2001). Although the resistance appeared to be expressed in the foliage, the authors claimed that it could reduce the inoculum produced on leaves and thereby reduce the risk of BFB development on fruits (Hopkins and Thompson, 2001). To address the challenge posed by BFB, Bahar *et. al.* (2009) screened commercially available and wild melon (*Cucumis melo*) lines for BFB resistance. The authors determined the response of the melon cultivars/lines to BFB in seedling transmission assays as well as assays that involved screening seedlings and mature plants (Bahar *et. al.*, 2009). The authors did not identify resistant melon cultivars/lines but they identified a
commercial cultivar, ADIR339, that was highly tolerant to BFB (Bahar et. al., 2009). In this study, melon cultivars/lines usually showed varying levels of tolerance to BFB depending on the assay used. For example commercial cultivar YC-17016 was the most susceptible in seed-to-seedling transmission assays, but was highly tolerant when mature plants were inoculated (Bahar et. al., 2009).

The molecular mechanisms involved in watermelon seed and seedling colonization by \textit{A. citrulli} have not been explored. Elucidating the host-pathogen interactions between \textit{A. citrulli} and cucurbit seed during germination may be important for developing a long term strategy for limiting BFB seed-to-seedling transmission. Studying the virulence systems involved in pathogenicity of other phytopathobacteria could facilitate a better understanding of the host-pathogen interactions involved in BFB. These systems include the type III and type II secretion apparati and cell-to-cell signaling by quorum sensing.

**THE TYPE III SECRETION SYSTEM**

Pathogenic bacteria can be distinguished from non-pathogenic relatives by pathogenicity-related genes that are usually arranged in clusters or islands (Hueck, 1998). These pathogenicity islands are thought to have evolved due to horizontal gene transfer (Hueck, 1998). The type III secretion system (T3SS) was first described in \textit{Yersinia enterocolitica} and is composed of approximately 20 proteins whose functions are conserved among different genera of phytopathobacteria (Hueck, 1998; Michiels et. al., 1990). Phytopathogenic bacteria are generally extracellular and the T3SS facilitates the secretion of effector proteins directly into the host plant cells (He et. al., 2004). These proteins are actively transported out of the cytoplasm across the bacterial inner and outer membranes and into the host cytoplasm via the hypersensitive response
and pathogenicity (hrp) pili. The T3SS is important for pathogenesis, and inactivation of critical T3SS genes usually results in reduction or loss of pathogenicity. The mechanism of secretion is conserved, but the proteins secreted by the T3SS are not, and this may explain the various disease symptoms observed on different hosts (Hueck, 1998).

The hypersensitive response (HR) is the manifestation of the incompatible plant defense response triggered by proteins translocated by the T3SS (Hueck, 1998). The HR, a type of programmed cell death, results in localized tissue necrosis at the site of infection and production of phenolics and other antimicrobial factors (Lindgren et al., 1986; Staskawicz et al., 1984). T3SS genes are required to elicit a HR on resistant or non-host plants and pathogenicity in susceptible plants and were termed hrp genes. hrp genes were first identified in Pseudomonas syringae pv. phaseolicola, a pathogen of bean (Lindgren et al., 1986). Nine genes are conserved between plant and animal pathogenic bacteria and they are termed hypersensitive response and pathogenicity genes conserved (hrc) (He et al., 2004). The production of a HR depends on the presence or absence of a corresponding plant resistance (R) gene whose protein product recognizes the activity of effectors secreted by the T3SS. This leads to initiation of defense responses that cause localized cell death and thereby prevent pathogen spread.

The T3SS has high similarity to the bacterial flagellar apparatus leading to the hypothesis that the two systems are evolutionarily linked (He et al., 2004). However, comparison of four conserved T3SS proteins to homologs found in the flagellum indicated that the T3SS did not evolve directly from flagella (Gophna et al., 2003). It is more likely that they diverged independently from a common ancestor (Gophna et al., 2003). The T3SS pilus assembles within the membrane of the bacterial cell forming a structure similar to the bacterial pilus. Hence, the T3SS pilus of plant pathogenic bacteria was termed the hrp pilus. It is similar in diameter to the
needle-like T3SS pilus found in animal bacterial pathogens, but is much longer. This is thought to be an adaptation for plant pathogenesis since animal cells lack cell walls (Roine et. al., 1997).

**Regulation and assembly of T3SS**

Regulation of type III secretion is closely monitored by the bacteria, because not only can effector proteins alert the plant to the presence of the pathogen, but synthesis of the T3SS is energy consuming. Therefore, regulation occurs at both transcriptional and post transcriptional levels (Tang et. al., 2006). Regulation is controlled by transcription factors and global regulatory networks that respond to environmental cues (Tang et. al., 2006). To ensure that the T3SS is turned on in the correct environmental conditions (inside the host), the T3SS can be induced by conditions that mimic the host environment. These include environmental factors such as osmolarity, concentration of oxygen, calcium, iron, nucleotides, pH, nutrient availability, host signals and cell density (Francis et. al., 2002; Galan and Collmer, 1999). In animal pathogens optimal expression occurs at 37ºC which is the internal temperature of mammals. In contrast, in phytopathogenic Psuedomonads the T3SS is expressed at lower temperatures (He et. al., 2004). Two component regulatory systems, members of the AraC transcription activators, quorum sensing, alternative sigma factors and RNA-binding proteins can act as regulators of T3SS (He et. al., 2004). In *R. solanacearum* the T3SS can be regulated by contact with the host cells, which ensures that effector proteins are transcribed and exported when there is maximum likelihood of successful infection (Aldon et. al., 2000).

Plant pathogenic bacteria can be allocated into two groups based on regulation of the T3SS. Group I *hrp* gene regulation has been described in pathogens such as *Pseudomonas syringae, Erwinia* spp. and *P. stewartii* and involves *hrpL*, a member of the ECF family of
alternative sigma factors (Tang et al., 2006). HrpL recognizes the hrp box (GGAACC-N15/16-CCACNNA) in the promoter region of regulated genes (Jin and He, 2001; Tang et al., 2006). Regulation of hrpL differs among these bacteria. In Erwinia spp. and P. stewartii regulation begins with the hrpX/Y operon. The HrpX sensor histidine kinase may perceive environmental signals and likely phosphorylates HrpY, the corresponding response regulator. HrpX/Y acts upstream of hrpS; however, the exact mechanism of hrpS induction by HrpY is not known. HrpS, a sigma-54 enhancer binding protein, in turn is required for induction of hrpL (Hutcheson et al., 2001; Tang et al., 2006). hrpL induction in P. syringae requires hrpS and hrpR, which can be regulated by multiple factors. This includes GacA/GacS, master regulators of multiple phenotypes, and hrpA, the major component of the T3SS pilus. Lon protease acts as a negative regulator of HrpR, a sigma-54 enhancer binding protein, by destabilizing the protein in non-inducing media (Hutcheson et al., 2001; Ortiz-Martín et al., 2010). hrpS is regulated by an anti-anti-activator mechanism that involves HrpV and HrpG. HrpV is thought to interact with HrpS in vivo thereby inhibiting proper function of the HrpS protein resulting in down regulation of T3SS (Ortiz-Martín et al., 2010). HrpG, a chaperone-like protein, likely suppresses HrpV by binding to it, thereby freeing HrpS (Wei et al., 2005).

In the group II organisms, Xanthomonas spp. and R. solanacearum, hrp operons are regulated by the AraC-like activators HrpX or HrpB, respectively. These proteins also recognize a PIP-box TTCGC-N15-TTCGC in Xanthomonas spp, and the hrpII box TTCG-N16-TTCG in R. solanacearum. hrpX and hrpB are regulated by HrpG a member of the OmpR family of two component response regulators (Brito et al., 1999; Wengelnik et al., 1996). In Ralstonia, hrpG is regulated by prhA/R, prhI, and prhJ, a multigene network that is induced by the presence of the plant cell (Brito et al., 2002). PrhA is a membrane bound protein that perceives the plant
signal (a cell wall component) and low nutrient availability (Yoshimochi et. al., 2009). PrhA transfers this signal to PrhR, a transmembrane protein that releases bound PrhI, an ECF sigma factor (Tang et. al., 2006). PrhI induces prhJ transcription, that in turn induces hrpG expression. The components upstream of hrpG in Xanthomonas spp. responsible for recognition of host and/or nutrient availability have yet to be identified.

**Proteins secreted by the T3SS**

Proteins secreted by the T3SS are effectors or chaperones. Effectors function inside the host cell and chaperones aid in the delivery of effectors into plant cells. The redundancy of these proteins makes mutational analysis studies difficult, as mutation in one gene may not lead to an easily recognizable phenotype. The avirulence phenotype can be used to identify avirulence (avr) genes. However, this depends on the presence of corresponding R genes in the host plant (Collmer et. al., 2002). Identification of effector genes based on the avirulence phenotype was first accomplished by expression of a cosmid library from an avirulent strain in a virulent strain of *P. syringae pv. glycinea* (Staskawicz et. al., 1984). The production of a HR in test plants led to isolation of the cosmid expressing the effector protein (Staskawicz et. al., 1987). Type III secreted proteins have also been identified based on their presence in pathogenicity islands or because they are flanked by other effectors (Jackson et. al., 1999). Identification of T3SS proteins can also be accomplished by genomic mining or secretion assays (Greenberg and Vinatzer, 2003; Guttman et. al., 2002). Effectors help bacteria colonize host tissues by interfering with host defense responses such as programmed cell death, strengthening of cell wall and non-host disease resistance (Grant et. al., 2006). Callose deposition around Arabidopsis cell walls, which occurs in response to bacterial colonization, can be inhibited by AvrPto of *P.*
syringae. The HR, triggered by Avr proteins injected into resistant cells, can decrease pathogen colonization. Some effectors like AvrB2 in *P. syringae* can repress programmed cell death induced by other avirulence proteins (Tsiamis *et. al.*, 2000).

The T3SS plays an important role in the pathogenesis of many Gram negative phytopathogens. Bioinformatic analysis has indicated the presence of a T3SS in *A. citrulli*; however, the role of the T3SS in *A. citrulli* pathogenesis and seed colonization has not been investigated.

**THE TYPE II SECRETION SYSTEM**

The type II secretion (T2S) system was first described in *Klebsiella pneumoniae*, where it was shown to be responsible for secretion of pullanase, a starch hydrolyzing protein (d'Enfert *et. al.*, 1987). Since then, the T2SS as been described in both human and plant bacterial pathogens such as *Vibrio cholerae, Legionella pneumophila, Pseudomonas, Xanthomonas campestris* and *Erwinia chrysanthemi* (Hales and Shuman, 1999; Iwobi *et. al.*, 2003). The T2SS secretes proteins that function in virulence and induction of plant defense responses (Jha *et. al.*, 2005). In most cases phytopathobacteria that lack type II secretion are less virulent. This has been shown for *R. solanacearum, E. carotovora* subsp. *atroseptica, X. campestris* pv. *campestris, X. oryzae* pv. *oryzae*, and *E. chrysanthemi* (Dow *et. al.*, 1987; Hinton *et. al.*, 1989; Kang *et. al.*, 1994; Sun *et. al.*, 2005). Protein secretion by the T2SS involves a two step process from inside the cell to the extracellular space. In the first step proteins are secreted into the periplasm via one of two pathways depending on the secretion signal within the protein sequence (Voulhoux *et. al.*, 2001). This may be the Sec-dependent or twin-arginine (Tat) pathway (Voulhoux *et. al.*, 2001). In the
second step the proteins are secreted across the outer membrane from the periplasm to the extracellular milieu.

The Sec system was initially thought to be the only method for translocation of T2S proteins across the inner membrane. The Sec system consists of the SecBDEFY and SecA (Akimaru et al., 1991). SecA is an ATP hydrolyzing protein that allows peptides with the correct signal sequence to pass (Lill et al., 1989). The SecB protein binds pre-secretory proteins in the cytosol (Watanabe and Blobel, 1989). SecDEFY proteins become integrated into the membrane to form the translocase (Pugsley, 1993). Proteins secreted via the Sec pathway are targeted to the Sec system by specific signal peptides. The signal peptide is located at the amino terminal of the protein with a short hydrophilic (N) region followed by a long hydrophobic (H) region (von Heijne, 1990). In Sec-dependent secretion, proteins are secreted unfolded into the periplasm (Voulhoux et al., 2001). However the protein has to be correctly folded for secretion out of the cell (Voulhoux et al., 2001). The Tat system is different from the Sec system in that it allows folded proteins across the inner membrane (Voulhoux et al., 2001). The Tat system is encoded by five genes organized in two loci, tatABCD and tatE, and as been extensively studied in E. coli. TatA is the smallest and most abundant protein and is thought to be the pore forming component of the TAT tranlocase (Voulhoux et al., 2001). TatE shares 50% identity and overlapping function with TatA. TatB and TatC are transmembrane proteins that function as the recognition complex for the TAT signal (Saier, 2006). TatD is a DNAse localized to the cytoplasm, whose role in the TAT secretory pathway is unknown. The Tat signal sequence composition is similar to that of the Sec system except for the presence of a twin-arginine motif in the N-terminal region and a positively charged Sec-avoidance signal in the C-terminal region (Cristobel et al., 1999). The Tat and Sec secretion signals target proteins to the bacteria.
periplasm, where select proteins may be retained or exported to the extracellular milieu. Since a secretion signal to target proteins in the periplasm to the T2SS pathway has not been identified, the identity of proteins secreted via the T2SS cannot be determined by bioinformatics.

The T2SS is usually encoded by 12 – 16 genes designated A - O and S (Filloux, 2004). Type II secretion structural protein D is a member of the secretin family, and is the outer membrane component of the T2SS. Proteins pass from the periplasmic space, through the pore created by this protein into the extracellular space (Kostakioti et. al., 2005). Protein D forms a stable multimer in the outer membrane (OM), with a conserved C-terminal and a variable N-terminal region (Kostakioti et. al., 2005). The N-terminal region extends into the periplasm and is believed to be involved in substrate recognition, interaction with other components of the T2SS, protein multimerization and possibly gating of the formed channel (Kostakioti et. al., 2005). The C-terminal portion of the protein is embedded within the OM forming the pore through which proteins are exported (Kostakioti et. al., 2005). Protein A and B when present, help with the translocation of protein D to the outer membrane (Hardie et. al., 1996). Prepilin proteins G, H, I, and J assemble into the pseudopilus (Filloux, 2004). Protein K is a possible prepilin whose inclusion in the T2 system might arrest elongation of the pilus (Jha et. al., 2005). Protein O is a prepilin peptidase, that methylates the new N-terminus produced after cleavage of the prepilin protein (Strom et. al., 1993). Protein E is a predicted ATPase located in the inner membrane facing the cytoplasm that provides energy for the translocation of prepilins across the inner membrane to the periplasm (Filloux, 2004). Proteins F, L, M and E are thought to form an inner membrane complex that forms a platform on which the pseudopilus is built (Jha et. al., 2005). Proteins C and N form interconnectors between the outer and inner membrane complex.
(Jha et. al., 2005). When present, protein S is a small outer membrane protein that stabilizes protein D and promotes protein D insertion into the outer membrane (Sandkvist, 2001).

The presence of more than one complete set of T2SS genes and atypical T2SS gene clusters have been described in some Gram-negative bacteria. Xanthomonas campestris pv vesicatoria, X. campestris pv. campestris and X. axonopodis pv. citri have two complete T2SS designated xcs and xps (da Silva et. al., 2002; Szczesny et. al., 2010). X. campestris pv. campestris strains with interruption in the xcs cluster were not affected in virulence, indicating that the xcs system does not significantly contribute to virulence in this pathogen. On the other hand, interruption of xps system led to reduced virulence on pepper plants (Qian et. al., 2005; Szczesny et. al., 2010). Interestingly a xcsD/xpsD double mutant was reduced in its ability to colonize pepper plant tissue compared to a xpsD mutant. This suggests that xcsD was could partially complement a xpsD mutation in X. campestris pv. campestris. In P. aeruginosa a second T2SS gene cluster, hxc (homologous to xcp) was described. Although hxc was not important to virulence, it was required for secretion of alkaline phosphatase LapA in phosphate-limiting conditions (Ball et. al., 2002). gspD and gspC homologs xphA and xqhA have been identified in the P. aeruginosa genome. These orphans genes are located in a distant part of the genome with regards to the T2SS gene clusters (Michel et. al., 2007). Incomplete gene clusters have also been described in R. solanacearum, where sequence analysis indicated the presence of one complete T2SS and three incomplete T2SS gene clusters (Genin and Boucher, 2004).

**Proteins secreted by the type II secretion system**

The plant cell wall acts as a barrier against phytopathogenic bacteria, but it can also serve as a potentially rich substrate on which bacteria can grow (Roberts et. al., 1999). The cell wall of
plants is composed primarily of complex carbohydrates such as cellulose, hemicellulose and pectin. Plant pathogens in turn secrete a battery of cell wall degrading enzymes such as pectinases (polygalacturonases, pectates lyases, pectin methyl esterases), cellulases, xylanases and proteases (Braun and Rodrigues, 1993; Kunkel and Chen, 2006; Saarilahti et al., 1990). These enzymes are capable of breaking down the plant cell wall allowing the release of nutrients, and promote pathogen spread between host cells (Kunkel and Chen, 2006). Type II secretion mutants lack the ability to secrete certain proteins and are usually attenuated in virulence (Jha et al., 2005; Liu et al., 2005; Szczesny et al., 2010). It is possible that exoproteins are needed for the breakdown of complex carbohydrates available to A. citrulli during watermelon seed germination and that they play a role in seed colonization and seedling transmission of BFB.

**ACYL HOMOSERINE LACTONE MEDIATED QUORUM SENSING**

Bacteria act cooperatively at certain population densities to coordinate certain activities via a process called quorum sensing (QS). Low molecular weight signal molecules diffuse out of cells between members of a population. Accumulation of these signals within cells activate cognate receptors (Beck von Bodman et al., 2003b). A range of compounds are employed as signaling molecules including 2-methyl-2,3,3,4-tetrahydrofuran (autoinducer 2), N-acyl homoserine lactones, 3-hydroxy palmitic acid methylester and cis-11-methyl-2-dodecenolic acid (diffusible signal factor) (González and Keshavan et al., 2006). Quorum sensing affects many biological functions including bioluminescence, swarming motility, biofilm formation, horizontal DNA transfer, production of antibiotics, extracellular polysaccharides and expression of pathogenicity factors (Atkinson et al., 2006; Choi and Greenberg, 1992; Flavier et al., 1997; Koutsoudis et al., 2006; Piper et al., 1993; Pirhonen et al., 1993). Intercellular communication
has been widely studied in the bacterium *Vibrio fischeri*, which uses QS to regulate its luminescence genes (Eberhard, 1972; Nelson, 1977). The genes for luminescence occur in two units, *luxR* and *luxICDABEG*, which are approximately 150 bp apart (Devine *et. al.*, 1989; Fuqua *et. al.*, 1994). The *luxI* gene encodes a protein that directs synthesis of the *V. fischeri* autoinducer (VAI), N-3-(oxohexanoyl) homoserine, whereas the other genes in this operon play mechanistic roles in light production (Devine *et. al.*, 1989). The bacterium is present at low cell density (less than $10^2$ cells per mL) when living free in the ocean and under these conditions *luxI* is transcribed at basal levels and VAI does not accumulate (Fuqua *et. al.*, 1994). When *V. fischeri* is present in jellyfish the population density increases and VAI accumulates (Eberhard, 1972; Nelson, 1977). The VAI interacts with the LuxR protein, a cell density-dependent transcriptional activator that activates transcription of the luminescence operon leading to bioluminescence (Devine *et. al.*, 1989; Fuqua *et. al.*, 1994).

Since the discovery of QS in *V. fischeri*, population density-dependent gene regulation has been identified in many other Gram-negative bacteria, such as *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Erwinia carotovora* and *Rhizobium leguminosa* (Cubo *et. al.*, 1992; Fuqua *et. al.*, 1994; Passador *et. al.*, 1993; Piper *et. al.*, 1993; Pirhonen *et. al.*, 1993). The LuxR protein binds to a 20-bp inverted repeat region termed the *lux* box that was first identified in the promoter region of the *lux* operon, *luxICDABEG* (Devine *et. al.*, 1989). At low autoinducer levels the LuxR protein may be in an inactive state due to interaction of the C-terminal HTH domain with the N-terminal domain in the absence of the AHL signal (Choi and Greenberg, 1992). Binding of the autoinducer to the LuxR protein causes a conformational change that allows the protein to interact with *lux*-box promoter sequences and activate transcription (Choi and Greenberg, 1992).
Beck von Bodman et al. (1998) described a QS system in *P. stewartii* subsp *stewartii*, in which the *luxR* homolog functioned as a repressor of transcription. In this system the *luxI* homolog was not regulated by the cognate LuxR protein but was constitutively expressed (Beck von Bodman et al., 1998). LuxR regulators with repressor function have been identified in plant pathogens such as *P. ananatis* and *E. carotovora* and in animal pathogens such as *Serratia plymuthica* and *Serratia marcescens* (Horng et al., 2002; Jones et al., 1993; Morohoshi et al., 2007; Van Houdt et al., 2007). LuxR repressors can bind DNA in the absence of AHLs. Addition of AHL reduces the ability of these repressors to bind DNA which allows activation of transcription (Andersson et al., 2000). LuxR homologs with repressor functions such as EsaR (*P. stewartii*) and EanR (*P. ananatis*) can also repress their own function (Minogue et al., 2002; Morohoshi et al., 2007). These repressors can also act as weak transcription activators. EsaR and ExpR (*E. carotovora*) can induce expression of a *luxI-lacZ* fusion in the absence of AHL (Beck von Bodman et al., 2003a). Addition of AHLs antagonizes ExpR/EsaR induced lacZ expression leading to reduced transcript production. ExpR/EsaR are believed to act as repressors of genes required at high density and activators of genes required at low density.

Bacteria may encode more than one *luxI/R* QS system that can regulate different phenotypes or act together. CarI/R and and ExpI/R are both encoded by *E. carotovora*; however, CarI/R function in the production of the antibiotic carbepenem at high population densities, while ExpI/R function in the regulation of several secreted virulence factors (Jones et al., 1993). *P. aeruginosa*, a pathogen of plants and animals, encodes *lasI/R* that regulate expression of *rhlI/R* and function to control the expression of virulence related genes such as elastase and exotoxin A.
Quorum sensing regulation was first believed to be relatively simple, however, further research has revealed a complex regulatory network. In *P. syringae* expression of the *luxI* homolog, *ahlI*, is depends on a novel regulator, AefR (*autoinducer and epiphytic fitness regulator*), and the GacA/GacS two component signal transduction pathway (Beck von Bodman *et. al.*, 2003b). In *A. tumefaciens* QS regulates Ti plasmid conjugal transfer. TraR is regulated by a truncated LuxR homolog TrlR and an anti-activator, TraM, both of which function by binding to TraR thereby preventing it from binding to target sequences (Pierson *et. al.*, 1998).

Bacteria are capable of producing more than one AHL from a single *luxI* encoded enzyme, with one AHL usually being the predominant species. *P. stewartii* esal allows production of 3-oxo-hexanoyl-homoserine lactone and smaller amounts of 3-oxo-octanoyl-homoserine lactone (Beck von Bodman and Farrand, 1995). *E. chryshamthemi* ExpI can catalyze production of both 3-oxoC₆HL and C₆HL (Nasser *et. al.*, 1998). Acyl homoserine lactones are produced from the formation of an amide bond between S-adenosylmethionine (SAM) and acyl-acyl carrier protein (acyl-ACP) (Miller and Bassler, 2001). This reaction is catalyzed by specific *luxI*-encoded acyl homoserine lactones and the homoserine lactones produced only differ in their acyl side chains (Miller and Bassler, 2001). *A. citrulli* is predicted to have *luxI* and *luxR* homologs based on genomic sequence analysis. It is predicted that these genes play a role in colonization and/or pathogenicity of *A. citrulli* on watermelon seed and seedling tissue. Elucidating the role of QS in BFB development can lead to a better understanding of the pathogenicity of this system.
SPECIFIC OBJECTIVES

The objectives of this project are aimed at understanding the molecular mechanisms involved in *A. citrulli* pathogenicity on watermelon seed and seedlings. Quorum sensing, type II secretion and type III secretion have been implicated in bacterial pathogenicity but their role in BFB have not been explored. Hence the role of these systems in *A. citrulli* pathogenicity and more importantly seed colonization and BFB seedling transmission will be investigated using the sequenced strain, AAC00-1.

**Characterize the role of T3SS in *A. citrulli* pathogenicity and use of a T3SS mutant as a biological control seed treatment for BFB.**

The role of T3SS in virulence on watermelon leaf tissue will be examined using a *hrcC* deletion mutant of *A. citrulli* strain 00-1. This T3SS mutant strain will be used to investigate whether the *A. citrulli* T3SS contributes to the ability of this bacterium to colonize seed and seedling tissue. Furthermore we will determine if this mutant can serve as a biocontrol seed treatment.

**Determine the role of T2SS in *A. citrulli* virulence and colonization of watermelon seed and seedling tissues during germination.**

The role of the T2SS in pathogenicity will be determined using a T2SS mutant of *A. citrulli*. This mutant lacks the major pseudopilin gene, *gspG*, and is unable to secrete cellulase. We will investigate the capacity of this T2SS mutant to colonize watermelon seed and the role of T2SS in virulence. The identity of proteins secreted via the T2SS will be identified using mass
spectrometry and we will also investigate the contribution of specific T2S proteins to watermelon seed colonization by *A. citrulli*.

**Determine the role of QS in *A. citrulli* virulence and seed-to-seedling transmission of BFB.**

*A. citrulli* encodes *luxI* and *luxR* homologs designated *aacI* and *aacR*, respectively. The role of QS in *A. citrulli* virulence will be investigated using strains with *aacI* and *aacR* gene deletions. Using these mutants the role of QS in seed colonization and seed-to-seedling transmission of BFB will be investigated.

**LITERATURE CITED**


CHAPTER 2

EFFICACY OF A NON-PATHOGENIC *ACIDOVORAX CITRULLI* STRAIN AS A BIOLOGICAL CONTROL SEED TREATMENT FOR BACTERIAL FRUIT BLOTCH OF CUCURBITS

ABSTRACT

Bacterial fruit blotch (BFB) caused by the Gram negative bacterium, *Acidovorax citrulli*, is a serious threat to cucurbit seed and fruit production worldwide. In this study we investigated the efficacy of a non-pathogenic strain of *A. citrulli* as a biological control seed treatment for BFB. A type III secretion system mutant of *A. citrulli*, AAC00-1Δ*hrcC*, was generated that was non-pathogenic on watermelon, but retained its ability to colonize watermelon seed during germination. On watermelon seed naturally infested with *A. citrulli*, AAC00-1Δ*hrcC* reduced BFB seed-to-seedling transmission by 81.8% relative to non-treated seed. In comparison, an antagonistic *A. avenae* strain, AAA 99-2, reduced BFB seedling transmission by 74.6%. Additionally, seedlots from fruit for which female blossoms were treated with AAC00-1Δ*hrcC* and subsequently challenged with AAC00-1 displayed 8% BFB seedling transmission compared to 36% for seed from blossoms protected with PBS. Seedlots from blossoms treated with AAA 99-2 displayed 4% BFB transmission. These results suggest that non-pathogenic *A. citrulli*, AAC00-1Δ*hrcC*, has potential as a biological control seed treatment component in a comprehensive BFB management program.

Keywords: *Acidovorax avenae* subsp. *citrulli*, *Citrullus lanatus*, Type III secretion system, *hrcC*, Watermelon fruit blotch, Seed pathology
INTRODUCTION

Bacterial fruit blotch (BFB) of cucurbits, caused by the Gram negative bacterium *Acidovorax citrulli* (33), (formerly *A. avenae* subsp. *citrulli* (42)), is a serious economic threat to cucurbit seed and fruit production worldwide (32). Bacterial fruit blotch was reported in commercial watermelon fields in Florida in 1989 and frequent outbreaks caused significant economic losses across the southeastern U.S. in the early 1990s (35). The disease continues to be a sporadic threat to cucurbit producers worldwide (4, 26, 29), as an outbreak can lead to 100% yield loss.

Seed are the most important source of primary inoculum for *A. citrulli* and infested seed can initiate BFB epidemics under field or transplant house conditions (18). Watermelon seed can become infested via infection of immature fruit through open stomata (up to three weeks after anthesis) or by bacterial penetration of the pistil of female flowers (10, 40). Commercial seed producers bear the responsibility for producing *A. citrulli*-free seed. To date, the most effective strategy for managing BFB is excluding the pathogen from seed, fruit and transplant production systems. This involves producing seed in regions of countries with cool, dry climates, combined with visual seed field inspection and rigorous seed health testing (40). Seed treatments including disinfectants and fermentation are also routinely employed for BFB management (15, 16); however, since it is likely that the pathogen is located under the seed coat (30), the efficacy of externally applied bactericides can vary (16, 30).

Currently, there are no commercially available BFB-resistant watermelon cultivars. Early reports of resistance in some watermelon cultivars were disputed and while five cultivars with BFB resistance were identified, the horticultural traits of these lines were undesirable (17). Identification of resistant lines is further complicated by the fact that *A. citrulli* can infect all
growth stages. This was supported by Bahar et. al. (2), who observed that melon cultivars had varying levels of susceptibility to BFB, depending on the organ being infected. While some cultivars had BFB-tolerant foliage, they had high seedling transmission rates (2). In the absence of resistance, there is a need to develop a comprehensive integrated disease management strategy for BFB. One environmentally sound component that could be incorporated into this strategy is biological control seed treatments.

Biological control is the use of one organism to limit the damage potential of a pest and it has been previously explored for BFB management (9, 25, 41). Fessehaie and Walcott (9) demonstrated that seed treatments with *A. avenae* (AAA) (formerly *A. avenae* subsp. *avenae* (33)) strain 99-2, a pathogen of maize, limited the seed-to-seedling transmission of BFB. Additionally, when applied as a protectant to female watermelon blossoms, AAA 99-2 significantly reduced seed infestation by *A. citrulli* (9). However, since AAA 99-2 was pathogenic on maize it was unsuitable as a commercial seed treatment for BFB. One approach for developing a more suitable BFB biocontrol candidate is the generation of a non-pathogenic strain of *A. citrulli*. Non-pathogenic strains of phytopathogens have been used to manage plant diseases (27, 37, 38). These non-pathogenic strains can occur naturally or be generated by mutagenesis. One strategy that can efficiently abolish pathogenicity in phytobacteria is mutagenesis of the type III secretion system (T3SS) (3, 5). Type III secretion (T3S) is important for pathogenesis because it facilitates the translocation of virulence proteins from the bacterial cell directly into the cytoplasm of the host plant cell (13). Mutations in critical T3SS genes result in loss of pathogenicity and the ability to induce the hypersensitive response (HR) in non-host plants (21). The use of T3SS mutants as biocontrol agents has been reported for *X. campestris* pv. *vesicatoria* and *E. amylovora* (27, 37), the causal agents of bacterial spot of pepper and
fireblight of pear and apple, respectively. However, there have been no previous attempts to develop a biocontrol seed treatment for BFB based on a T3SS mutant of *A. citrulli*. Hence, the objective of this study was to develop and assess the efficacy of a T3SS mutant of *A. citrulli* as a biocontrol seed treatment for BFB.

**MATERIALS AND METHODS**

**Bacterial cultures, media and inoculum preparation.** *A. citrulli, A. avenae* and *Escherichia coli* strains used in this study are listed in Table 2.1. *A. citrulli* and *A. avenae* strains were routinely grown on nutrient agar (Difco, Becton Dickinson and Co., Sparks MD) with appropriate antibiotics for 48 h at 28°C. *E. coli* strains were grown in Luria Bertani (LB) broth or agar (Difco) with appropriate antibiotics for 24 h at 37°C. To prepare *A. citrulli* inoculum, bacterial cells were pelletted from 1.5 mL of overnight LB broth culture by centrifugation at 13,000 rpm for 1 min. Cells were washed with and resuspended in phosphate buffered saline (PBS, 137 mM NaCl, 5.6 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄). Bacterial suspensions containing ~1 × 10⁸ CFU/mL were estimated using a spectrophotometer (OD₆₀₀ ~ 0.3) (Spectronic 20, Baush and Lomb, Rochester, NY) and ten-fold serially diluted with PBS to generate cell suspensions with desired concentrations. When used, antibiotics were incorporated into media at the following concentrations; rifamycin (100 μg/mL, *A. citrulli*), spectinomycin (100 μg/mL, *A. citrulli, E. coli*), kanamycin (50 μg/mL, *A. citrulli, E. coli*), and cycloheximide (200 μg/mL, *A. citrulli*) (Sigma Aldrich, St. Louis, MO).

**DNA manipulations.** A non-pathogenic *A. citrulli* T3SS mutant (AAC00-1ΔhrcC) was generated by deletion of the *hrcC* gene that encodes the T3SS outer membrane pore (22). Briefly, a 3.6-kb fragment containing the *hrcC* gene was identified in the genome library of
AAC00-1. The 3.6-kb fragment was digested from the cosmid clone containing the hrcC gene using BamHI (Promega, Madison, WI), cloned into pBluescript and transformed into E. coli strain DH5α. Six-hundred and seventy three base pairs were deleted from the cloned fragment by restriction digestion with StuI and BstEII (Promega, Madison, WI). The fragment was re-ligated resulting in deletion of hrcC. The flanking open reading frames, SAM methyl transferase and hrpD5 remained unaffected. The deleted hrcC gene construct was cloned into the suicide vector pOK1, which carried the levansucrase SacB gene, to generate pOK-HRCC. Plasmid pOK-HRCC was transformed into E. coli strain S17-1 (λpir), which was mated with AAC00-1 and single-crossover transconjugants were selected based on resistance to spectinomycin. A second homologous recombination was promoted by growth on M9 agar plates containing sucrose, which retards the growth of bacteria expressing SacB. Generation of the deletion mutation was confirmed by polymerase chain reaction (PCR) amplification using primers, HrcC.for (5’-TGT ATT TCT ACC CGG GCA AGT CC-3’) and HrcC.rev (5’-TCC TCG ATG TAG AGA CTC AGC TTG-3’). One hrcC deletion mutant, designated AAC00-1.1 was found to be suitable for our studies. The hrcC deletion mutant was complemented by sub-cloning the 3.6-kb BamHI fragment containing the hrcC gene into the cosmid, pUFR043, to produce pUFR034-hrcC. pUFR034-hrcC was subsequently transformed into E. coli strain S17-1 (λpir) for use in triparental mating. pUFR034-hrcC was subsequently transformed into the T3SS mutant, AAC00-1ΔhrcC, to create strain AAC00-1ΔhrcCcomp.

**Pathogenicity and hypersensitive response assays.** The effect of the hrcC gene deletion on the ability of A. citrulli to induce a HR was evaluated using six-week-old tobacco plants (Nicotiana tabacum). Using a hypodermic syringe with no needle, ~ 1 × 10⁸ CFU/mL of AAC00-1, AAC00-1ΔhrcC, AAC00-1ΔhrcCcomp or PBS was infiltrated into the intercellular
spaces of leaves between the major veins of a fully expanded tobacco leaf. Plants were incubated under conditions of approximately 75% relative humidity (RH) with 12 h florescent light at 28ºC in a growth chamber (Percival, Perry, IA) for 2 days and subsequently visually observed for cell death associated with a typical HR. To determine the effect of the T3SS mutation on A. citrulli pathogenicity, two-week-old watermelon seedlings (cv. Sugar Baby) were infiltrated with \( \sim 1 \times 10^6 \) CFU/mL of AAC00-1\( \Delta hrcC \), AAC00-1\( \Delta hrcC \)comp (\( hrcC \) mutation complement), AAC00-1 (wild-type) or PBS as a negative control. The cotyledons of three seedlings were inoculated and plants were incubated for 2 days at 28ºC with approximately 75% RH and 12 h fluorescent light in a growth chamber. Plants were then visually observed for BFB symptoms (necrosis and water soaking). This experiment was repeated three times.

**Role of T3SS in A. citrulli colonization of watermelon seed during germination.** The ability of the T3SS mutant, AAC00-1\( \Delta hrcC \), to colonize watermelon seed (cv. Sugar Baby) during the early stages of germination was determined by infiltrating seed with \( \sim 1 \times 10^3 \) CFU of AAC00-1, AAC00-1\( \Delta hrcC \) or AAC00-1\( \Delta hrcC \)comp as previously described (39). Watermelon seed (n = 35) were incubated in plastic boxes (Tri-state plastics, Dixon KY) on moist blotter paper (Hoffman Manufacturing, Albany, OR) at 30ºC with 100% RH and continuous fluorescent light. Seed samples (n = 5 seed) were collected at 0, 6, 12, 24, 48, 72, 96 h after planting and bacterial populations were estimated by macerating each seed individually in 900 \( \mu \)L of PBS in a sterile microcentrifuge tube. Appropriate ten-fold serial dilutions of each seed homogenate were spotted onto NA plates with appropriate antibiotics and incubated for 48-72 h at 28ºC. Subsequently, A. citrulli colonies were enumerated. The experiment was repeated three times and mean bacterial populations were plotted over time to generate area under population dynamics curves (AUPDC). Analysis of variance was conducted on AUPDC data using JMP statistical software.
Role of T3SS in *A. citrulli* virulence. To determine the role of T3S in virulence, a suspension with ~ $1 \times 10^3$ CFU/mL of AAC00-1, AAC00-1Δ*hrcC* or AAC00-1Δ*hrcC*comp was infiltrated separately into the cotyledons of three, two-week-old watermelon seedlings (cv. Sugar Baby). Infiltrated leaves were allowed to air-dry for 1 h, then six leaf discs, 3 mm each (1 from each cotyledon) were collected at 0, 12, 24, 48, 72, 96 h after inoculation. Each leaf disc was macerated in a separate microcentrifuge tube containing 900 $\mu$L of PBS and appropriate ten-fold serial dilutions were spotted onto NA amended with appropriate antibiotics to enumerate bacterial populations. The experiment was repeated three times and bacterial populations were plotted over time and to generate AUPDC data. AUPDC data were used to compare the impact of the T3SS mutation on *A. citrulli* virulence using Tukey-Kramer’s HSD.

Efficacy of AAC00-1Δ*hrcC* as a biocontrol seed treatment for BFB. To evaluate the efficacy of the T3SS mutant as a biocontrol agent for BFB, watermelon seed naturally infested with *A. citrulli* were used. Naturally infested seed were extracted by hand from symptomatic watermelons from a natural BFB outbreak in Reidsville, GA in 2005. After extraction, seed were air-dried at 25°C and stored at 4°C until they were used. To assess the efficacy of the biocontrol seed treatment, naturally infested seed (n = 100) were incubated in 20 mL of cell suspension containing ~ $1 \times 10^8$ CFU/mL of AAC00-1Δ*hrcC*, AAA 99-2 or PBS, as a negative control. Watermelon seed were then subjected to continuous vacuum for 20 min, which allowed the biocontrol agents to be deposited under the testae. After inoculation seed were air-dried at ~ 25°C for 24 h and then assayed for seed-to-seedling BFB transmission using modified seedling
grow-out assay. Briefly, seed (n = 100) were incubated in transparent plastic boxes on two layers of blotter paper (Hoffman Manufacturing) saturated with distilled water. Germination boxes were incubated in a growth chamber (Percival, Perry, IA), under conditions of 100% RH, 30°C, and continuous fluorescent light for 12 days. Seed were monitored daily and germination percentage (number of seed that produced radicles and plumules divided by the total number of seed planted × 100) and BFB seedling transmission (number of seedlings displaying typical BFB symptoms divided by the number of seedlings that germinated × 100) percentages were recorded daily. The experiment was repeated twice and mean BFB seedling transmission percentage data were plotted over time to generate area under disease progress (AUDPC) data. AUDPC data were used to conduct ANOVA and to determine the significance of the effect of AAC00-1ΔhrcC on BFB seedling transmission using Tukey-Kramer HSD. Seed germination data were used to determine the effect of AAC00-1ΔhrcC on seed physiology. The ability of AAC00-1ΔhrcC and AAA 99-2 to reduce BFB incidence compared to the PBS negative control was determined using the formula described by Moss et. al. (27).

**Evaluation of AAC00-1ΔhrcC as a biocontrol seed treatment under greenhouse conditions.** Naturally infested watermelon seed (cv. Crimson Sweet) were produced by hand pollinating, then inoculating blossoms of greenhouse-grown plants with AAC00-1. Fruits from inoculated blossoms were allowed to develop to maturity (35 days after anthesis) and seed were extracted by hand. After extraction, seed were air-dried at 25°C for 24 h and samples (n= 100 seed) were treated with ~ 1 × 10⁸ CFU/mL of AAC00-1ΔhrcC or PBS as a negative control as described previously. Treated seed were air-dried at room temperature for 24 h and then assayed for seed-to-seedling BFB transmission under greenhouse conditions as follows. Seed (n = 100) were planted in 128-cell polystyrene flats (Speedling Inc. Sun City Fl.) filled with sterile potting mix
(90% composted pine bark and 10% vermiculite). Flats were incubated in the greenhouse in plastic chambers used to generate conditions of 30°C and ~100% RH for 21 days. Seedlings were monitored daily and germination and BFB seedling transmission percentages were recorded. The experiment was repeated three times and mean BFB seedling transmission percentages were plotted over time to generate AUDPC data. AUDPC data were used to conduct ANOVA and to determine the significance of the effect of AAC00-1ΔhrcC on seedling transmission of BFB using the Student’s t test.

**Efficacy of AAC00-1ΔhrcC as a biocontrol blossom protectant to prevent watermelon seed infestation by *A. citrulli***. Watermelon plants (cv. Crimson Sweet) were maintained under standard greenhouse conditions in 15-L pots containing 90% composted pine bark and 10% vermiculite until anthesis. At anthesis, female blossoms were manually pollinated by rubbing pollen from male blossoms onto the stigmatic surfaces, and then were immediately inoculated by depositing 10 μL of a suspension containing ~1 × 10^8 CFU/mL of AAC00-1ΔhrcC, AAA 99-2 or PBS, as a negative control onto the stigmas. Five hours after inoculation, blossoms were challenged with 10 μL of a cell suspension containing 1 × 10^9 CFU/mL of AAC00-1. Each treatment was applied to 10 blossoms, each on a separate plant, and fruits were allowed to develop for 35 days after pollination. At harvest maturity, fruits were surface sterilized and seed were manually extracted (seed from each fruit were maintained as a separate lot), air-dried at 25°C for 24 h and stored at 4°C. To determine the level of seed infestation by *A. citrulli*, samples (*n = 100 seed*) from each lot were subjected to the modified seedling grow-out test as described above. Seed were monitored daily for 14 days and germination and BFB seedling transmission percentages were recorded daily. This experiment was repeated twice, and mean BFB seedling transmission percentage data were plotted over time and used to generate AUDPC data.
Germination data were also collected and used to determine the effect of biocontrol blossom treatments on seed physiology. AUDPC data were analyzed using the Student’s \( t \) test to determine the significance of blossom protection on seed infestation by *A. citrulli*.

**Inoculation of watermelon seed with AAC00-1\(\Delta hrcC\) via blossom treatment.** To determine if blossom inoculation was an effective approach to inoculate seed with biocontrol agents, female blossoms were inoculated with AAC00-1\(\Delta hrcC\) and the resulting seed were assayed for the bacterium by quantitative real-time PCR. Watermelon plants (cv. Crimson Sweet) were grown under greenhouse conditions in 15-L pots as described above. At anthesis, female blossoms were hand pollinated and stigmas were immediately inoculated with 10 \( \mu \)L of a \( \sim 1 \times 10^8 \) CFU/mL suspension of AAC00-1\(\Delta hrcC\) or AAC00-1 as described above. Inoculated blossoms were allowed to develop to harvest maturity. For each treatment, five mature fruit were harvested and seed were extracted and stored at 4ºC. The level of seed infestation was determined by extracting total microbial DNA from seed and conducting quantitative real-time PCR. Seed \((n = 50)\) were macerated for 5 min using a homogenizer (Bioreba Homex 6, Bioreba, Gilroy, Ca) and suspended in 10 mL of PBS. One milliliter of the suspension was pelleted by centrifugation at 13,000 rpm for 3 min. The PBS was decanted and total microbial DNA was extracted from the pellet using a Mobio Ultraclean Microbial DNA isolation kit (Mobo Laboratory Inc, Carlsbad, CA), according to the manufacturers instructions. Extracted DNA was dissolved in 20 \( \mu \)L of water and 5 \( \mu \)L was used for real-time PCR. For real-time PCR the mastermix was comprised of 12.5 \( \mu \)L of Biorad real-time PCR mastermix (Biorad Laboratories, Hercules, CA), 7.5 pM of BOXAACF and BOXAACR2 primers and 5 pM TaqMan probe (11). Thermal cycling conditions included denaturation at 95ºC for 2 min, 35 cycles of denaturation at 95ºC for 15 sec. and annealing and extension at 55ºC for 45 sec. The experiment was repeated.
twice and mean cycle threshold (Ct) values were recorded. A standard curve was generated using ten-fold serial dilutions of *A. citrulli* cell suspensions (1 × 10^8 - 1 × 10^6 CFU/mL). An amplification threshold value of 30 fluorescence units was routinely used, and the experiment was repeated four times. The standard curve was constructed by plotting mean Ct values against Log_{10} *A. citrulli* cell dilutions and it was used to estimate *A. citrulli* populations in seed. The effect of the T3SS mutation on colonization of watermelon seedling tissue was determined using the Student’s t test.

**RESULTS**

**Generation and characterization of a T3SS mutant of *A. citrulli***. Deletion of the AAC00-1 *hrcC* gene was confirmed by PCR amplification (Fig. 2.1). Using primers for the *hrcC* gene, a 1.08 Kb DNA fragment was amplified from wild-type AAC00-1, and the complemented strain, AAC00-1Δ*hrcC*comp. However, the 1.08 Kb fragment was absent from the *hrcC* deletion mutant, AAC00-1Δ*hrcC*. A smaller 0.4 Kb fragment was amplified from AAC00-1Δ*hrcC* and AAC00-1Δ*hrcC*comp indicating deletion of the *hrcC* gene (Fig. 2.1). *hrcC* deletion resulted in the loss of T3S in AAC00-1, which was confirmed by lack of an HR when AAC00-1Δ*hrcC* was infiltrated into tobacco leaves (Fig. 2.2A). In contrast, AAC00-1 (wild-type) and AAC00-1Δ*hrcC*comp (*hrcC* complement) induced a HR (Fig. 2.2A) on tobacco leaves. As expected, leaf infiltration with PBS yielded no HR on tobacco. When infiltrated into watermelon seedlings AAC00-1 and AAC00-1Δ*hrcC*comp produced typical watersoaked and necrotic lesions by two days post inoculation (dpi) (Fig. 2.2B). In contrast seedlings infiltrated with AAC00-1Δ*hrcC* did not develop BFB symptoms and were comparable to seedlings inoculated with PBS (Fig. 2.2B).
Role of T3S in *A. citrulli* colonization of watermelon seed during germination. Both AAC00-1Δ*hrcC* and AAC00-1Δ*hrcC*comp colonized watermelon seed at wild-type levels during the first 96 h of germination. For all strains, population increases on watermelon seed were observed by 6 h after planting (Fig. 2.3A). AAC00-1Δ*hrcC* and AAC00-1Δ*hrcC*comp increased continuously over the course of the experiment (96 h), similar to AAC001-1. By 96 h after planting the mean populations of AAC00-1, AAC00-1Δ*hrcC* and AAC00-1Δ*hrcC*comp were 1.5 × 10⁸, 3.4 × 10⁷, and 5.4 × 10⁸ CFU/g of seed, respectively (Fig. 2.3A). For seed inoculated with AAC00-1 and AAC00-1Δ*hrcC*comp seedlings emerged and developed typical BFB symptoms on cotyledons by 14 days after planting (dap). In contrast, seedlings from seed inoculated with AAC00-1Δ*hrcC* emerged and remained asymptomatic by 14 dap. ANOVA conducted on AUPDC data indicated that the effect of experiment was not statistically significant (P = 0.39), therefore data from three experiments were pooled and analyzed. There was no significant difference in the colonization of watermelon seed by AAC00-1Δ*hrcC*, AAC00-1 and AAC00-1Δ*hrcC*comp according to Tukey-Kramer’s HSD (P = 0.17) (Fig. 2.3B).

Role of T3S in the virulence of *A. citrulli*. Populations of AAC00-1 and AAC00-1Δ*hrcC*comp increased continuously over the 96 h period after cotyledon infiltration. AAC00-1 and AAC00-1Δ*hrcC*comp populations increased 100-fold from ~ 1 x 10¹ CFU/disc at 0 hours post inoculation (hpi) to 1 x 10³ CFU/disc by 24 hpi. On the other hand, populations of AAC00-1Δ*hrcC* remained at 10¹ CFU/disc for the first 72 hpi. AAC00-1 and AAC00-1Δ*hrcC*comp colonized watermelon cotyledons at similar rates reaching mean population levels of 2.2 x 10⁷ CFU/disc and 4.3 × 10⁶ CFU/disc, respectively, by 96 hpi (Fig. 2.4A). In contrast, AAC00-1Δ*hrcC* only reached a population of 2.0 x 10² CFU/disc by 96 hpi (Fig. 2.4A). The effect of the
T3SS on *A. citrulli* colonization watermelon cotyledon tissue was statistically significant (*P* = 0.0004) (Fig. 2.4B).

**Efficacy of AAC00-1Δ*hrcC* as a biocontrol seed treatment.** Watermelon seed naturally infested with *A. citrulli* displayed a mean BFB seedling transmission percentage of 55 (± 23, (standard deviation)) % (Fig. 2.5A). Seed treated with the biocontrol agents, AAC00-1Δ*hrcC* and AAA 99-2, displayed BFB seedling transmission percentages of 10 (±9) and 14 (±10) %, respectively by 12 dap (Fig. 2.5A). The reduction in BFB incidence was approximately 5.5- fold for AAC00-1Δ*hrcC* and 3.6-fold for AAA 99-2. Tukey-Kramer’s HSD analysis indicated that BFB seedling transmission percentages for seed treated with AAA 99-2 and AAC00-1Δ*hrcC* were significantly lower than for seed treated with PBS. However, BFB seedling transmission for seed treated with AAC00-1Δ*hrcC* was not significantly different to that of seed treated with AAA 99-2 (*P* = 0.8) (Fig. 2.5B). Treatment of naturally infested seed with AAC00-1Δ*hrcC* and AAA 99-2 delayed BFB onset by 2 and 1 day, respectively. When seed were treated with AAC00-1Δ*hrcC* and AAA 99-2 BFB seedling transmission was reduced by 81.8% and 74.6%, respectively compared to PBS. BFB seedling transmission was observed in 100% of the repetitions of the experiment when seed were treated with PBS or AAA 99-2. However, BFB seedling transmission was observed in 75% of the repetitions when AAC00-1Δ*hrcC* was used.

**Evaluation of AAC00-1Δ*hrcC* as a biocontrol seed treatment in the greenhouse.** To determine the applicability of AAC00-1Δ*hrcC* as a biocontrol seed treatment under transplant house conditions, seeds naturally infested with AAC00-1 were treated and planted under greenhouse conditions that were highly conducive to BFB development. AAC00-1Δ*hrcC* reduced BFB seedling transmission and epidemic development relative to the PBS negative control. Seed naturally infested with AAC00-1 were treated with PBS as a negative control, these
displayed a mean BFB seedling transmission percentage of 37 ± 3% (Fig. 2.6A). In contrast, seed treated with AAC00-1ΔhrcC displayed 23 ±7% BFB incidence by 21 dap (Fig. 2.6A). AAC00-1ΔhrcC seed treatments reduced BFB seedling transmission by 37.8%. Statistical analysis using the students t test indicated that BFB seed-to-seedling transmission percentage for seed treated with AAC00-1ΔhrcC was significantly lower than for seed treated with PBS (P = 0.02) (Fig. 2.6B). BFB development on AAC00-1ΔhrcC-treated seed was delayed by 5 days in the greenhouse assay compared to 2 days in the growth chamber assay.

**Efficacy of AAC00-1ΔhrcC as a biocontrol blossom protectant to prevent watermelon seed infestation.** Watermelon fruit produced from blossoms protected with AAC00-1ΔhrcC or AAA 99-2 and subsequently challenged with AAC00-1 did not develop BFB symptoms at harvest maturity. Based on ANOVA, the effect of experiment on seed infestation was not significant (P = 0.64); hence the data for the two experiments were pooled for analysis. Blossom protection with biocontrol agents reduced seed infestation by *A. citrulli* and this effect was statistically significant (P < 0.01). Approximately 36% of seed produced from blossoms protected with PBS developed BFB symptoms in the modified seedling grow-out assay. On the other hand seedlots produced from blossoms protected with AAA 99-2 and AAC00-1ΔhrcC had mean BFB seedling transmission percentages of 4 and 8%, respectively (Fig. 2.7). Tukey-Kramer’s HSD analysis indicated that seed infestation via blossoms treated with AAA 99-2 or AAC00-1ΔhrcC was significantly less than for blossoms treated with PBS (P < 0.0002) (Fig. 2.7). However, there was no significant difference between the abilities of AAC00-1ΔhrcC and AAA 99-2 to limit seed infection by *A. citrulli* when applied as a blossom treatment (P = 0.8). ANOVA of seed germination percentage data indicated that the effect of experiment was statistically significant (P = 0.002). Hence the data for the two experiments were analyzed.
separately. In experiment 1 mean germination percentage was 91, 86, and 81% for PBS, AAA 99-2 and AAC00-1ΔhrcC, respectively. In experiment 2, seed from blossoms treated with PBS, AAA 99-2 or AAC00-1ΔhrcC displayed 98, 97 or 89% germination, respectively. Differences between germination percentages for seed produced from blossoms treated with PBS, AAA 99-2 or AAC00-1ΔhrcC were not significant in experiment 1 (P = 0.095) or 2 (P = 0.08). Germination percentage data from experiment 1 and 2 were presented together, because there was no significant difference between the treatments in both experiments (Fig. 2.7).

Efficiency of watermelon seed inoculation with a biocontrol agent via blossom colonization. AAC00-1ΔhrcC retained its ability to invade blossoms and colonize watermelon seed relative to wild-type pathogenic AAC00-1. One hundred percent of the seedlots produced in fruits from AAC00-1 or AAC00-1ΔhrcC inoculated watermelon blossoms were infested. AAC00-1ΔhrcC infested seedlots had population densities that ranged from 10^2-10^4 CFU/g of seed. In comparison, AAC00-1 infested seedlots had population densities that ranged from 10^3-10^4 CFU/g. The mean \emph{A. citrulli} population on seedlots infested with AAC00-1 (2.1 x 10^4 CFU/g) was similar to that of AAC00-1ΔhrcC (1.2 x 10^4 CFU/g) infested seedlots. \emph{A. citrulli} populations on seedlots produced from blossoms inoculated with AAC00-1ΔhrcC were not significantly different to those on seed produced from blossoms inoculated with AAC00-1 (P = 0.33).

DISCUSSION

In this study we confirmed that \emph{A. citrulli} has a functional T3SS encoded by the \emph{hrp} gene cluster. \emph{hrcC} encodes a multimeric protein that forms the outer membrane pore of the T3SS (6). Truncation of \emph{hrcC} in AAC00-1 abolished pathogenicity on watermelon cotyledon tissue and the
ability to induce a HR on tobacco plants. This observation suggests that the T3SS is required for foliar pathogenicity. As expected, complementation of the deleted hrcC gene restored functionality of the T3SS. While this is the first report of a functional T3SS in *A. citrulli*, it was expected as T3SS have been reported for other phytopathogenic bacteria (1, 24, 34).

Biological control of plant diseases usually involves competition for nutrients or colonization sites, and/or antibiosis (20, 31). The ability of a T3SS mutant of AAC00-1ΔhrcC to colonize seed was assessed to determine if a non-pathogenic strain could compete with wild-type bacteria during seed germination. AAC00-1ΔhrcC retained its ability to colonize the germinating watermelon seed and by 96 h after planting AAC00-1ΔhrcC populations were not significantly different to those of AAC00-1 or AAC00-1ΔhrcCcomp. The ability of a T3SS mutant of a phytopathogen to colonize seed at wild-type rates has been previously reported for *Pseudomonas syringae* pv. *syringae* B728a on bean (14). Similar to this study, Hirano *et. al.* reported that disruption of the T3SS did not affect colonization of the germinating bean seed (14). Based on these observations, we conclude that type III effector proteins are not essential for seed colonization by *A. citrulli* during the early stages of germination. It is logical to conclude that during the initial phases of seedling emergence, seedborne phytopathogens grow as epiphytes rather than as plant pathogens.

When applied as a seed treatment to naturally infested watermelon seed AAC00-1ΔhrcC suppressed BFB development at levels similar to AAA 99-2, a strain previously reported to be antagonistic to *A. citrulli*. When the biocontrol efficacy of AAC00-1ΔhrcC was evaluated in the greenhouse, AAC00-1ΔhrcC was able to delay BFB onset. Reduction of BFB seedling transmission was lower in the greenhouse assay (1.6 fold) than in the growth chamber assay (5.5 fold), which suggests that the biocontrol efficacy of AAC00-1ΔhrcC declined as the seedlings...
matured. This was likely due to the inability of AAC00-1ΔhrcC to colonize seedling tissue as a phytopathogen.

Pollination of blossoms leads to the production of stigmatic exudates, including sugars and proteins, that make the blossom an ideal environment for A. citrulli colonization (12). A. citrulli can colonize and penetrate watermelon female blossoms, which can lead to seed infestation without typical BFB fruit symptoms (18, 36). Interestingly, hrcC is not critical for blossom colonization and penetration as AAC00-1ΔhrcC was not affected in its ability to infest seed via this pathway. In two blossom inoculation experiments, 100% of seedlots generated were contaminated with AAC00-1ΔhrcC. Seed infestation levels for blossoms inoculated with AAC00-1ΔhrcC were not significantly different to those for seed from blossoms inoculated with AAC00-1. This suggests that the T3SS does not play a role in seed infection via blossoms. In contrast however, Darsonval et. al. reported that T3SS mutants of X. fuscans subsp. fuscans were impaired in their ability to be transmitted to bean seed via blossoms (7). This discrepancy might be explained by differences in the morphology of watermelon and bean flowers and the unique nature of the host-pathogen interactions. It is likely that in the case of watermelon and A. citrulli the interaction is non-specific and colonization and invasion of blossoms is passive and appears to be nonselective. This explanation is supported by the observation that Pantoea ananatis, a seedborne bacterium that is not pathogenic on watermelon was observed to colonize blossoms and infest watermelon seed (40). Lessl et. al. also reported that P. syringae cit7, an epiphyte recovered from tomato foliage could also actively colonize watermelon stigmas (23). It would be interesting to determine if bean flowers could be colonized and invaded by bacteria that were not bean pathogens.
The ability of T3SS mutants of phytobacteria to limit plant diseases caused by their pathogenic progenitors has been previously reported for *X. campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* (27, 43). Moss *et al.* (27) found that while the *hrp* mutants reduced disease severity, regulatory mutants were more effective than structural mutants. This is thought to be due to the induction of host defense responses that are greater in the presence of regulatory mutants (27). Because watermelon blossom and seed colonization does not require the T3SS, it is unlikely that *hrp* regulatory mutants of *A. citrulli* would improve biocontrol activity as seed or blossom treatments.

The use of chemical seed treatments has been limited in effectiveness with regards to BFB control because *A. citrulli* may be present under the seed coat (18, 30). One advantage of AAC00-1Δ*hrcC* is its ability to infest seed in a similar manner to wild-type *A. citrulli* and become localized in similar tissues of the seed. While the mechanism of biological control is unknown, we suspect that AAC00-1Δ*hrcC* competes with wild-type *A. citrulli* for nutrients and colonization sites on the germinating seedling. Suppression of BFB development has been reported using an epiphytic yeast, *Pichia anomala*, on hami melon seed and *Bacillus* sp RAB9 on watermelon seed, further supporting the potential of biological control as a part of an integrated BFB management approach (25, 41). AAC00-1Δ*hrcC* can also be used as a blossom protectant in seed production fields to limit seed infestation. In hybrid seed production, lyophilized AAC00-1Δ*hrcC* inoculum could be introduced into female blossoms during hand pollination. Unfortunately, it is unlikely that blossom protection with AAC00-1Δ*hrcC* could be a stand-alone treatment for BFB since it resulted in low levels of seed infestation. Nevertheless, blossom protection followed by seed treatment with AAC00-1Δ*hrcC* might represent an important component in a comprehensive integrated BFB management strategy that seeks to
deemphasize the use of xenobiotic pest management chemicals, i.e. organic cucurbit seed production. Benefits may also be gained by developing and exploring more effective strategies and techniques for treating seed or blossoms with biocontrol agents.

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LITERATURE CITED


Table 2.1. Bacterial strains and plasmids used in this study.

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<th>Strains and plasmids</th>
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<tr>
<td><em>Acidovorax citrulli</em> (AAC)</td>
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<tr>
<td>00-1</td>
<td>Wild-type, Rf^6</td>
<td>(9)</td>
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<tr>
<td>00-1ΔhrcC</td>
<td>hrcC deletion mutant</td>
<td>This study</td>
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<tr>
<td>00-1ΔhrcCcomp</td>
<td>hrcC complemented mutant</td>
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<tr>
<td><em>A. avenae</em> (AAA)</td>
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<td>99-2</td>
<td>Maize pathogen</td>
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<td><strong>Escherichia coli</strong></td>
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<td>DH5α</td>
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<td>Invitrogen</td>
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<tr>
<td>S17-1 (λpir)</td>
<td>ΔlacU169 (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir</td>
<td>Dr. Jeff Jones</td>
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<td>pUFR043-hrcC</td>
<td>Complete hrcC gene in pUFR043</td>
<td>This study</td>
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Figure 2.1. Polymerase chain reaction confirmation of hrcC gene deletion in Acidovorax citrulli strain 00-1. Lane: 1) Fermentas 1kb DNA ladder; 2) AAC00-1ΔhrcC (non-pathogenic hrcC mutant of AAC00-1); 3) AAC00-1ΔhrcCcomp (complemented hrcC mutant of AAC00-1); 4) AAC00-1 (wild-type A. citrulli); 5) water (negative control).
Figure 2.2. Pathogenicity and hypersensitive response assays. **A.** Hypersensitive response induced in tobacco (*Nicotiana tabacum*) by different *Acidovorax citrulli* strains used in this study. Leaves were infiltrated with 1 x 10^6 CFU/mL of: 1. AAC00-1Δ*hrcC* (non-pathogenic *hrcC* mutant of AAC00-1); 2. AAC00-1Δ*hrcC*comp (complemented *hrcC* mutant of AAC00-1); 3. AAC00-1 (wild-type *A. citrulli*); and 4. phosphate buffered saline (PBS, negative control). **B.** Response of two-week-old watermelon (cv. Crimson Sweet) cotyledons infiltrated with different *A. citrulli* strains including: 1. PBS (negative control); 2. AAC00-1Δ*hrcC*; 3. AAC00-1Δ*hrcC*comp; and 4. AAC00-1.
Figure 2.3A. Colonization of watermelon seed (cv. Sugar Baby) by wild-type *A. citrulli* (AAC00-1), the non-pathogenic *hrcC* mutant (AAC00-1Δ*hrcC*) or the complemented *hrcC* mutant (AAC00-1Δ*hrcC*comp) during the first 96 h of seed germination. The experiment was repeated three times and each datum point represents the mean of the three experiments (n = 5 seed). Lines represent the standard deviation of the mean. B. Bar chart of area under population dynamics curve (AUPDC) calculated for seed infiltrated with $10^3$ CFU of AAC00-1, AAC00-1Δ*hrcC* or AAC00-1Δ*hrcC*comp and germinated for 96 h. Bars represent mean AUPDC and lines indicate standard deviation of the means. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference.
**Treatments**

Area under the population dynamics curve (AUPDC)

- **AAC00-1**
- **AAC00-1ΔhrcC**
- **AAC00-1ΔhrcCcomp**

**Graph A**

Log$_{10}$ CFU/g watermelon seed vs. Time (hours post inoculation)

**Graph B**

Area under the population dynamics curve (AUPDC) for different treatments.
Figure 2.4A. Colonization of watermelon seedlings (cv. Crimson Sweet) by AAC00-1 (wild-type \textit{A. citrulli}), AAC00-1\textDelta hrcC (the \textit{hrcC} deletion mutant) and AAC00-1\textDelta hrcCcomp (the complemented \textit{hrcC} mutant). The experiment was repeated three times and each datum point represents the mean of three experiments (\(n = 6\) leaf discs/treatment). Lines represent the standard deviation of the means. B. Bar chart of area under population dynamics curve calculated for watermelon seedlings infiltrated with AAC00-1, AAC00-1\textDelta hrcC or AAC00-1\textDelta hrcCcomp and incubated for 96 h. Bars represent the mean AUPDC and lines indicate standard deviation of the means. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference.
Treatments

Area under the population dynamics curve (AUPDC)

Log$_{10}$ CFU/leaf disc

Time (hours post inoculation)

AAC00-1
AAC00-1ΔhrcC
AAC00-1ΔhrcCcomp

Area under the population dynamics curve (AUPDC)

AAC00-1
AAC00-1ΔhrcC
AAC00-1ΔhrcCcomp

Treatments
Figure 2.5A. Comparison of the effects seed treatments with *A. avenae* (AAA 99-2), *A. citrulli* T3SS mutant (AAC00-1ΔhrcC) or phosphate buffered saline (PBS, negative control) on BFB seedling transmission for watermelon seed naturally infested with *A. citrulli*. Each datum point represents the mean BFB seedling transmission percentage of two experiments (n=100 seed/treatment) and the lines represent the standard deviation of the means. B. Bar chart of area under disease progress curves calculated for naturally infested seed treated with AAC00-1, AAC00-1ΔhrcC or AAC00-1ΔhrcCcomp. Bars represent mean AUDPC values and lines indicate standard deviation of the means. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference.
Area under the disease progress curve (AUDPC)

- AAC00-1ΔhrcC
- AAA 99-2
- PBS

Time (days after planting)

BFB seedling incidence (%)

A

B

Treatments

AAC00-1ΔhrcC
AAA 99-2
PBS

Area under the disease progress curve (AUDPC)

A

A

B
**Figure 2.6A.** Effect of seed treatment with AAC00-1ΔhrcC (the T3SS mutant) or phosphate buffered saline (PBS, negative control) on BFB seedling transmission for watermelon seed naturally infested with *Acidovorax citrulli* under greenhouse conditions. Each datum point represents the mean BFB seedling transmission for three experiments (n = 100 seed per treatment) and the lines represent the standard deviation of the means. **B.** Bar chart of area under disease progress curves (AUDPC) calculated for *A. citrulli* infested watermelon seed treated with AAC00-1 or AAC00-1ΔhrcC germinated and grown under greenhouse conditions for 21 days. Bars represent mean AUDPC values and lines indicate standard deviation of the means. Treatments with different letters are significantly different according to the Student’s t test.
Area under the disease progress curve (AUDPC)

Treatments

AAC00-1ΔhrcC
PBS

A

AAC00-1ΔhrcC
PBS

B
Figure 2.7. Effect of female blossom protection with the T3SS mutant (AAC00-1ΔhrcC), *A. avenae* (AAA 99-2) or phosphate buffered saline (PBS, negative control) on watermelon seed infestation by *Acidovorax citrulli* and seed germination. The experiment was repeated two times and bars represent the mean disease incidence or germination of two experiments (n = 100 seed/treatment). Lowercase letters represent the comparison of effects of blossom treatments on bacterial fruit blotch seedling transmission, while uppercase letters indicates the results for comparing the effects of biological control seed treatments on germination. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference.
CHAPTER 3

ROLE OF *ACIDOVORAX CITRULLI* TYPE II SECRETED PROTEINS IN SEED COLONIZATION AND SEEDLING TRANSMISSION OF BACTERIAL FRUIT BLOTCH OF CUCURBITS

Johnson, K. L., Chebrolu, P., Denny, T., and Walcott, R. R. 2010. To be submitted to *Molecular Plant Microbe Interaction*
ABSTRACT

*Acidovorax citrulli* is a seedborne bacterium that causes bacterial fruit blotch (BFB) of cucurbits. Here we provide evidence for a functional type II secretion system (T2SS) in *A. citrulli* strain 00-1, and demonstrate its importance in virulence, seed colonization and seed-to-seedling transmission of BFB. The AAC00-1 genome includes one complete T2SS gene cluster and one incomplete cluster designated *gsp1* and *gsp2*. A T2SS mutant of *A. citrulli*, designated AAC00-1Δ*gspG1*:Δ*gspG2*, was generated by deleting the *gspG* gene homologs from the *gsp1* and *gsp2* gene clusters. AAC00-1Δ*gspG1*:Δ*gspG2* retained pathogenicity but was reduced in its ability to colonize watermelon seed and seedling tissue compared to the wild-type strain. There was a significant reduction in BFB seed-to-seedling transmission for AAC00-1Δ*gspG1*:Δ*gspG2* (33%) relative to AAC00-1 (62%). Deletion of a gene encoding endoglucanase from AAC00-1 significantly reduced its ability to colonize watermelon seed. However, xylanase and pectate lyase mutants of AAC00-1 were not affected in their ability to colonize watermelon seed. These data suggest that both the T2SS and endoglucanase play a role in watermelon seed colonization by *A. citrulli*.

Additional keywords: General secretory pathway, *Acidovorax avenae* subsp. *citrulli*, Seed pathology, Endoglucanase, Watermelon fruit blotch, Seed pathology, Cell wall degrading enzymes
INTRODUCTION

*Acidovorax citrulli* (Schaad *et al*., 2008), (formerly *A. avenae* subsp. *citrulli* (Willems *et al*., 1992) = *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (Schaad *et al*., 1978)), is the causal agent of bacterial fruit blotch (BFB) of cucurbits. BFB affects all agronomically important cucurbit crops including watermelon (*Citrullus lanatus*), honeydew (*Cucumis melo* var. *inodorus*), pumpkin (*Cucurbita pepo*), and cucumber (*Cucumis sativus*) (Wall *et al*., 1990; Isakeit *et al*., 1997; Langston *et al*., 1999a; Martin and O'Brien, 1999). The pathogen was first reported in Florida in 1989 on watermelon (Somodi *et al*., 1991). However, the seedborne nature of the bacterium has facilitated its spread, and the disease has since been reported in many cucurbit producing states in the United States including Texas, Georgia, Oklahoma, South Carolina, and Oregon (Jacobs *et al*., 1992; Black *et al*., 1994; Hamm *et al*., 1997; Langston *et al*., 1999b). The disease has also been reported in China, Turkey, Israel, Brazil, Japan and Australia (O'Brien and Martin, 1999; Shirakawa *et al*., 2000; Macagnan *et al*., 2003; Burdman *et al*., 2005; Mirik *et al*., 2006; Ren *et al*., 2006).

The seedborne nature of *A. citrulli* has mandated production of cucurbit seed in regions of countries with cool, dry climates that limit BFB development. For disease management, seed fields are visually inspected at harvest maturity and seed treatments and rigorous seed health testing are employed (Walcott *et al*., 2003). Despite these management tactics, infested seed still initiate sporadic and costly BFB outbreaks around the world annually. Neither seed treatments nor seed health testing is 100 percent effective and since there is zero tolerance for BFB in cucurbit production systems, there is a need for more effective BFB management strategies. One potentially effective disease management strategy is prevent of seed-to-seedling transmission *A. citrulli*. However, at present little is known about the seed-pathogen interactions that occur
during the early stages of seed germination and contribute to BFB seedling transmission. To address this lack of information, we employed a random mutagenesis approach to identify potential virulence factors that might be involved in this critical process. One mutant that displayed reduced levels of seed colonization had an insertion in a homolog of the prepilin peptidase gene, *pulO*, and was designated AAC00-1::*pulO*. PulO allows for maturation of pilins of the type IV pilus and pseudopilins of the type II secretion system (T2SS), by cleavage of leader sequences (Pugsley and Dupuy *et al*., 1992). Type IV pili are required for biofilm formation and motility in *A. citrulli* (Bahar *et al*., 2009). *A. citrulli* strains lacking type IV pili were also reduced in virulence and seed-to-seedling transmission (Bahar *et al*., 2009). However the contribution of the T2SS to *A. citrulli* virulence is still largely unknown. Based on these observations, we hypothesized that the T2SS plays a key role in the ability of *A. citrulli* to colonize watermelon seed during germination and be transmitted to seedlings. Seed can be a rich source of nutrients that support complex microbial communities (Nelson, 2004). Compounds commonly secreted from seed during germination include galactomannan, galactose, sucrose, glucose, mannose, amino acids, (Roberts *et al*., 1999). Currently, the *A. citrulli* genes required for colonization of the watermelon spermosphere are unknown.

The T2SS is a bacterial virulence-associated mechanism that allows secretion of proteins including β-1, 4-endoglucanase, xylanases, cellobiose and pectinase (Kunkel and Chen, 2006). These proteins are important for the hydrolysis of complex carbohydrates. Mutations in T2SS structural genes or individual type II secreted (T2S) enzymes has been shown to reduce the virulence of phytopathogens such as *Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *vesicatoria*, *Erwinia chrysanthemi* and *Ralstonia solanacearum* (Andro *et al*., 1984; Ray *et al*., 2000; Liu *et al*., 2005; Szczesny *et al*., 2010). A T2SS mutant of *X. oryzae* pv. *oryzae* produced
smaller lesions on rice leaves compared to wild-type bacteria (Ray et. al., 2000), and T2SS mutants of *R. solanacearum* were reduced in their ability to induce wilting on tomato plants (Liu et. al., 2005). Here we report the presence of a functional T2SS system in *A. citrulli*, and provide evidence for its role in virulence, seed colonization and seed-to-seedling transmission of BFB. We also identified T2S proteins of *A. citrulli* using mass spectrometry and determined the role of select T2S proteins (endoglucanase, xylanase and pectate lyase) in virulence on watermelon.

**RESULTS**

**Characterization of a T2SS mutant of AAC00-1.**

All *A. citrulli* strains used in this study were derived from the wild-type strain 00-1 (Table 3.1). Two sets of general secretory pathway (*gsp*) genes were identified in AAC00-1 based on available genomic sequence data (Table 3.2). These were designated *gsp1* and *gsp2* for gene cluster1 and 2, respectively (Fig. 3.1). The pilus-like structure of the T2SS apparatus is created by oligomerization of the psuedopilins GspG, H, I, J and K (Filloux, 2004; Kuo et. al., 2005). GspG encodes the major pseudopilin of the T2SS (Sauvonnet et. al., 2000). The GspG1 and GspG2 proteins of *A. citrulli* share 50% identity as determined by BLAST sequence alignment. To create a T2SS mutant of *A. citrulli*, the *gspG* genes encoded by loci Aave_4158 (*gspG1*) and Aave_0925 (*gspG2*) were disrupted. *gspG1* was disrupted by homologous recombination with a pOK1 suicide vector carrying a deleted version of *gspG1* to create AAC00-1∆*gspG1*. However, AAC00-1∆*gspG1* still secreted endoglucanase. Hence, *gspG2* was deleted from AAC00-1∆*gspG1* by homologous recombination with pOK1-*gspG2* carrying a deleted version of *gspG2*. Deletion of the *gspG1* and *gspG2* genes was confirmed by PCR amplification (Fig. 3.2A). PCR fragments amplified with primers specific for *gspG2*
(gspG2.For1/gspG2.Rev4), amplified a 1.3-kb fragment from AAC00-1, and a 1.1-kb fragment from the T2SS mutant, AAC00-1ΔgspG1:ΔgspG2. Using primers, (gspG1.For1/gspG1.Rev4), specific for the gspG1 gene a 1.3-kb fragment was amplified from AAC00-1, while a 1.08-kb fragment was amplified from AAC00-1ΔgspG1:ΔgspG2. Loss of T2SS was confirmed by the loss of ability to secrete endoglucanase as determined by the carboxymethyl cellulose assay (Hueck, 1998) (Fig. 3.2B). When 10⁶ CFU/mL of bacteria was infiltrated into the cotyledons of watermelon seedlings, AAC00-1 and AAC00-1ΔgspG1:ΔgspG2 produced typical watersoaked and necrotic lesions by two days after inoculation (Fig. 3.3). These observations indicated that AAC00-1ΔgspG1:ΔgspG2 retained pathogenicity on watermelon leaves.

Role of T2S in *A. citrulli* virulence on watermelon seedlings.

The role of the T2SS in colonization of watermelon seedlings was determined in three independent experiments using cotyledon infiltration assays. Similar to AAC00-1, populations of AAC00-1ΔgspG1:ΔgspG2 increased continuously over 96 h after infiltration of watermelon cotyledons (Fig. 3.4). However, AAC00-1ΔgspG1:ΔgspG2 populations were less than those of AAC00-1. AAC00-1 increased from 10³ CFU/disc at 0 hours post inoculation (hpi) to 10⁵ CFU/disc at 24 hpi, while, AAC00-1ΔgspG1:ΔgspG2 did not increase significantly over the first 24 hpi. At 96 hpi. AAC00-1ΔgspG1:ΔgspG2 populations (~2.0 × 10⁸ CFU/disc) were still lower than those of AAC00-1 (~3.2 × 10¹⁰ CFU/disc) (Fig. 3.4A). The effect of experiment on AUPDC data was not significant (P = 0.95), therefore data from the three experiments were pooled for analysis. The effect of the T2SS on *A. citrulli* colonization of watermelon cotyledons was statistically significant (P = 0.0005) (Fig. 3.4B).
Role of the T2SS in *A. citrulli* colonization of watermelon seed during germination.

The role of the T2SS in the colonization of watermelon seed by *A. citrulli* was determined by enumerating bacterial populations on artificially inoculated seed during the initial 96 h of germination. Individual seed were infiltrated with either AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 and bacterial populations per seed were estimated at 24 h intervals. AAC00-1ΔgspG1:ΔgspG2 was reduced in its ability to colonize seed relative to AAC00-1 (Fig. 3.5A). The difference in seed colonization between AAC00-1ΔgspG1:ΔgspG2 and AAC00-1 was apparent by approximately 48 hpi when populations of AAC00-1ΔgspG1:ΔgspG2 (~6.3 × 10⁵ CFU/seed) were 10-fold less than those of AAC00-1 (~7.9 × 10⁶ CFU/g of seed). By 96 h after seed germination AAC00-1ΔgspG1:ΔgspG2 populations (4.0 × 10⁷ CFU/g of seed) were 100-fold lower than AAC00-1 (~4.0 × 10⁹ CFU/g of seed) (Fig. 3.5A). ANOVA of AUPDC data indicated that the effect of experiment was not significant (P = 0.97), therefore data from different experiments were pooled for analysis. The difference between AUPDC data for the two strains was statistically significant according to the Student’s t test (P = 0.018) (Fig. 3.5B).

Effect of T2SS on seed-to-seedling transmission of the BFB.

The reduction in watermelon seed colonization by AAC00-1ΔgspG1:ΔgspG2 prompted investigation of the role of T2SS in seed-to-seedling transmission of BFB. Individual seed (n=25) were inoculated with ~1 × 10³ CFU/mL of AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 and planted individually in sterile test tubes to prevent cross-contamination. BFB seed-to-seedling transmission was observed for 14 days. Of the seed inoculated with AAC00-1, 62% developed seedlings with BFB symptoms. In contrast 33% of seedlings from seed inoculated with AAC00-1ΔgspG1:ΔgspG2 displayed symptoms (Fig. 3.6A). The decrease in BFB seed-to-seedling
transmission for the AAC00-1ΔgspG1:ΔgspG2 was significant according to the Student’s t test (P = 0.0004) (Fig. 3.6B).

**Generation of endoglucanase, xylanase and pectate lyase mutants of AAC00-1 and elucidation of their role in watermelon seed colonization.**

AAC00-1 putatively encodes β-1, 4-endoglucanase, xylanase and pectate lyase enzymes and these genes were deleted to assess their role in seed colonization by *A. citrulli*. Deletion and complementation of endoglucanase, xylanase and pectate lyase genes of *A. citrulli* were confirmed by PCR analysis (Fig. 3.7A). PCR amplification with cel.del.for/cel.rev4 resulted in a 2.75-kb fragment from AAC00-1 and AAC00-1ΔEgl comp. While a 1.36-kb fragment was amplified from AAC00-1ΔEgl and AAC00-1ΔEgl comp. The endoglucanase mutant, AAC00-1ΔEgl, was unable to degrade cellulose in CMC medium, while, AAC00-1 and AAC00-1ΔEgl comp degraded cellulose (Fig. 3.7B). Xylanase and pectate lyase activity for AAC00-1 were not detected *in vitro*. Deletion of the xylanase gene was confirmed using primers xyl.del.For/xyl.del.Rev that resulted in a 1.4-kb fragment from AAC00-1ΔXyl DNA and a 2.3-kb fragment from AAC00-1 DNA. Both fragments were amplified with genomic DNA from AAC00-1ΔXyl comp. PCR confirmation of pectate lyase deletion resulted in fragments of 1.4-kb and 2.4-kb from AAC00-1ΔPec, and AAC00-1, respectively, while both fragments were amplified AAC00-1ΔPec comp DNA (Fig. 3.7A). Even though endoglucanase was the only protein confirmed to be secreted by the T2S system of AAC00-1 in *vitro*, the roles of xylanase and pectate lyase in watermelon seed colonization were investigated. Xylanase (AAC00-1ΔXyl) and pectate lyase (AAC00-1ΔPec) mutants of *A. citrulli* were not significantly reduced in their ability to colonize seed compared to AAC00-1. The differences between bacterial populations
achieved on seed by 96 hours after planting were not statistically significant compared to AAC00-1 (P = 0.0569 and 0.5748 for AAC00-1ΔXyl and AAC00-1ΔPec, respectively) (Fig. 3.8A and 3.8C). These data suggest that xylanase and pectate lyase did not contribute to watermelon seed colonization by \textit{A. citrulli}. In contrast, the endoglucanase mutant, AAC00-1ΔEgl, was reduced in its ability to colonize watermelon seed (Fig. 3.8B). Mean populations of AAC00-1ΔEgl were 100-fold less than AAC00-1 and 10-fold less than the complemented strain at 96 h after planting. At 96 h after planting the populations of AAC00-1ΔEgl (~2.0 \times 10^8 \text{ CFU/g of seed}) were lower than for AAC00-1 (~1.5 \times 10^{10} \text{ CFU/g of seed}) and AAC00-1ΔEgl/comp (~3.5 \times 10^9 \text{ CFU/g of seed}). The difference in seed colonization by AAC00-1ΔEgl was statistically significant (P = 0.024) compared to AAC00-1 and AAC00-1ΔEgl/comp.

**Effect of T2S on \textit{A. citrulli} virulence on watermelon fruit tissue.**

The ability of AAC00-1, AAC00-1ΔgspG1:ΔgspG2, AAC00-1ΔXyl, AAC00-1ΔEgl and AAC00-1ΔPec to induce BFB symptoms on watermelon fruit rind was determined in three independent experiments by infiltrating fruit rind with bacterial cell suspensions. Pathogenicity tests indicated that T2SS is important in \textit{A. citrulli} virulence on fruit tissue. All treatments were significantly different from the negative control, which produced no lesions on the watermelon rind. Watersoaked lesions typical of BFB developed on rind infiltrated with all strains tested; however, the mean lesion diameter varied based on the strain tested (Fig. 3.9). Mean lesion diameters produced by the pectate lyase (3.4 mm) and xylanase (3.6 mm) deletion mutants were not significantly different from AAC00-1 (4.6 mm) or the T2SS mutant (2.7 mm). However, mean diameter of lesions produced by the endoglucanase mutant (3.1 mm) was significantly less than for those produced by AAC00-1 (P = 0.026) (Fig. 3.9). The mean diameter of lesions
produced by AAC00-1ΔgspG1:ΔgspG2 (2.7 mm) was significantly different to that produced by AAC00-1 (4.6 mm) (P = 0.004).

**DISCUSSION**

Based on our observations, we concluded that the *A. citrulli* T2SS contributed to virulence, seed colonization and seed-to-seedling transmission of BFB. The T2SS facilitates the transport of proteins from the periplasm of bacterial cells to the extracellular milieu. Disruption of the T2SS in pathogens results in the accumulation of T2SS substrates within the periplasm of bacterial cells and reduced virulence (Andro *et. al.*, 1984; Filloux *et. al.*, 1987; Sun *et. al.*, 2005). Bioinformatic analysis indicated the presence of two general secretory protein (gsp) gene clusters in AAC00-1 genome, designated gsp1 and gsp2 (Fig. 3.1). The presence of multiple functional T2SS was reported for *Psuedomonas aeruginosa* PAO1 (Wretlind and Pavlovskis, 1984; Ball *et. al.*, 2002) and the systems were designated xcp and hxc (Wretlind and Pavlovskis, 1984; Ball *et. al.*, 2002). Interestingly, these secretory systems exhibited substrate specificity. The presence of multiple T2SS gene clusters (xps and xcs) was also reported for *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri* and *X. campestris* pv. *vesicatoria* (Dums *et. al.*, 1991; da Silva *et. al.*, 2002; Qian *et. al.*, 2005). In the case of *A. citrulli*, there are 11 genes in each cluster with the gspF gene being present only in the gsp1 cluster, and the prepepilin peptidase being located elsewhere in the genome. GspF is an essential inner membrane protein that interacts with GspM, N, and E proteins to form a stable inner membrane platform upon which the T2SS pseudopilus is built (Py *et. al.*, 2001). The absence of gspF in the gsp2 cluster suggests that this system is nonfunctional.

The T2SS pseudopilins GspG, H, I, J, and K are produced as precursors with leader peptides that are cleaved by a prepepilin peptidase to produce the mature protein (Johnson *et. al.*, 2005).
GspG is the major pseudopilin capable of forming a multifibular structure that spans the bacterial envelope, and deletion of the gspG gene interrupts the T2SS (Durand et al., 2003; Shi et al., 2008). Deletion of the gspG gene of Shewalla oneidensis disrupted T2S and transport of MtrC and OmcA across the outer membrane (Shi et al., 2008). Further evidence that the gspG would be a good target for deletion was our observation with a random transposon mutant that lacked prepilin peptidase and failed to produce functional pseudopilins (data not shown). These data suggested that the gspG homologs of A. citrulli would be suitable targets to produce a T2SS mutant. Deletion of the gspG1 gene resulted in a strain (AAC00-1ΔgspG1) that secreted endoglucanase (data not shown). It is possible that GspG2 can complement the gspG1 deletion in AAC00-1ΔgspG1 or GspG1 has no role in secretion of endoglucanase. In X. campestris pv. campestris, which also has two T2S gene clusters, the XcsD protein partially complemented the xpsD mutation (Szczesny et al., 2010). Since our goal was to identify A. citrulli T2S proteins it was necessary to abolish T2S so gspG2 was also deleted. Deletion of both gspG1 and gspG2 from A. citrulli resulted in AAC00-1ΔgspG1:ΔgspG2 in which endoglucanase secretion was abolished, as confirmed by loss of endoglucanase activity on CMC plates.

In this study we demonstrated that the T2SS mutant of A. citrulli displayed reduced virulence on watermelon cotyledons. The role of T2S in virulence has been reported for the soft rotting Erwinias that produce a range of plant cell wall degrading enzymes (PCWDEs) including endoglucanase, polygalacturonase, pectate lyase and pectin methylesterases (Barras et al., 1994). The T2SS was also shown to be important for vascular pathogens such as Ralstonia solanacearum and X. campestris pv. campestris. R. solanacearum PCWDEs contribute to systemic infection of tomato, leading to wilting and lateral spread of the pathogen (Liu et al., 2005; Tsujimoto et al., 2008). T2SS mutants of R. solanacearum are reduced in ability to induce
wilting (Liu et al., 2005; Tsujimoto et al., 2008). The importance of T2S proteins in virulence was further demonstrated in *R. solanacearum* where virulence was correlated to the number and type of PCWDEs mutated (Liu et al., 2005). In *A. citrulli* the T2SS mutant was reduced in its ability to colonize watermelon seed, which supports the hypothesis that the T2SS contributes to epiphytic growth during the early stages of seed germination. AAC00-1ΔgspG1:ΔgspG2 was also reduced in seed-to-seedling transmission of BFB, which suggests that T2S proteins may be important in achieving critical cell densities necessary for BFB development.

The plant cell wall is composed of cellulose, a complex polysaccharide comprised of 1,4 β-D-glucose units, hemicellulose which may be xyloglucans, arabinoxylan or glucomannan and pectins, which are comprised of 1,4-linked α-D-galacturonic acids (Keegstra et al., 1973; Ebringerová, 2006; Minic and Jouanin, 2006). Enzymes such as endoglucanase, pectate lyase and xylanase facilitate hydrolysis of these carbohydrates (Minic and Jouanin, 2006). Using bioinformatic analysis xylanase, pectate lyase and endoglucanase genes were identified in the *A. citrulli* genome and were predicted to be exported across the inner membrane based on the presence of a Sec secretion signal in the polypeptide sequence. The putative xylanase identified in *A. citrulli*’s genome has 66, 66 and 69% identity to the xylanases of *X. oryzae* pv. *oryzae* (accession number YP_971915), *X. campestris* pv. *vesicatoria* (accession number YP_366089.1), and *X. fuscans* subsp. *aurantifolii* (accession number ZP_06732701,1), respectively (Ray et al., 2000). Unexpectedly, xylanase was not identified by mass spectrometry of extracellular proteins nor was it identified *in vitro* assays with either the T2S mutant or wild-type *A. citrulli* strain. The predicted *A. citrulli* xylanase was not important for watermelon seed colonization or virulence on watermelon fruit tissue. These results were unexpected as xylanase was reported to be important in phytopathogenicity. For example
xylanase mutants of *X. oryzae* pv. *oryzae* produced significantly smaller lesions on rice leaves (Ray *et. al.*, 2000). It is also possible that xylanase may be induced only in the presence of plant tissue like *X. oryzae* pv. *oryzae* or that the protein is not expressed (Rajeshwari *et. al.*, 2005).

We also investigated the role of pectate lyase in pathogenicity, virulence and watermelon seed colonization by *A. citrulli*. Pectate lyases depolymerize polygalacturonate to cause cell wall degradation (De Lorenzo *et. al.*, 1991). The putative pectate lyase has a pectate lyase C domain but has low similarity to other proteins classified as pectate lyase using BLAST sequence analysis. The *A. citrulli* putative pectate lyase had 37, 29 and 34% similarity to *Bacillus subtilis* subsp. *subtilis* (accession number NP_388637), *Erwinia chrysanthemi* (accession number P0C1A2), *Pseudomonas syringae* pv. *glycinea* (accession number AAL56657) (Tamaki *et. al.*, 1988; Soriano *et. al.*, 2006). The role of pectate lyase in virulence of other phytopathobacteria is well established and genomic data suggested that it was present in *A. citrulli*. However, this protein was not detected in *A. citrulli* cell free extract. Our inability to detect pectate lyase in cell free extracts produced in minimal media could be due to lack of induction of the enzyme under the test conditions. For some pectate lyases, pectate is required for gene induction; however, this was not the case for *A. citrulli* as pectate lyase activity was not detected when media containing polygalacturonic acid was used (data not shown). In *Erwinia chrysanthemi* EC16 pectate lyase activity was detected on pectate containing media (Kelemu and Collmer *et. al.*, 1993). However upon deletion of pectate-induced pectate lyase enzymes Kelemu and Collmer (1993) reported that *E. chrysanthemi* EC16 still degraded pectin in cell wall media. These observations indicated that *E. chrysanthemi* EC16 encoded plant inducible pectate lyases (Kelemu and Collmer *et. al.*, 1993). Our inability to detect pectate lyase could also be due to the gene not being transcribed or the protein being non-functional. The *A. citrulli* pectate lyase mutant was not affected in
virulence on watermelon fruit rind, nor ability to colonize germinating watermelon seeds. Our observations suggest that pectate lyase is not important for BFB seed-to-seedling transmission.

*A. citrulli* encodes an endoglucanase (Aave_2102) and a degenerate endoglucanase (Aave_2924), both of which are members of the glycoside hydrolase family 5 (pFAM00150). The degenerate endoglucanase (Aave_2942) has a poorly conserved cellulase domain and did not produce detectable cellulolytic activity on CMC plates in an endoglucanase deletion background (AAC00-1ΔEgl). The lack of cellulolytic activity suggests that this degenerate endoglucanase is not be secreted into the extracellular matrix. It is also possible that the degenerate endoglucanase may not be expressed or it does not encode a functional protein. The endoglucanase, Aave_2102, encoded by *A. citrulli* contains a cellulose binding domain (CBD) that is not required for the catalysis of CMC and is believed to increase the activity of the catalytic domain of the enzyme (Jahr et. al., 2000). The *A. citrulli* endoglucanase has 66, 65 and 64% identity to the endoglucanase genes from *X. campestris* pv. *campestris* (accession number CAC18529), *Xylella fastidiosa*, (accession number NP_298108), and *X. oryzae* pv. *oryzae* (accession number YP_001912992) (Gough et. al., 1990; Wulff et. al., 2006; Hu et. al., 2007). Bacterial endoglucanases have been previously implicated in virulence. Mutation of the endoglucanase, CelA in *Clavibacter michiganensis* subsp. *michiganensis*, yielded a strain that was unable to induce wilting (Jahr et. al., 2000). Reduction in virulence was also observed for *X. oryzae* pv. *oryzae* with the mutation of eglXoB that encoded endoglucanase (Hu et. al., 2007). This reduced virulence was in the form of decreased lesion length on rice leaves (Hu et. al., 2007). Similar observations were made for *A. citrulli* when mutation of the endoglucanase gene resulted in reduced lesion size on watermelon rind and reduced watermelon seed colonization.
While the role of PCWDE in rhizosphere colonization has been previously reported (Ahmad and Baker, 1986; Mostajeran et. al., 2007), to our knowledge this is the first report that endoglucanase contributes to watermelon seed colonization by a foliar phytopathogenic bacterium. Increased production of endoglucanase resulted in *Tricoderma harzianum* mutants that were more competent rhizosphere colonizers of buried wheat straw (Ahmad and Baker, 1986). These mutants produced significantly higher amounts of endoglucanase allowing the fungi to use cellulose on or near the root, while maintaining a saprophytic relationship with the plant (Ahmad and Baker, 1986). A similar trend was observed with *Azospirillum brasilense* where wheat roots treated with endoglucanase supported higher populations of the bacterium than roots treated with pectinase or non-treated control roots (Mostajeran et. al., 2007). Roots treated with both endoglucanase and pectinase supported the highest bacterial populations, suggesting the effect of the enzymatic treatments were additive. Endoglucanase might be enhancing the survival of *A. citrulli* on the germinating seed by allowing it to hydrolyze β-1, 4-glycosidic bonds of cellulose in the spermosphere. We suspect that as a foliar pathogen, *A. citrulli* is a poor soil competitor; hence, endoglucanase could allow *A. citrulli* to establish populations during the epiphytic phase of colonization that results in seed-to-seedling transmission of BFB.

Overall we have demonstrated that the T2SS, and endoglucanase, are important virulence factors for *A. citrulli*. Both are involved in seed colonization during the early stages of germination as well as virulence on fruit and seedling tissues. Despite this observation it is imperative to continue to study these T2SS mutants to gain a better understanding of the molecular interactions that contribute to seedling transmission of phytobacterial diseases.
MATERIALS and METHODS

Bacterial strains, plasmids and media.

*A. citrulli* and *Escherichia coli* strains used in this study are listed in Table 3.1. *A. citrulli* strains were routinely grown on nutrient agar (Difco, Becton Dickinson, Sparks MD) with appropriate antibiotics for 48 h at 28°C. *E. coli* strains were grown in Luria Bertani (LB) broth or agar (Difco) with appropriate antibiotics for 24 h at 37°C. To prepare *A. citrulli* inoculum, bacterial cells were pelleted from 1.5 mL of overnight LB broth culture by centrifugation at 13,000 rpm for 1 min. Cells were washed with and resuspended in phosphate buffered saline (PBS, 137 mM NaCl, 5.6 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄). Bacterial suspensions containing ~ 1 × 10⁸ CFU/mL were estimated using a spectrophotometer (OD₆₀₀ = 0.3) (Spectronic 20, Bausch and Lomb, Rochester, NY) and ten-fold serially diluted with PBS to generate cell suspensions with desired concentrations. Bacterial matings were routinely carried out on M9 minimal medium (10 mM Na₂PO₄, 10 mM KH₂PO₄, 9.4 mM NH₄Cl, 4.3 mM NaCl) agar plates (Ausubel et. al., 1999). When used, antibiotics were incorporated into media at the following concentrations; rifamycin (100 µg/mL, *A. citrulli*), spectinomycin (100 µg/mL, *A. citrulli*, *E. coli*), kanamycin (50 µg/mL, *A. citrulli*, *E. coli*), cycloheximide (200 µg/mL, *A. citrulli*) (Sigma Aldrich, St. Louis MO). Carboxymethyl cellulose (CMC, 1g NaNO₃, 1g K₂HPO₄, 0.5g KCl, 0.5g MgSO₄, 0.5g yeast extract, 4g Carboxymethyl cellulose, 15g agar)(Laurent et. al., 2000) plates were used to detect cellulolytic activity.

DNA manipulations.

Based on the complete genomic sequence available for AAC00-1 (NC_008752), *A. citrulli* has two T2SS gene clusters designated *gsp1* and *gsp2*. To create a T2SS mutant of *A.
citrulli the gspG genes of both clusters were deleted. To delete the gspG1 gene, sequences upstream and downstream (~ 500 bp in each direction) of the gene were amplified by PCR and subjected to splice overlap extension (SOEing) PCR in two steps to generate one fragment (Shevchuk et. al., 2004). The upstream and downstream regions of the gene were amplified using primer pairs gspG1.For1/gspG1.Rev2 and gspG1.For3/gspG1.Rev4, respectively (Table 3.3). The fragments were separated by agarose gel electrophoresis and the fragments extracted from the agarose using a commercially available kit, according to the manufacturer’s instructions (Qiagen, Valencia, CA). The first step of the SOEing PCR included 100 ng of each fragment, 300 mM dNTP, 2 mM MgSO4, 1.25U Platinum Taq, in a 50 μL volume. PCR conditions were as follows; denaturation at 95°C for 2 min, 15 cycles of denaturation at 95°C for 45 sec., annealing at 60°C for 15 min and extension at 72°C for 2 min and 30 sec. For the second step of the splicing reaction the PCR reagents included 2 μL of product from the first reaction, 200 mM dNTP, 2 mM MgSO4, 0.5U Platinum Taq, 40 nM of gspG2.For1 and gspG2.Rev4 in a 25 μL reaction mixture. PCR conditions were similar to those used for the first reaction with the exception that 35 cycles were used rather than 15. The PCR amplicon was separated by gel electrophoresis and the fragment was extracted from the gel as described above. The deletion fragment was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and subsequently digested with the respective restriction enzymes listed in Table 3.3. The digested fragments were cloned into the similarly digested suicide vector, pOK1, to generate pOK-gspG1, which was transformed into E. coli strain DH5α (λpir) (Table 3.1). pOK-gspG1 was transferred into AAC00-1 by tri-parental mating with an E. coli strain carrying the helper plasmid, pRK2073. Strains in which homologous recombination occurred were selected for on M9 minimal medium amended with spectinomycin. Individual colonies were transferred to M9 minimal medium
plates with sucrose to allow excision of the plasmid. AAC00-1 strains in which gspG1 was replaced with the deletion construct were identified based on sensitivity to spectinomycin. Spectinomycin-sensitive transformants were further screened by PCR, using primers gspG1.For1 and gspG1.Rev4, to confirm deletion of gspG1. The gspG1 deletion mutant of A. citrulli was designated AAC00-1ΔgspG1.

The gspG2 gene was deleted from AAC00-1ΔgspG1 using the protocol described above. Upstream and downstream regions of the gspG2 gene were amplified using primer pairs gspG2.For1/ gspG2.Rev2 and gspG2.For3/gspG2.Rev4 (Table 3.3). The fragments were spliced together by SOEing PCR as previously described and the gspG2 deletion fragment was cloned into pOK1 to yield pOK-gspG2 (Table 3.1). E. coli carrying pOK-gspG2 was mated to AAC00-1 to facilitate deletion of the gspG2 gene. Target gene deletion was verified by PCR, using primers gspG2.For1 and gspG2.Rev4, and the strain with both gspG1 and gspG2 deleted was designated AAC00-1ΔgspG1:ΔgspG2.

Characterization of the T2SS mutant of A. citrulli.

To assess the functionality of the T2SS, AAC00-1ΔgspG1:ΔgspG2 was assayed for its ability to secrete endoglucanase using CMC plates. To determine endoglucanase activity, 20 µL of a 10^6 CFU/mL suspension of AAC00-1ΔgspG1:ΔgspG2 or AAC00-1 was spotted onto a CMC plate and incubated at 28ºC for 24 h. After incubation endoglucanase activity was observed by staining plates with a 0.1% congo red dye solution (Sigma Aldrich, St. Louis, MO) for 15 min. Plates were then washed with 1M NaCl and celullase activity was visualized as an orange halo on a red background (Laurent et. al., 2000). To determine the effect of the T2SS mutation on A. citrulli pathogenicity, cotyledons of two-week-old watermelon seedlings (cv. Crimson
Sweet) were infiltrated with ~$1 \times 10^6$ CFU/mL of AAC00-1ΔgspG1:ΔgspG2, AAC00-1 or PBS as a negative control (Neipold et. al., 1985) and plants were incubated for 48 h at 28°C with approximately 75% relative humidity in a growth chamber (Percival, Perry, IA). Plants were then visually observed for BFB symptoms (necrosis and watersoaking).

**Role of T2S in *A. citrulli* virulence on watermelon seedlings.**

To determine the role of the T2SS in the colonization of watermelon seedling tissue, ~ $1 \times 10^3$ CFU/mL of AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 were infiltrated separately into the cotyledons of three, two-week-old watermelon seedlings (cv. Crimson Sweet). Infiltrated tissues were allowed to air-dry, and six leaf discs, 3 mm each (1 from each cotyledon) were collected at 0, 24, 48, 72, 96 h after inoculation. Each leaf disc was macerated in a separate microcentrifuge tube containing 900 μL of PBS and appropriate ten-fold serial dilutions were plated onto nutrient agar amended with rifamycin and cycloheximide to enumerate bacterial populations. The experiment was repeated three times and bacterial populations were plotted over time. These graphs were used to generate area under population dynamics curve (AUPDC) data, which in turn were used to conduct analysis of variance (ANOVA) using JMP statistical software (version 8 for windows; SAS Institute Inc, Cary NC). The effect of the T2SS mutation on colonization of watermelon seedling tissue was determined using the Student’s t test.

**Effect of T2S on watermelon seed colonization by *A. citrulli*.**

The role of T2S in watermelon seed colonization during the early stages of germination was determined by infiltrating seed (cv. Sugar Baby) with ~$1 \times 10^3$ CFU of AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 as previously described (Walcott et. al., 2006). Watermelon seed
25) were incubated in plastic boxes (Tri-state plastics, Dixon KY) on moist blotter paper (Hoffman Manufacturing, Albany, OR) at 30ºC with 100% relative humidity. Seed samples (n = 5 seed) were collected at 0, 24, 48, 72, 96 h after planting and bacterial populations were estimated by macerating each seed, separately in 900 μL of PBS in a sterile microcentrifuge tube. Appropriate ten-fold serial dilutions of each seed homogenate was spotted onto NA with appropriate antibiotics and incubated for 48-72 h at 28ºC. Subsequently, the A. citrulli colonies were enumerated. The experiment was repeated three times and mean bacterial populations were plotted over time to generate area under population dynamics curve (AUPDC) data which was used for ANOVA analysis. The effect of the T2SS mutation on colonization of watermelon seed was determined using the Student’s t test.

**Effect of T2SS on seed-to-seedling transmission of BFB.**

To determine the effect of T2S on seed-to-seedling transmission of BFB, watermelon seed (cv. Sugar Baby) were individually vacuum-infiltrated with ~1 x 10³ CFU of AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 as described above. Seed were allowed to air-dry overnight and then incubated individually in moist chambers comprised of sterile, stoppered test tubes containing sterilized cotton balls saturated with 3 mL of 0.03% Captan (Hi-yield, Bonham, TX) to limit the growth of saprophytic fungi. Each seed was planted in a separate test tube to prevent cross-contamination between seedlings, and test tubes were incubated at 28ºC for 14 days with 12 h fluorescent light daily in an incubator (Percival, Perry, IA). The proportion of seedlings displaying BFB symptoms was recorded daily and this experiment was repeated three times. The area under the disease progress curve (AUDPC) data were generated and used for ANOVA.
The Student’s t test was used to compare the effect of the T2SS on seed-to-seedling transmission of BFB.

**Generation of endoglucanase, xylanase and pectate lyase mutants of AAC00-1.**

The role of specific carbohydrate degrading enzymes in the colonization of watermelon seed (cv. Sugar Baby) by *A. citrulli* was determined. Xylanase, endoglucanase and pectate lyase deletion mutants were created using the protocol described above. To create constructs for deletion primers; cel.For1/cel.Rev2 and cel.For3/cel.Rev4 were used for endoglucanase, xyl.For1/xyl.Rev2 and xyl.For3/xyl.Rev4 were used for xylanase, and pec.For1/pec.Rev2 and pec.For3/pec.Rev4 were used for pectate lyase (Table 3.3). These mutants were designated AAC00-1ΔXyl (xylanase deletion mutant), AAC00-1ΔEgl (endoglucanase deletion mutant), AAC00-1ΔPec (pectate lyase deletion mutant). Deletion of target genes was confirmed by PCR analysis using gene-specific primers; Del.cel.For/cel.Rev4 for endoglucanase, xyl.del.For/xyl.del.Rev for xylanase, and pec.del.For/pec.Rev4 (Table 3.3). To complement the gene deletions, the open reading frame of each gene including ~ 500 bp upstream and downstream flanking sequences was amplified using the appropriate primer sets (Table 3.3). The primers used for complementation were; Cel.comp.for/Cel.comp.rev for the endoglucanase gene, xyl.For1/xyl.Rev4 for the xylanase gene, and pec.For1/pec.Rev4 for the pectate lyase gene. Each gene fragment was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and subsequently digested with the appropriate restriction enzymes (Table 3.3), to facilitate subcloning into a similarly digested pUFR043 cosmid. pUFR043 carrying either the endoglucanase, xylanase or pectate lyase gene was transformed into *E. coli* DH5α (λpir) and *E. coli* DH5α (λpir) carrying pUFR043-Xyl, pUFR043-Cel, or pUFR043-Pec were mated to AAC00-1ΔXyl, AAC00-1ΔEgl,
or AAC00-1ΔPec, respectively. This gave rise to AAC00-1ΔXylcomp (complemented xylanase deletion mutant), AAC00-1ΔEglcomp (complemented endoglucanase deletion mutant), AAC00-1ΔPeccomp (complemented pectate lyase deletion mutant). The presence of the complemented gene in each strain was confirmed by PCR using Del.cel.For/cel.Rev4 for endoglucanase, xyl.del.For/xyl.del.Rev for xylanase, and pec.del.For/pec.Rev4 (Table 3.3). Functional complementation of endoglucanase deletion mutant was confirmed using a CMC plate assay as described above.

The role of endoglucanase, xylanase and pectate lyase in watermelon seed colonization by

*A. citrulli*.

The role of endoglucanase, xylanase and pectate lyase in seed colonization was determined by infiltrating watermelon seed (cv. Sugar Baby) individually with the mutated strain or the complemented strain (for each gene), or wild-type strain AAC00-1. Bacterial populations were monitored over time as described above. AUPDC data were collected and Tukey-Kramer’s honestly significant difference (HSD) analysis was conducted to determine if proteins were important for watermelon seed colonization by *A. citrulli*.

Effect of type II secretion on *A. citrulli* virulence on watermelon fruit rind.

To determine the role of CWDE in *A. citrulli* virulence on watermelon fruit tissues, AAC00-1ΔXyl, AAC00-1ΔEgl, and AAC00-1ΔPec were infiltrated separately into the rind of mature fruits. Using a 1 mm needle, a small wound was made in the rind of the fruit and 2 μL of a $1 \times 10^8 \text{ CFU/mL}$ suspension of each strain were infiltrated into the wound using a micropipettor (Rainin, Oakland, CA). Watermelon fruits were incubated at 100 % relative
humidity and 30°C in an incubator (Percival) for 1 wk. After incubation, the diameter of each watersoaked lesion was measured at its widest point. Lesion diameter data were collected and used to determine the effect of the T2SS and T2S proteins on *A. citrulli* virulence. The experiment was repeated twice and the data were analyzed by ANOVA and by Tukey-Kramer’s HSD.

**LITERATURE CITED**


Table 3.1. Bacterial strains and plasmids used in this study.

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<th>Strains, plasmids</th>
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Table 3.2. Predicted genes in the *Acidovorax citrulli* gsp1 and gsp2 type II secretion system gene clusters.

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<td>Aave_4160</td>
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<tr>
<td>Outer membrane pore</td>
<td>gspD</td>
<td>Aave_4150</td>
</tr>
<tr>
<td>Interconnector between outer and inner membrane</td>
<td>gspN</td>
<td>Aave_4150</td>
</tr>
<tr>
<td>Platform on which pseudopilus is built</td>
<td>gspM</td>
<td>Aave_4152</td>
</tr>
<tr>
<td>Platform on which pseudopilus is built</td>
<td>gspL</td>
<td>Aave_4153</td>
</tr>
<tr>
<td>Psuedopilus, inclusion arrests elongation</td>
<td>gspK</td>
<td>Aave_4154</td>
</tr>
<tr>
<td>Psuedopilus</td>
<td>gspJ</td>
<td>Aave_4155</td>
</tr>
<tr>
<td>Psuedopilus</td>
<td>gspI</td>
<td>Aave_4156</td>
</tr>
<tr>
<td>Psuedopilus</td>
<td>gspH</td>
<td>Aave_4157</td>
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<td>Psuedopilus</td>
<td>gspG</td>
<td>Aave_4158</td>
</tr>
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<td>Interconnector between outer and inner membrane</td>
<td>gspC</td>
<td>Aave_4151</td>
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<tr>
<td>Platform on which pseudopilus is built</td>
<td>gspF</td>
<td>Aave_4159</td>
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**Table 3.3. Oligonucleotide primers used in this study**

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<th>Function</th>
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<td>SalI</td>
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</tr>
<tr>
<td>gspG1.Rev2</td>
<td>GCTTCACCCTCATCGACTAGTACCGCTACGCCAAATC</td>
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</tr>
<tr>
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<tr>
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<td>XbaI</td>
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</tr>
<tr>
<td>gspG2.Rev2</td>
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<td></td>
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<tr>
<td>gspG2.For3</td>
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<td></td>
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<tr>
<td>gspG2.Rev4</td>
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<td>xyl.del.rev</td>
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<tr>
<td>pec. Rev4</td>
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<td><em>XbaI</em></td>
<td><em>A. citrulli</em> pectate lyase gene downstream reverse primer/ pectate lyase complementation reverse primer</td>
</tr>
<tr>
<td>pec.del.For</td>
<td>ATCCGAGAAGCGACTCTTGAGGA</td>
<td></td>
<td><em>A. citrulli</em> pectate lyase gene deletion check forward primer</td>
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<td><em>A. citrulli</em> endoglucanase gene deletion check forward primer</td>
</tr>
</tbody>
</table>

* Restriction site in bold
**Figure 3.1.** Organization of the type II secretion system gene clusters of *Acidovorax citrulli*.

Letters indicate the common terms used to describe the general secretory pathway components.
gsp1

D C M L K J I H G F E

gsp2

E D N M L K J I H G C
Figure 3.2. Confirmation of gspG1 and gspG2 deletion in Acidovorax citrulli type II secretion system mutant (AAC00-1ΔgspG1:ΔgspG2). A. Polymerase chain reaction confirmation of gspG2 deletion in Acidovorax citrulli type II secretion system mutant (AAC00-1ΔgspG1:ΔgspG2). Lanes 1-4 represent PCR amplification of the gspG2 gene; 1) 1-kb DNA ladder; 2) AAC00-1ΔgspG1:ΔgspG2 (T2SS mutant); 3) AAC00-1 (wild-type A. citrulli); 4) water (negative control). Lanes 5-8 represent PCR amplification of the gspG1 gene. Lane: 5) AAC00-1ΔgspG1:ΔgspG2 (T2SS mutant); 6) AAC00-1 (wild-type A. citrulli); 4) water (negative control). B. Carboxymethyl cellulose assay for endoglucanase activity of wild-type A. citrulli. Colony1 represents AAC00-1 and colony 2 represents the type II secretion system mutant, AAC00-1ΔgspG1:ΔgspG2. An orange halo around the colony indicates the ability to hydrolyze cellulose.
Figure 3.3. Watermelon seedling (cv. Crimson Sweet) cotyledons infiltrated with different strains of *A. citrulli*. Cotyledons were inoculated with $10^6$ CFU/mL of wild-type bacteria (AAC00-1), the type II secretion system mutant (AAC00-1ΔgspG1:ΔgspG2), or phosphate buffered saline (PBS), as a negative control.
**Figure 3.4A.** Colonization of watermelon cotyledons (cv. Crimson Sweet) by AAC00-1 (wild-type *A. citrulli*) or AAC00-1ΔgspG1:ΔgspG2 (*A. citrulli* T2SS mutant). The experiment was repeated three times and each datum point represents the mean of three experiments (*n* = 6 leaf discs/treatment). Lines represent the standard error of the means. **B.** Bar chart of area under population dynamics curve calculated for watermelon seedlings infiltrated with AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 and incubated for 96 hours. Bars represent the mean AUPDC and lines indicate standard error of the means. Treatments with different letters are significantly different according to the Student’s t test (*P* < 0.05).
**Figure A**

Graph showing the log_{10} CFU/disc over time (hours post inoculation) for different treatments:

- **AAC00-1**
- **AAC00-1ΔgspG1:ΔgspG2**

**Figure B**

Bar chart comparing the area under the population dynamics curve (AUPDC) for different treatments:

- **AAC00-1**
- **AAC00-1ΔgspG1:ΔgspG2**

Legend:

- Solid dot: AAC00-1
- Open circle: AAC00-1ΔgspG1:ΔgspG2
Figure 3.5A. Colonization of watermelon seed by wild-type *A. citrulli* (AAC00-1) or the T2SS mutant (AAC00-1ΔgspG1:ΔgspG2) during the first 96 h of germination. The experiment was repeated three times and each datum point represents the mean of the three experiments (*n* = 5 seed). Lines represent the standard error of the means. B. Bar chart of area under population dynamics curve (AUPDC) calculated for seed infiltrated with 10³ CFU of AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 and germinated for 96 h. Bars represent mean AUPDC and lines indicate standard error of the means. Treatments with different letters are significantly different according to the Student’s t test (*P* < 0.05).
**Time (hours after planting)**

**Log$_{10}$ CFU/g seed tissue**

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<th>Log$_{10}$ CFU/g seed tissue</th>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
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</tbody>
</table>

**A**

**Treatments**

- AAC00-1
- AAC00-1ΔgspG1:ΔgspG2

**Area under the population dynamics curve (AUPDC)**

<table>
<thead>
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<th>Treatments</th>
<th>Area under the population dynamics curve (AUPDC)</th>
</tr>
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<tbody>
<tr>
<td>AAC00-1</td>
<td>A</td>
</tr>
<tr>
<td>AAC00-1ΔgspG1:ΔgspG2</td>
<td>B</td>
</tr>
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</table>

B
Figure 3.6A. Bacterial fruit blotch incidence on seedlings germinated from seed infiltrated with $10^3$ CFU of wild-type *A. citrulli* (AAC00-1) or the type II secretion system mutant (AAC00-1ΔgspG1:ΔgspG2). Each datum point represents the mean BFB seedling transmission for three experiments (n = 25 seed per treatment) and the lines represent the standard error of the means. 

**B.** Bar chart of area under disease progress curve for changes in BFB incidence over time. Lines indicate standard error of the means. Treatments with different letters are significantly different according to Student’s t test (P < 0.05).
Treatments

Area under disease progress curve (AUDPC)

Mean bacterial fruit blotch incidence (%)

Time (days after planting)

A

AAC00-1
AAC00-1ΔgspG1:ΔgspG2

AAC00-1 ΔgspG1:ΔgspG2

B

A

B

AAC00-1
AAC00-1ΔgspG1:ΔgspG2

Treatments
Figure 3.7. Confirmation of xylanase, endoglucanase and pectate lyase gene deletions in *A. citrulli*. A. Polymerase chain reaction confirmation of xylanase, endoglucanase and pectate lyase gene deletions in *A. citrulli*. Lanes 1-4 represent xylanase gene PCR; Lane: 1) AAC00-1ΔXyl; 2) AAC00-1ΔXylcomp; 3) AAC00-1; 4) water (negative control). Lanes 5-8 represent PCR amplification of the endoglucanase gene; Lane: 5) AAC00-1ΔEgl; 6) AAC00-1ΔEglcomp; 7) AAC00-1; 8) water (negative control). Lanes 9-12 represent PCR amplification of the pectate lyase gene; Lane: 9) AAC00-1ΔPec; 10) AAC00-1ΔPeccomp; 11) AAC00-1; 12) water (negative control); 13) 1-kb DNA ladder (Fermentas). B. Carboxymethyl cellulose assay indicating endoglucanase activity for the different of *A. citrulli* strains. An orange halo around the colonies indicates the ability to utilize cellulose. Colonies 1, 2 and 3 represent AAC00-1, AAC00-1ΔEgl and AAC00-1ΔEglcomp, respectively.
<table>
<thead>
<tr>
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<th>Pectate lyase</th>
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</tr>
<tr>
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</tr>
<tr>
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</table>

2.5 kb

1 kb

A

B

1  2  3
Figure 3.8. Colonization of watermelon seed (cv. Sugar Baby) by *A. citrulli* strains in the first 96 h of germination. Seed were infiltrated with $10^3$ CFU of bacteria and 5 seed were collected at different timepoints. Experiments were repeated three times and each datum point represents the mean of the three experiments. Lines represent the standard error of the mean. **A.** Watermelon seed colonization by wild-type *A. citrulli* (AAC00-1), the xylanase deletion mutant (AAC00-1ΔXyl) and the complemented strain (AAC00-1ΔXyl/comp). **B.** Colonization of watermelon seed by wild-type *A. citrulli* (AAC00-1), the endoglucanase deletion mutant (AAC00-1ΔEgl) and the complemented strain (AAC00-1ΔEgl/comp). **C.** Colonization of watermelon seed by wild-type *A. citrulli* (AAC00-1), the pectate lyase deletion mutant (AAC00-1ΔPec) and the complemented strain (AAC00-1ΔPec/comp).
Figure 3.9. Effects of mutations in the type II secretion system and type II secreted proteins on lesion diameter produced by *A. citrulli* on mature watermelon fruit rind. Fruit rind was infiltrated with $1 \times 10^7$ CFU/mL of AAC00-1, AAC00-1ΔgspG1:ΔgspG2 (the type II secretion system mutant), AAC00-1ΔXyl (xylanase mutant), AAC00-1ΔEgl (endoglucanase mutant), AAC00-1ΔPec (pectate lyase mutant), and phosphate buffered saline (PBS) as a negative control. The experiment was repeated twice. Bars represent mean lesion size and lines indicate standard error of the means. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference (P < 0.05).
Lesion size (mm)

Treatments

AAC00-1
AAC00-1ΔXyl
AAC00-1ΔPec
AAC00-1ΔEgl
AAC00-1Δgspg1Δgspg2
PBS
CHAPTER 4

ROLE OF QUORUM SENSING IN SEED-TO-SEEDLING TRANSMISSION OF
BACTERIAL FRUIT BLOTCH OF CUCURBITS

Johnson, K. L., and Walcott, R. R. 2010. To be submitted to Molecular Plant Microbe Interaction
ABSTRACT

Based on the observation that *A. citrulli* must switch from epiphytic to pathogenic growth for seed-to-seedling transmission of bacterial fruit blotch (BFB), we hypothesized that quorum sensing (QS) was involved in the regulation of this process. Using *aacI* (*luxI* homolog) and *aacR* (*luxR* homolog) mutants of AAC00-1, we investigated the role of QS in watermelon seed colonization and seed-to-seedling transmission of BFB. *aacR* and *aacI* mutants of AAC00-1 colonized germinating watermelon seed at wild-type levels; however, BFB seedling transmission was affected in a cell density-dependent manner. There were no significant differences in BFB seedling transmission between watermelon seed infiltrated with $\sim 1 \times 10^6$ CFU of AAC00-1, the *aacR* or *aacI* deletion mutants, (95.2, 94.9 and 98.3% BFB incidence, respectively). In contrast, when seed inoculum was reduced to $\sim 1 \times 10^3$ CFU, BFB seedling transmission percentage declined to 34.3% for the *aacI* which was significantly less than the wild-type AAC00-1 (78.6%). Interestingly, BFB transmission for the *aacR* mutant was not significantly different to the wild-type strain. These data suggest that QS plays a role in regulation of genes involved in seed-to-seedling transmission of BFB.

Additional keywords: *Acidovorax avenae* subsp. *citrulli*, Watermelon fruit blotch, Seed pathology, Cell-to-cell communication.
INTRODUCTION

Bacterial fruit botch of cucurbits (BFB) caused by Acidovorax citrulli (Schaad et. al., 2008) (formerly A. avenae subsp. citrulli (Willems et. al., 1992) = Pseudomonas pseudoalcaligenes subsp. citrulli (Schaad et. al., 1978)) is a serious threat to cucurbit production worldwide. BFB was first reported in commercial watermelon fields in Florida in 1989 (Somodi et. al., 1991) and has since become a major economic concern for US cucurbit seed producers (Walcott, 2008). A. citrulli-infested cucurbit seed is the most important source of primary inoculum for BFB, which can cause up to 100% cucurbit yield loss under favorable field conditions (Latin and Hopkins, 1995). While the geographical center of origin for this pathogen is unknown, global seed production and trade has facilitated worldwide distribution of the pathogen. Therefore, rigorous seed health testing is necessary to exclude seedborne inoculum from commercial cucurbit production systems.

Despite significant disease management efforts, including zero tolerance for A. citrulli in cucurbit seed and transplant production, sporadic but costly BFB outbreaks still occur worldwide (Walcott, 2008). Since pathogen exclusion and seed treatments are not 100% effective, new approaches must be sought to limit losses caused by BFB. Unfortunately, at present, limited information is available on the host-pathogen interactions that facilitate seedling transmission of A. citrulli, especially during the early stages of seed germination. In preliminary studies, we observed that a non-pathogenic A. citrulli type three secretion system mutant (AAC00-1ΔhrcC), retained its ability to colonize seed during the first 96 h of germination (Johnson et. al., unpublished data). Interestingly, by 6 days after planting, AAC00-1ΔhrcC population growth declined significantly relative to the wild-type strain. Additionally, we observed that a type II secretion system mutant of A. citrulli, retained pathogenicity on watermelon leaf tissue but was
limited in its ability to colonize watermelon seed during the first 96 h of germination (Johnson et al., unpublished data). Taken together, these observations suggest that during initial stages of seed germination *A. citrulli* grows as an epiphyte, relying on the degradation of complex carbohydrates released in seed exudates. Subsequently, the bacterium switches to pathogenic growth on seedling tissues, which requires type III secreted effector proteins. Based on these observations, we hypothesized that quorum sensing (QS) plays a role in the shift from epiphytic to pathogenic survival of *A. citrulli* on germinating watermelon seed, and contributes to seed-to-seedling transmission of BFB.

Quorum sensing is the phenomenon by which bacteria communicate and respond collectively to environmental cues (Waters and Bassler, 2006). It is involved in the regulation of many biological functions including bioluminescence, swarming motility, biofilm formation and horizontal DNA transfer (Hwang et al., 1994; Meighen, 1994; Quinones et al., 2005). Acyl homoserine lactone mediated QS is comprised of a two component system that includes a *luxI* homolog, which encodes an acyl homoserine lactone (AHL) synthase that produces the AHL signaling molecule (Federle and Bassler, 2003; Williams et al., 2007). The QS system also includes a *luxR* homolog that encodes a transcriptional regulator, which can act as a repressor or, in complex with AHL acts as a transcriptional activator (Federle and Bassler, 2003; Williams et al., 2007). AHLs accumulate in a population density dependent manner and in complex with the LuxR protein regulate target gene transcription (Waters and Bassler, 2006). The paradigm for this system is *Vibrio fischeri*, a bacterium in which QS mediates light production in the symbiotic host, *Euprymna scolopes* (Nelson, 1977). Another QS model was proposed for *Pantoea stewartii* subsp. stewartii, the causal agent of Stewart’s wilt of maize (Beck von Bodman et al., 1998). According to this model, the LuxR protein acts as a transcriptional
repressor and binding of AHLs to LuxR removes repression (Minogue et al., 2002). Evidence for quorum sensing in A. citrulli was provided by Tao et al. (2009) who demonstrated that quorum quenching of AHL molecules produced by A. citrulli resulted in reduced virulence on watermelon fruit rind. At present little is known about the genes regulated by QS or the role of luxI and luxR homologs in A. citrulli virulence. Hence, the goal of this research was to investigate the role of QS in seed-to-seedling transmission of BFB.

RESULTS

Confirmation and characterization of aacI and aacR mutants of A. citrulli.

To characterize the role of QS in A. citrulli virulence, the genes encoding the luxI and luxR homologs, aacI and aacR, respectively, were deleted by homologous recombination. Deletion of the aacI and aacR genes was confirmed by PCR analysis (Fig. 4.1A and 4.1B, respectively) and by the A. tumefaciens biosensor assay (Fig. 4.2). Using primers AacI.comp.For/AacI.comp.Rev (Table 4.2), a 2.0-kb DNA fragment was amplified from wild-type AAC00-1, and the complemented strain, AAC00-1ΔaacIcomp. However, these primers only amplified a 1.3-kb fragment from the aacI deletion mutant, AAC00-1ΔaacI, which was also identified in AAC00-1ΔaacIcomp (Fig.4.1A). PCR amplification of the aacR gene region using aacR primers (AacR.comp.For/AacR.Rev4; Table 4.2) yielded a 2.86-kb amplicon with template DNA from AAC00-1, and a 2.3-kb fragment from the aacR mutant, AAC00-1ΔaacR. As predicted, culture supernatant from AAC00-1ΔaacI did not induce β-galactosidase expression in Agrobacterium tumefaciens NTI (Fig. 4.2). This phenotype was similar to the A. tumefaciens NTI indicator strain that does not produce AHL (Fig. 4.2). Culture supernatant from AAC00-1ΔaacR induced β-galactosidase expression that resulted in the breakdown of X-gal and
production of a blue color (Fig. 4.2). This indicated that AAC00-1ΔaacR produced AHLs in the absence of the AacR transcriptional regulator. β-galactosidase was also produced by AAC00-1 and the AHL-producing A. tumefaciens strain. These data suggest that AHL production in AAC00-1 is conferred by aaci and is not influenced by aacR.

The aaci and aacR genes are transcribed in tandem with the aaci gene located downstream of aacR. The genes do not appear to be located in an operon because they are separated by a 186 bp intergenic region. aaci encodes a 211 amino acid protein that shares 29% identity to EanI (P. ananatis, accession number BAF69065), 34% identity to PmII (Burkholderia pseudomallei, accession number YP_110894), 36% identity to CepI (B. cepacia, accession number AAK70348), and 24% identity to EsaI (P. stewartii subsp. stewartii, accession number AAA82096) (Lewenza et. al., 1999; Valade et. al., 2004; Beck von Bodman et. al., 1998; Morohoshi et. al., 2007). Pairwise alignment of the AacR amino acid sequence to proteins of other LuxR homologs indicated that the protein is 27, 43, 35 and 28% identical to EanR (accession number BAF69065), PmII (accession number YP_106160), CepR (accession number AAG61131), and EsaR (accession number AAA82097), respectively (Lewenza et. al., 1999; Beck von Bodman et. al., 1998; Valade et. al., 2004; Morohoshi et. al., 2007). BLAST analysis of the AacR protein indicated that it has an AHL binding domain located at residues 15-160, and a DNA binding domain located at residues 179-222, characteristic of the LuxR protein family.

Role of QS in A. citrulli pathogenicity on watermelon seedlings.

Since QS affected virulence of A. citrulli on watermelon fruit rind (Tao et. al., 2009), we investigated the influence of population density and QS on A. citrulli virulence on watermelon seedlings. Two week old watermelon seedlings (cv. Crimson Sweet) were infiltrated with ~1 ×
10^3 CFU/mL or ~1 × 10^6 CFU/mL AAC00-1, AAC00-1ΔaacI or AAC00-1ΔaacR and observed for BFB symptoms. When infiltrated with ~1 × 10^6 CFU/mL of AAC00-1, AAC00-1ΔaacI or AAC00-1ΔaacR BFB symptoms (necrosis) were observed on seedlings by 2 days post inoculation (dpi). When similar inoculations were conducted with ~1 × 10^3 CFU/mL of each strain, seedlings infiltrated with AAC00-1 or AAC00-1ΔaacR developed watersoaked lesions by two dpi and necrosis by 3 dpi (Fig. 4.3). On the other hand, seedlings inoculated with ~1 × 10^3 CFU/mL of AAC00-1ΔaacI remained asymptomatic at 3 dpi, and small necrotic lesions were observed by 4 dpi.

**Role of QS in *A. citrulli* virulence on watermelon seedlings.**

The delay in BFB symptom development on seedlings inoculated with AAC00-1ΔaacI led us to investigate the role of QS in *A. citrulli* virulence on watermelon seedlings. Watermelon cotyledons were infiltrated with ~1 × 10^3 CFU/mL of AAC00-1, AAC00-1ΔaacI or AAC00-1ΔaacR. AAC00-1ΔaacI was impaired in its ability to colonize cotyledons relative to AAC00-1 and AAC00-1ΔaacR (Fig. 4.4A). At 48 h post inoculation (hpi) AAC00-1ΔaacI populations were 10-fold less than AAC00-1ΔaacR and 100-fold less than AAC00-1. By 96 hpi AAC00-1ΔaacI populations increased but were 100-fold less than AAC00-1 and AAC00-1ΔaacR. By the end of the study AAC00-1 and AAC00-1ΔaacR attained mean populations of 2.1 × 10^{10} and 3.7 × 10^{10} CFU/leaf disc, respectively, while AAC00-1ΔaacI reached populations of 4.3 × 10^8 CFU/leaf disc (Fig. 4.4A). The overall colonization of watermelon cotyledons by AAC00-1ΔaacI (mean AUPDC = 519 ± 15) was significantly less than AAC00-1 (mean AUPDC = 663 ± 47) and AAC00-1ΔaacR (mean AUPDC = 618 ± 24) using Tukey-Kramer’s Honestly Significant Difference (P = 0.0036) (Fig. 4.4B). Even though by 48 hpi the mean populations of
AAC00-1ΔaacR on cotyledons were 10-fold less than AAC00-1, AAC00-1ΔaacR populations reached wild-type levels by 72 hpi. Overall, the difference between the abilities of AAC00-1ΔaacR and AAC00-1 to colonize watermelon cotyledons was not significant (P = 0.26).

**Effect of QS on watermelon seed colonization by A. citrulli.**

Individual watermelon seed were infiltrated with ~1 × 10³ CFU of AAC00-1, AAC00-1ΔaacI or AAC00-1ΔaacR and incubated in germination boxes (30°C and 100% RH). Five seed were collected each day for 8 days and populations of bacteria on each seed were estimated by dilution plating on selective media plates. Overall, both A. citrulli QS mutants colonized watermelon seed at wild-type levels (Fig. 4.5A). There was a 10³-fold increase in the mean populations of AAC00-1ΔaacI/g of seed 2 days after planting (dap), and similar increases were observed for AAC00-1 and AAC00-1ΔaacR. AAC00-1ΔaacI, AAC00-1ΔaacR, and AAC00-1 attained mean populations of ~7.5 × 10¹², 4.5 × 10¹², 2.0 × 10¹³ CFU/g of seed tissue, respectively, by 8 dap (Fig. 4.5A). Based on analysis of AUPDC data there were no significant differences between the abilities of AAC00-1ΔaacI and AAC00-1ΔaacR to colonize seed relative to the wild-type strain (P = 0.28) (Fig. 4.5B). These data indicate that QS did not play a role in A. citrulli colonization of watermelon seed during germination.

**Effect of QS on seed-to-seedling transmission of BFB.**

Preliminary observations that A. citrulli grows as an epiphyte during the initial stages of seed germination prompted us to investigate the role of QS in the putative switch to pathogenic growth on emerging watermelon seedlings. Individual seed were infiltrated with ~1 × 10³ or ~1 × 10⁶ CFU of bacteria and incubated on germination paper in separate test tubes to prevent
seedling-to-seedling contamination. Seed-to-seedling transmission of BFB was influenced by the initial level of seed inoculum (Fig. 4.6A). Seed infiltrated with ~1 × 10^6 CFU had significantly higher levels of BFB seed-to-seedling transmission relative to seed infiltrated with ~1 × 10^3 CFU of bacteria with all strains (P < 0.05) (Fig. 4.6B). Mean BFB seed-to-seedling transmission percentages were 95.2, 94.9 or 98.3% for seed infiltrated with ~1 × 10^6 CFU of AAC00-1ΔaacI, AAC00-1ΔaacR, or AAC00-1, respectively by 14 dap. In comparison, seed infiltrated with ~1 × 10^3 CFU of AAC00-1ΔaacI, AAC00-1ΔaacR, or AAC00-1 displayed mean seed-to-seedling transmission percentages of 34.3, 61.9 or 78.6%, respectively (Fig. 4.6A). Interestingly, when seed were inoculated with ~1 × 10^6 CFU of bacteria the differences in seed-to-seedling transmission between AAC00-1ΔaacI, AAC00-1ΔaacR and AAC00-1 were not statistically significant (P < 0.05) (Fig. 4.6B). However, when seed were inoculated with 10^3 CFU AAC00-1ΔaacI, BFB seedling transmission (34.3%) was significantly less than AAC00-1ΔaacR (61.9%) and AAC00-1 (78.6%) (P = 0.05). These data suggest that aacI plays a role in seed-to-seedling transmission of BFB at low A. citrulli seed populations.

**Effect of complementation of the aacI mutation on seed-to-seedling transmission of BFB.**

To determine if the reduction in seed-to-seedling BFB transmission observed for AAC00-1ΔaacI was due to aacI deletion a complemented strain, AAC00-1ΔaacIcomp, was generated. Complementation of aacI deletion mutation in AAC00-1ΔaacIcomp was confirmed by PCR analysis (Fig. 4.1A). Watermelon seed were infiltrated with ~1 × 10^3 CFU of AAC00-1, AAC00-1ΔaacI or AAC00-1ΔaacIcomp as described above and BFB seedling transmission percentages was monitored for 14 dap. Seed infiltrated with AAC00-1ΔaacI, AAC00-1ΔaacIcomp, and AAC00-1 displayed mean seed-to-seedling BFB transmission levels of 75.5, 49, or 72 %,
respectively (Fig. 4.7A). There was no significant difference in BFB seedling incidence between seed infiltrated with AAC00-1 and AAC00-1Δaaclcomp (P = 0.71). In contrast seed-to-seedling BFB transmission for seed infiltrated with AAC00-1ΔaacI was significantly lower than for AAC00-1, (P = 0.0042) and AAC00-1Δaaclcomp (P = 0.0023) (Fig. 4.7B). Based on these data wild-type levels of seed-to-seedling BFB transmission were restored by reintroduction of aacI to AAC00-1ΔaacI, suggesting that QS is involved in seed-to-seedling transmission of BFB. It also appears that AHL is critical for this process.

DISCUSSION

Acyl-homoserine lactone (AHL)-mediated quorum sensing is employed by a diverse group of Gram negative α, γ, and β- proteobacteria (Fuqua et. al., 1994; Swift et. al., 1999). Bioinformatic analysis indicated the presence of luxI and luxR gene homologs in the AAC00-1 genome, which were designated aacI and aacR. Blast sequence analysis of the AacI/R proteins revealed low identity to other LuxI/R homologs in the NCBI database (http://www.ncbi.nlm.nih.gov/pubmed/). This was not surprising as there can be a high degree of variability in LuxI/R sequences amongst strains within bacterial species (Gray and Garey, 2001).

Tao et. al. (2009) used quorum quenching with an acyl homoserine lactonase encoded by aiiA to demonstrate that A. citrulli produces AHLs (3-oxo-octanoly-L-homoserine lactone) and has a functional QS system (Tao et. al., 2009). However, Tao et. al. (2009) did not explore the role of the AacR transcriptional regulator in A. citrulli pathogenicity. To study the role of QS in seed-to-seedling transmission of BFB, we generated separate aacI and aacR gene deletion mutants. In the aacI deletion mutant AHLs were not produced; however, in the absence of aacR, AHLs were detected using the AHL indicator strain, A. tumefaciens NTI. These observations
suggest that *A. citrulli* AHL production was not controlled by the LuxR protein. This is different from QS systems in *Vibrio fischeri, A. tumefaciens*, and *Psuedomonas aeruginosa* where the *luxI* gene is regulated by the LuxR/AHL complex resulting in a positive feedback loop (Piper *et. al.*, 1993; Latifi *et. al.*, 1995; Stevens and Greenberg, 1997). Alternatively, *A. citrulli* AHL production is similar to systems described for *P. stewartii* subsp. *stewartii*, *Serratia plymuthica* and *P. ananatis* (Beck von Bodman *et. al.*, 1998; Morohoshi *et. al.*, 2007; Van Houdt *et. al.*, 2007). In these organisms the LuxR protein acts as a negative regulator of transcription and AHL production is constitutively active. To our knowledge, *A. citrulli* is the first seedborne β-proteobacteria for which independent regulation of the *luxI* homolog has been reported.

In the current study QS influenced the virulence of *A. citrulli* on watermelon seedlings. We observed a delay in BFB symptom development on watermelon cotyledons with an *aacI* deletion mutant relative to the wild-type and the *aacR* mutant strains. This delay in symptom development was not observed when seedlings were inoculated ~1 × 10⁶ CFU/mL. The role of QS in *A. citrulli* virulence was supported by the reduction in colonization when ~1 × 10³ AAC00-1ΔaacI CFU/mL was infiltrated into watermelon cotyledons. This suggested that QS affects expression of *A. citrulli* virulence related genes.

Regulation of virulence related genes by QS has been reported for both plant and animal pathogens. In *E. carotovora* QS regulates the production of pectinase, cellulase and proteases that contribute to cell wall degradation (Jones *et. al.*, 1993). Deletion of the *E. carotovora luxI* homolog, *expI*, resulted in a strain that was unable to secrete exoenzymes and was reduced in virulence. QS as also been implicated in the regulation of the T3SS. In *Pectobacterium atrosepticum* QS regulates T3SS structural genes, *hrpE* and *hrpN* and the T3SS regulators *hrpS* and *hrpL* (Liu *et. al.*, 2008). The T3SS is important for virulence and colonization of watermelon
seedling tissue by *A. citrulli* (*data not shown*). A reduction in T3S or translocation of certain effectors could account for the reduction in virulence of the *aacI* mutant on watermelon seedlings. However, at high concentrations of *A. citrulli* the QS-regulated genes may not be required. Expression of non-QS regulated virulence related genes are likely able to allow *A. citrulli* to colonize watermelon seedling tissue at wild-type levels.

The *aacR* deletion mutant, AAC00-1ΔaacR, colonized watermelon cotyledons and induced BFB symptoms at wild-type levels. The lack of difference in virulence of the *aacR* deletion mutant and the wild-type *A. citrulli* strain may be a result of the AacR protein acting as a transcriptional repressor as observed with EsaR of *P. stewartii* subsp. *stewartii* (Beck von Bodman and Farrand, 1995). EsaR can bind to DNA in the absence of AHLs and function as a repressor by preventing transcription. At threshold levels, AHLs bind to EsaR and modify its configuration, which decreases EsaR DNA-binding capacity and results in removal of transcription repression (Minogue *et. al.*, 2002). In fact, over expression of luxR transcription regulators with repressor functions led to even greater reductions of virulence factor production (Andersson *et. al.*, 2000). If the AacR protein is acting as a repressor, in its absence, genes under its control will be constitutively expressed, allowing the AacR mutant to remain virulent. In contrast, for the *aacI* mutant in which there is a functional AacR protein and no AHLs, QS regulated genes would be constantly repressed. This would explain the reduction in virulence that was observed for the *A. citrulli aacI* deletion mutant.

Data generated in this study suggests that QS does not play a significant role in seed colonization by *A. citrulli* in the early stages of germination. However, the *aacI* mutant was significantly reduced in its ability to be transmitted from infested seed to germinating seedlings.
at low bacteria inoculum densities. The reduction in BFB incidence suggests that QS plays a role in seed-to-seedling transmission and is required for the switch to pathogenic survival.

This is the first report of the role of QS in seed-to-seedling transmission of a phytopathogen. It appears that this effect is population density-dependent, whereby low initial pathogen populations result in seedling transmission levels that are less than those observed for seed inoculated with AAC00-1. These data have furthered our understanding of the host-pathogen interactions that govern seed-to-seedling transmission of BFB. Further exploration of the role of QS in seedling transmission of BFB might yield avenues for better disease management. For example, it may be feasible to interrupt QS and thereby disrupt the coordination of genes necessary for seed-to-seedling transmission. Dong et al. (2000) identified a gene, aiIA, which encodes an acyl homoserine lactonase protein that functions to inactivate AHLs. AHL production is required for secretion of virulence proteins in E. carotovora, mutagensis of acyl homoserine synthase, expI, reduces pathogenicity (Pirhonen et al., 1993). E. carotovora expressing aiIA had reduced levels of AHL activity (Dong et al., 2000). When assayed for production of extracellular pectolytic enzymes, the activity of pectate lyase, pectin lyase and polygalacturonase were 3-10 fold less than wild-type (Dong et al., 2000). Interruption of QS can also be adapted to increase plant resistance to pathogens. Expression of aiIA in Amorphophallus konjac attenuated the virulence of E. carotovora, by inactivating AHLs (Ban et al., 2009). Interruption of QS in A. citrulli might yield a durable and environmentally sound approach for managing this and similar seedborne phytopathological diseases.
MATERIALS and METHODS

Bacterial strains, plasmids and media.

*A. citrulli*, *Agrobacterium tumefaciens* and *Escherichia coli* strains used in this study are listed in Table 4.1. *A. citrulli* and *A. tumefaciens* strains were routinely grown on nutrient agar (Difco, Becton Dickinson and Co., Sparks MD) with appropriate antibiotics for 48 h at 28°C. *E. coli* strains were grown in Luria Bertani (LB) broth or agar (Difco) with appropriate antibiotics for 24 h at 37°C. To prepare *A. citrulli* inoculum, cells were pelleted from 1.5 mL of overnight culture by centrifugation at 13,000 rpm for 1 min. Cells were washed with and resuspended in phosphate buffered saline (PBS, 137 mM NaCl, 5.6 mM Na$_2$HPO$_4$, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$). Bacterial suspensions containing ~ $1 \times 10^8$ CFU/mL were estimated spectrophotometrically (OD$_{600}$ =~ 0.3) (Spectronic 20, Baush and Lomb, Rochester, NY) and ten-fold serially diluted with PBS to generate desired cell suspensions. Bacterial matings were routinely carried out on M9 minimal medium (10 mM Na$_2$PO$_4$, 10 mM KH$_2$PO$_4$, 9.4 mM NH$_4$Cl, 4.3 mM NaCl) agar plates (Ausubel *et al.*, 1999). When used, antibiotics were incorporated into media at the following concentrations; rifamycin (100 μg/mL, *A. citrulli*), spectinomycin (100 μg/mL, *A. citrulli* and *E. coli*), kanamycin (50 μg/mL, *A. citrulli, A. tumefaciens* and *E. coli*), ampicillin (100 μg/mL, *E. coli*), cycloheximide (200 μg/mL, *A. citrulli*) (Sigma Aldrich, St. Louis MO). For blue colony screening during TOPO cloning, bromo-chloro-indolyl-galactopyranoside (X-gal, Sigma Aldrich, St. Louis, MO) was added to media at a final concentration of 40 μg/mL.
DNA manipulations.

To delete the *A. citrulli luxI* gene homolog, *aacI* (Aave_3811), flanking sequences (~ 500 bp in each direction) were amplified by PCR. The upstream and downstream regions of the gene were amplified using primer pairs AacI.For1/AacI.Rev.2 and AacI.For.3/AacI.Rev4, respectively (Table 4.2). Amplicons were analyzed by gel electrophoresis and extracted from the agarose using a gel extraction kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). The purified PCR products were subjected to splice overlap extension (SOEing) PCR to generate one fragment (Shevchuk *et. al*., 2004). The PCR amplicon was analyzed by gel electrophoresis and the band was extracted using a Qiagen gel extraction kit, as described above. The construct was cloned into the TOPO-XL vector (Invitrogen, Carlsbad, CA) and subsequently digested with *SalI* and *XbaI* to facilitate cloning into the suicide vector, pOK1 (Huguet *et. al*., 1998), to generate pOK-AacI (Table 4.1), which was transformed into *E. coli* DH5α (*λpir*). Plasmid pOK-AacI was transferred into AAC00-1 by tri-parental mating with an *E. coli* strain carrying the helper plasmid, pRK2073. Strains in which homologous recombination occurred were selected for on M9 plates with spectinomycin. Individual colonies were transferred to M9 minimal medium plates supplemented with sucrose to allow excision of the plasmid. AAC00-1 mutants in which the *aacI* gene was replaced with the deletion construct were isolated based on sensitivity to spectinomycin. Subsequently, spectinomycin-sensitive transformants were screened by PCR to confirm the presence of the truncated *aacI* gene. The *aacI* deletion mutant of *A. citrulli* was designated AAC00-1ΔaacI.

The *A. citrulli luxR* gene homolog, *aacR* (Aave_3810), was deleted using the same approach. The flanking sequences of the *aacR* gene homolog were amplified using primer pairs AacR.For1/AacR.Rev2 and AacR.For3/AacR.Rev4 (Table 4.2). The amplicons were spliced
together by PCR, as previously described, and the luxR deletion fragment was cloned into pOK1 to yield pOK-AacR (Table 4.1). E. coli carrying pOK-AacR was mated to A. citrulli to facilitate deletion of the aacR gene. Target gene deletion was verified by PCR as described. The aacR deletion mutant of A. citrulli was designated AAC00-1ΔaacR.

**Assay for acyl homoserine lactone production.**

Ten milliliters of M9 minimal medium were inoculated with a 100 μL aliquot of an overnight culture of AAC00-1, AAC00-1ΔaacI, AAC00-1ΔaacR, Agrobacterium tumefaciens, or A. tumefaciens NTI (AHL biosensor strain, (Cook et. al., 1997), and incubated at 30ºC overnight in a rotary shaker at 300 rpm. Subsequently, bacterial cells were pelleted by centrifugation at 13,000 rpm for 5 min. and the broth was passed through a 25 μm mixed cellulose ester syringe filter (Fisherbrand, Pittsburgh, PA). One milliliter of an overnight culture of A. tumefaciens was pelleted and washed with PBS before being resuspended in 1 ml of PBS. One hundred microliters of the cell-free filtrate was mixed with 2 μL of an overnight culture of A. tumefaciens NTI grown in nutrient broth and 1 μL of a 40 mg/mL solution of X-gal and incubated for 24 h at 28ºC. The presence of appropriate AHL molecules in the media induced β–galactosidase production which degrades X-gal to yield a blue color. The experiment was repeated three times.

**Pathogenicity tests.**

To determine the role of QS in A. citrulli pathogenicity, ~ 1 × 10³ and ~1 × 10⁶ CFU/mL of AAC00-1, AAC00-1ΔaacR, AAC00-1ΔaacI or PBS as a negative control were infiltrated separately into the cotyledons of ten, two-week-old watermelon seedlings (cv. Crimson Sweet) per treatment. The seedlings were incubated at 28ºC with 12 h of fluorescent light daily for three
days in a Percival Intellus incubator (Percival, Perry, IA). The experiment was repeated three times and the development of watersoaking and necrosis typical of seedling BFB symptoms was recorded.

**Effect of QS on *A. citrulli* virulence on watermelon leaf tissue.**

To determine the role of QS in *A. citrulli* colonization of watermelon seedling tissue, ~1 × 10³ CFU/mL of AAC00-1, AAC00-1ΔaacR or AAC00-1ΔaacI were infiltrated separately into the cotyledons of three, two-week-old watermelon seedlings (cv. Crimson Sweet) per treatment. Infiltrated tissues were allowed to air-dry for 1 h, and six leaf discs, (~3 mm in diameter), each from separate cotyledons were collected at 0, 12, 24, 48, 72, 96 h after inoculation. Each leaf disc was macerated in a separate microcentrifuge tube containing 900 μL of PBS and appropriate ten-fold serial dilutions were plated onto nutrient agar amended with rifamycin and cycloheximide to enumerate bacterial populations. Plates were incubated for 2-3 days at 28ºC in a Percival Intellus incubator (Percival, Perry, IA). The experiment was repeated three times. Bacterial populations were plotted over time and these graphs were used to generate area under population dynamics curves (AUPDC) data. AUPDC data were used to compare the effects of the mutations on tissue colonization using Tukey-Kramer’s Honestly Significant Difference (HSD).

**Effect of QS on seed colonization by *A. citrulli*.**

The effect of QS on watermelon seed colonization was determined by infiltrating 50 watermelon seed (cv. Sugar Baby) individually with ~1 × 10³ CFU of *A. citrulli* strains AAC00-1, AAC00-1ΔaacR or AAC00-1ΔaacI. Briefly, ten microliters of a suspension containing ~1 ×
$10^5$ CFU/mL of each strain were aspirated into each seed (Walcott et. al., 2006). Watermelon seed were incubated in transparent plastic boxes (34 cm long by 24 cm wide by 6 cm high) (Tri-state plastics, Dixon, KY) on moist blotter paper (Hoffman Manufacturing, Albany, OR) at 28°C and 100% relative humidity. Watermelon seed (n=5) were collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 days after planting and bacterial populations were estimated at each time interval by macerating individual seed in 9 mL of PBS in a sterile microcentrifuge tube. To enumerate bacterial populations, appropriate ten-fold serial dilutions of the seed macerate were spread onto nutrient agar amended with rifamycin and cycloheximide, and plates were incubated for 2-3 days at 28°C in a Percival Intellus incubator (Percival, Perry, IA). This experiment was repeated twice and bacterial populations were plotted over time. AUPDC data were generated and used to compare the effects of $aacI$ and $aacR$ mutations on $A. citrulli$ colonization of watermelon seed.

**Effect of QS on seed-to-seedling transmission of BFB.**

To determine the effect of QS on seed-to-seedling transmission of BFB, watermelon seed (cv. Sugar Baby) were individually inoculated with $\sim 1 \times 10^3$ or $\sim 1 \times 10^6$ CFU of $A. citrulli$ strains AAC00-1, AAC00-1ΔaacR or AAC00-1ΔaacI as described above. Seed were allowed to air-dry overnight and then incubated individually in moist chambers comprised of sterile, stoppered test tubes containing sterilized cotton saturated with 3 mL of 0.03% Captan (Hi-yield, Bonham, TX) to limit the growth of saprophytic fungi. Each seed was incubated in a separate test tube to prevent BFB cross-contamination between seedlings. Test tubes were incubated in a Percival Intellus incubator at 28°C for 14 days with 12 h fluorescent light daily. The number of seedlings displaying BFB symptoms was recorded daily. This experiment was repeated three times and the area under the disease progress curve (AUDPC) data were generated based on
these observations. Tukey-Kramer’s HSD was used to compare the effect of aacI and aacR mutations on seed-to-seedling transmission of BFB.

**Complementation of the aacI mutation.**

To confirm that the reduction in seed-to-seedling transmission of BFB observed for AAC00-1ΔaacI was due to deletion of the aacI gene, the aacI gene deletion was complemented. For complementation the aacI ORF and its promoter region were amplified using the AacI.comp.For/AacI.comp.Rev primer set (Table 4.2) which allowed introduction of restriction sites at the 5’ and 3’ ends of the PCR amplicon. The amplicon was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and subsequently digested with the appropriate restriction enzymes (Table 4.2), to facilitate subcloning into similarly digested pUFR043 cosmid. pUFR043-AacI, which carried the aacI gene was transformed into E. coli DH5α (λpir), which was subsequently mated to AAC00-1ΔaacI as described above. The resulting complemented strain was designated AAC00-1ΔaacIcomp and gene complementation was confirmed by PCR. Watermelon seed (cv. Sugar Baby) (n = 25) were individually inoculated with ~1 × 10³ CFU of AAC00-1, AAC00-1ΔaacI or AAC00-1ΔaacIcomp. The proportion of seedlings displaying BFB symptoms was recorded daily over a period of 14 days and seed-to-seedling transmission was determined as described above. This experiment was repeated four times.

**LITERATURE CITED**


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<th>Bacteria, plasmids</th>
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<td><em>A. tumefaciens</em></td>
<td>Wild-type, AHL producer</td>
<td>Tim Denny, UGA</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>F− Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α (λpir)</td>
<td>TpR SmR recA, thi, pro, hsdR-M°RP4: 2-Tc:Mu: Km Tn7 λpir</td>
<td>Jeff Jones, UFL</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>TOPO-XL</td>
<td>Km&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>pCR2.1-TOPO</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRK2073</td>
<td>ColEl replicon, Tra&lt;sup&gt;+&lt;/sup&gt;, Mob&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;f&lt;/sup&gt;, helper plasmid</td>
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</tr>
<tr>
<td>pUFR043</td>
<td>Gm&lt;sup&gt;f&lt;/sup&gt;, Km&lt;sup&gt;f&lt;/sup&gt;, complementation vector</td>
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<tr>
<td>pOK1</td>
<td>Sp&lt;sup&gt;f&lt;/sup&gt;, SacB&lt;sup&gt;+&lt;/sup&gt;, gene replacement vector</td>
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<tr>
<td>pOK-AacI</td>
<td>pOK1 containing the <em>aacI</em> deletion construct</td>
<td>This study</td>
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<tr>
<td>pOK-AacR</td>
<td>pOK1 containing the <em>aacR</em> deletion construct</td>
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Table 4.2. Oligonucleotide primers used in this study and their functions.

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<th>Function</th>
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<td><em>KpnI</em></td>
<td>A. citrulli upstream aacI forward primer</td>
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<tr>
<td>AacI.Rev.2</td>
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<td>AacI.For.3</td>
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<td><em>SalI</em></td>
<td>A. citrulli aacI downstream reverse primer</td>
</tr>
<tr>
<td>AacI.comp.For</td>
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<td><em>SalI</em></td>
<td>A. citrulli aacI complement forward primer</td>
</tr>
<tr>
<td>AacI.comp.Rev</td>
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<td><em>SalI</em></td>
<td>A. citrulli aacI complement reverse primer</td>
</tr>
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<td>AacR.For1</td>
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<td>A. citrulli upstream aacR reverse primer</td>
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<tr>
<td>AacR.For3</td>
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<td><em>SalI</em></td>
<td>A. citrulli aacI complement forward primer</td>
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</table>

* Restriction site in bold
Figure 4.1A. Polymerase chain reaction confirmation of aacI gene deletion in Acidovorax citrulli strain 00-1 Lane: 1) 1 Kb DNA ladder (Fermentas); 2) AAC00-1\(\Delta\)aacI (aacI deletion mutant); 3) AAC00-1\(\Delta\)aacIcomp (complemented strain of the aacI deletion mutant); 4) AAC00-1 (wild-type A. citrulli); 5) water (negative control). B. Confirmation of aacR gene deletion using PCR analysis. Lane: 1) 1 Kb DNA ladder; 2) AAC00-1\(\Delta\)aacR (aacR deletion mutant); 3) AAC00-1 (wild-type A. citrulli); 4) water (negative control).
Figure 4.2. Induction of β-galactosidase activity by acyl homoserine lactones (AHL) present in culture supernatants from quorum sensing mutants of *Acidovorax citrulli* and *Agrobacterium tumefaciens*. Well contents include: 1) *A. tumefaciens* (AHL producing strain); 2) *A. tumefaciens* NTI (AHL indicator strain); 3) AAC00-1 (wild-type *A. citrulli*); 4) AAC00-1ΔaacR (*aacR* deletion mutant); and 5) AAC00-1ΔaacI (*aacI* deletion mutant).
Figure 4.3. Pathogenicity assays indicating the ability of *A. citrulli* quorum sensing mutants to infect two-week old watermelon seedlings (cv. Crimson Sweet). Pathogenicity assays for; 1) AAC00-1 (wild-type *A. citrulli*); 2) AAC00-1ΔaacI (aacI deletion mutant); 3) AAC00-1ΔaacR (aacR deletion mutant); 4) phosphate buffered saline (PBS, negative control). Necrotic lesions developed three days after inoculation with ~ 1 x 10³ CFU/mL of bacteria.
**Figure 4.4A.** Effect of quorum sensing on the growth of *Acidovorax citrulli* strains AAC00-1, AAC00-1ΔaacI (aacI mutant) and AAC00-1ΔaacR (aacR mutant) in watermelon seedling (cv. Crimson Sweet) tissue. Watermelon cotyledons were infiltrated with ~1 x 10³ CFU/mL of bacteria. The experiment was repeated three times and each datum point represents the mean of three experiments (n = 6 leaf discs/treatment). Lines represent the standard error of the means. **B.** Bar chart of area under population dynamics curve for *A. citrulli* strains AAC00-1, AAC00-1ΔaacI and AAC00-1ΔaacR. Bars represent mean AUPDC and lines indicate standard error of the means. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference.
Figure A shows the Log10 CFU/disc over time for different treatments. The treatments include AAC00-1, AAC00-1ΔaacI, and AAC00-1ΔaacR. The area under the population dynamics curve (AUPDC) is presented in Figure B for the same treatments. The AUPDC values are compared between AAC00-1, AAC00-1ΔaacI, and AAC00-1ΔaacR.
Figure 4.5A. Effect of quorum sensing on *A. citrulli* colonization of watermelon seedlings (cv. Sugar Baby) during the first eight days of germination. *A. citrulli* strains included in this study were AAC00-1 (wild-type), AAC00-1ΔaacI (*aacI* mutant) and AAC00-1ΔaacR (*aacR* mutant). Watermelon seed were infiltrated with ~1 x 10^3 CFU/mL of bacteria. The experiment was repeated twice and each datum point represents the mean of the two experiments (n = 5 seed). Lines represent the standard error of the means. B. Bar chart of area under population dynamics curve for different *A. citrulli* strains. Bars represent mean AUPDC and lines indicate standard error of the means. Treatments with similar letters are not significantly different according to Tukey-Kramer’s Honestly Significant Difference.
Time (days after planting)

0 2 4 6 8

Log10 CFU/g of watermelon seed

0 2 4 6 8 10 12 14

A

Area under the population dynamics curve (AUPDC)

0 20 40 60 80 100

B

Treatments

AAC00-1 AAC00-1ΔaacI AAC00-1ΔaacR

AAC00-1 AAC00-1ΔaacI AAC00-1ΔaacR
**Figure 4.6A.** Effect of quorum sensing on seed-to-seedling transmission of bacterial fruit blotch (BFB). Graph shows the seed-to-seedling transmission over time for watermelon seed (cv. Sugar Baby) inoculated with AAC00-1 (wild-type), AAC00-1ΔaacI (aacI mutant) and AAC00-1ΔaacR (aacR mutant) at 10³ and 10⁶ CFU/seed. The experiment was repeated three times and each datum point represents the mean BFB seedling transmission for three experiments (n = 25 seed per treatment). **B.** Bar chart of area under disease progress curve for changes in BFB incidence over time. Bars represent mean AUDPC and lines indicate standard error of the means. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference.
Treatments

- Area under the disease progress curve (AUDPC)
- Bacterial fruit blotch incidence (%)

Graph A shows the bacterial fruit blotch incidence (%) over time (days after planting) for different treatments.

Graph B illustrates the area under the disease progress curve (AUDPC) for treatments at two different concentrations: 10^6 CFU/mL and 10^3 CFU/mL.

The treatments include:
- AAC00-1
- AAC00-1 ΔaacR
- AAC00-1 ΔaacI

The graph compares the effectiveness of these treatments in controlling the disease progression.
**Figure 4.7A.** Role of *aadI* in the seed-to-seedling transmission of bacterial fruit blotch (BFB).

Graph shows bacterial fruit blotch incidence over time for watermelon seed (cv. Sugar Baby) inoculated with wild-type (AAC00-1), the *aadI* deletion mutant (AAC00-1Δ*aadI*) or the complemented strain (AAC00-1Δ*aadI*comp) at 10³ CFU/seed. The experiment was repeated twice and each datum point represents the mean BFB seedling transmission of the four experiments (n = 25 seed per treatment). **B.** Bar chart of area under disease progress curve for changes in BFB incidence over time. Bars represent mean AUDPC and lines indicate standard error of the means. Treatments with different letters are significantly different according to Tukey-Kramer’s Honestly Significant Difference.
Figure A: Bacterial fruit blotch incidence (%) over time (days after planting). The graph shows the progression of disease incidence for AAC00-1, AAC00-1ΔlaacI, and AAC00-1ΔaacIcomp.

Figure B: Area under the disease progress curve (AUDPC) for different treatments. AAC00-1, AAC00-1ΔaacI, and AAC00-1ΔaacIcomp are compared in terms of their AUDPC values.
Bacterial fruit blotch (BFB) is an economically important disease of cucurbits caused by *Acidovorax citrulli* (Schaad et al, 2008) (formerly *Acidovorax avenae* subsp. *citrulli* (Willems et. al., 1992) = *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (Schaad et. al., 1978)). *A. citrulli* can affect all parts of cucurbit plants and causes water-soaked lesions on cotyledons and distinct reddish-brown lesions along veins of true leaves (Latin and Hopkins, 1995). On fruit, the disease results in water-soaked lesions with irregular margins and as fruit lesions age, they can crack and lead to fruit rot (Somodi et. al., 1991). *A. citrulli* is a seedborne bacterium, and infested seed is the most important source of primary inoculum for BFB. Seed can become infested with *A. citrulli* via invasion of the pistils of blossoms or through the open stomata of immature fruit (Frankle et. al., 1993; Walcott et. al., 2003). Once planted, infested seed can give rise to infected seedlings.

Currently, little is known about the molecular mechanisms involved in the seed-to-seedling transmission of BFB, or the factors that influence *A. citrulli* virulence. As a seedborne pathogen, seedling colonization and seed-to-seedling transmission are important for BFB development. The type three secretion system (T3SS), type two secretion system (T2SS) and quorum sensing (QS) are well studied and important virulence systems in many Gram negative bacteria. The aim of this research was to investigate the role of the aforementioned systems in
BFB. More specifically, the objectives of this research were to; 1) characterize the role of T3SS in *A. citrulli* pathogenicity and efficacy of the T3SS mutant as a biocontrol seed treatment for BFB, 2) determine the role of the type two secretion system in *A. citrulli* virulence and seed-to-seedling transmission of BFB and, 3) determine the role of quorum sensing in *A. citrulli* virulence and seed-to-seedling transmission of BFB.

A T3SS mutant of *A. citrulli* was generated by truncation of the *hrcC* gene of the wild-type strain AAC00-1. The resultant T3SS mutant, AAC00-1Δ*hrcC*, was non-pathogenic on watermelon tissue but retained its ability to colonize seed at wild-type levels. Based on this observation, we explored the possibility of using this mutant as a biological control agent to limit seed transmission of BFB. The use of non-pathogenic mutants as biocontrol agents was previously reported bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* and fire blight of apple caused by *Erwinia amylovora* (Tharaud *et al*., 1997; Moss *et al*., 2007). Because watermelon seed can become infested with *A. citrulli* via blossoms, it was important that a potential biocontrol agent could protect blossoms and the resultant seed from infestation. AAC00-1Δ*hrcC* protected watermelon blossoms as well as *A. avenae* (formerly *A. avenae* subsp. *avenae* (Schaad *et al*., 2008) strain 99-2, a previously reported biocontrol agent of BFB (Fessehaie and Walcott, 2005). The T3SS mutant also significantly reduced BFB seedling transmission for naturally infested watermelon seed. BFB seedling transmission for naturally infested seed was reduced both in the growth chamber and the greenhouse by 81.8 and 37.8 %, respectively. Unfortunately seed treatment with AAC00-1Δ*hrcC* did not eliminate BFB seedling transmission. This indicated that while biocontrol seed treatment with AAC00-1Δ*hrcC* could be beneficial, it would be of greater practical use if incorporated into a comprehensive integrated disease management strategy.
*A. citrulli*’s genome includes two-type two secretion system gene clusters, designated *gsp1* and *gsp2*. Deletion of the *gspG1* gene did not affect endoglucanase secretion by AAC00-1. A T2SS mutant of AAC00-1 was generated only after deletion of both *gspG1* and *gspG2*. While the T2SS mutant retained pathogenicity, it was significantly reduced in its ability to colonize seed and leaf tissue. At 96 h after planting, populations of the T2SS mutant, AAC00-1Δ*gspG1*:Δ*gspG2*, were 100-fold less than for wild-type bacteria on watermelon seed and seedling tissue. Based on genomic sequence data, we hypothesized that *A. citrulli* produced and secreted xylanase, pectate lyase and endoglucanase and that these enzymes were important for seed colonization. However, comparative proteomics data revealed that only endoglucanase was secreted by the T2SS of AAC00-1. Xylanase and pectate lyase mutants of AAC00-1 were not affected in their ability to colonize watermelon seed or their virulence on watermelon fruit rind. In contrast, the endoglucanase mutant was significantly reduced in its ability to colonize watermelon seed during germination. Pathogenicity of the T2SS and the endoglucanase mutants on watermelon fruit rind was also reduced compared to the wild-type strain. These data indicate that the T2SS contributes to the ability of *A. citrulli* to colonize seed and that endoglucanase, and possibly other type two secreted proteins facilitate seed colonization during the epiphytic phase of seedling transmission of BFB.

Quorum sensing (QS) is the ability of bacteria to coordinate gene expression at certain population densities. *A. citrulli* employs QS which is facilitated by the *luxI* and *luxR* homolgs, *aacI* and *aacR*; however, in our studies QS did not affect *A. citrulli* colonization of watermelon seed. In the absence of the *aacR* gene, AHLs were still produced; therefore *aacI* was not auto-regulated by the AacR/AHL complex as in the case of *V. fischeria* (Nelson, 1977). Both *aacI* and *aacR* mutants of *A. citrulli* were pathogenic on watermelon seedlings; however, the *aacI* mutant
was reduced in virulence. When watermelon seedlings were infiltrated with low inoculum densities (10^3 CFU/mL) of the aacI mutant (AAC00-1ΔaacI) there was a delay in BFB symptom development. In contrast, the aacR mutant (AAC00-1ΔaacR) was comparable to the wild-type strain. When low inoculum densities were used, AAC00-1ΔaacI was less virulent on watermelon seedling tissue. There was no significant difference between the abilities of AAC00-1ΔaacI and AAC00-1ΔaacR to be transmitted from infested seed to seedlings compared to AAC00-1 when a high initial inoculum density (10^6 CFU/mL) was used. However, AAC00-1ΔaacI was significantly reduced in its ability to be transmitted to seedlings compared to AAC00-1 and AAC00-1ΔaacR at low initial inoculum densities. 34.3% of seedlings from seed inoculated with 10^3 CFU/mL of AAC00-1ΔaacI, developed BFB. In contrast, seed infested with 10^3 CFU/mL of AAC00-1 or AAC00-1ΔaacR had BFB seedling transmission percentages of 78.6 and 61.9%, respectively. These data suggest that QS is important for seed-to-seedling transmission of BFB. More specifically the production of AHLs is important for BFB development when the pathogen is present at low inoculum densities. Additionally, QS is not important for A. citrulli colonization of the seed during the early stages of germination.

This research has shown the contribution of the T3SS, T2SS and QS in A. citrulli virulence and seed-to-seedling transmission of BFB. The T2SS allows bacterial population growth that might enable the A. citrulli to compete with other saprophytes in the spermosphere. Once high populations are achieved, QS appears to be involved in regulating genes that are required for seed-to-seedling transmission of BFB. The T3SS becomes relevant once seedling tissues emerge. The knowledge gained from this study may be important in developing environmentally sound methods for BFB management.
LITERATURE CITED


APPENDIX A

APPENDIX TO CHAPTER 2

RESULT

Identification of proteins secreted by the T2SS of *A. citrulli* using mass spectrometry.

To identify potential T2S proteins of *A. citrulli* mass spectrometry was performed on proteins precipitated from the cell free extracts of AAC00-1, and AAC00-1ΔgspG1:ΔgspG2. The T2SS mutant secreted approximately 50% fewer proteins than AAC00-1 and there was a visible difference in the protein profiles of the two strains (data not shown). Two hundred and fifty-five proteins were detected in the secretomes of AAC00-1 and AAC00-1ΔgspG1:ΔgspG2. Of these, 34 were exclusively found in the supernatant of AAC00-1 and 97 were found exclusively in the AAC00-1ΔgspG1:ΔgspG2 supernatant (Table A.1). Forty three proteins were secreted from both strains but were detected at higher concentrations in the supernatant of AAC00-1ΔgspG1:ΔgspG2. Fifty-eight percent of proteins present at higher concentrations in the AAC00-1ΔgspG1:ΔgspG2 supernatant had no secretion signal, and proteins with secretion signals were mostly predicted to localize to the periplasm using BLAST analysis. Seventy six percent of the proteins that were found only in AAC00-1ΔgspG1:ΔgspG2 had no secretion signal. Forty-one proteins were observed in the supernatant of the T2S mutant and the wild-type strain, with lower quantities of protein being secreted from AAC00-1ΔgspG1:ΔgspG2 (Table A.2). The secretion of four proteins was between 20-30-fold higher for AAC00-1 than AAC00-
1ΔgspG1:ΔgspG2 (Table A.2). These included a hypothetical protein (Aave_0783) (relative abundance ratio = 30), and a type 4 pilus related protein, pilY (Aave_3551) (relative abundance ratio = 28). A TonB-dependent siderophore receptor was also identified with an abundance ratio of 27, and a gamma-glutamyltransferase with an abundance ratio of 23.5. Six proteins were identified in AAC00-1 that had abundance ratios between three and nine compared to AAC00-1ΔgspG1:ΔgspG2. These included a degenerate glycoside hydrolase which was 8.8-fold higher in AAC00-1 than in AAC00-1ΔgspG1:ΔgspG2. Even though this protein is described as a degenerate endoglucanase, cellulolytic activity was not detected for AAC00-1ΔgspG1:ΔgspG2 colonies or supernatant on CMC plates. Some proteins identified in the AAC00-1 supernatant were predicted to localize to the cell surface such as flagellar proteins. Flagellar proteins have been shown to be easily disrupted from the bacterial cell surface especially during shaking incubation (Kazemi-Pour et. al., 2004). Similar to proteins that were at a higher concentration in AAC00-1ΔgspG1:ΔgspG2, some proteins that were localized to the cell membrane, such as porins, Ton B siderophore receptors, carbohydrate transport associated proteins, and the general secretory protein D were present in the supernatant of AAC00-1. The presence of membrane associated proteins and cytoplasmic proteins could be explained by cell lysis which allowed release of intracellular proteins into the supernatant. Of the 34 proteins observed only in the supernatant of AAC00-1, five are thought to be secreted by the T2S, (Table A.2). However, further analysis is required to confirm if these proteins are T2S proteins. Of these, three have putative functions including a VirK family protein, an arginyl endoproteinase (endoproteinase Arg-C), and an endoglucanase enzyme. Interestingly the VirK family protein shares 43% identity to that of Agrobacterium tumefaciens. VirK is an important virulence factor in bacteria like Salmonella enterica where it is required for cell to cell spread and in A. tumefaciens where it
functions as a part of the *vir* regulon to facilitate DNA transfer (Detweiler *et. al.*, 2003).

Endoproteinase Arg-C proteases specifically cleave at the carboxyl end of arginine residues contributing to protein degradation (Wright *et. al.*, 1998). Possible cell membrane-associated transport related proteins such as extracellular solute binding proteins (Aave_1937) that function in carbohydrate transport were also observed in the AAC00-1 secretome.

**METHOD**

**Identification of proteins secreted by the T2SS of *A. citrulli.*

Mass spectrometry was employed to identify substrates of the T2S machinery of AAC00-1. This was done by comparing the secretome of AAC00-1 with that of AAC00-1ΔgspG1:ΔgspG2, the T2S mutant. AAC00-1 and AAC00-1ΔgspG1:ΔgspG2 were inoculated into M9 minimal medium broth, at a final concentration of ~1 × 10⁴ CFU/mL, and incubated at 30ºC for 24 h with shaking (300 rpm) in a rotary shaker (New Brunswick Scientific, Edison, NJ) to induce the production of T2S proteins. Bacterial cells were removed from 300 mL of culture by centrifugation, at 4ºC and 8000 x g for 30 min, and vacuum filtration using a polyethersulfone (PES) membrane (0.2 μM pore, VWR, West Chester, PA). Potential polysaccharides in the media were removed by addition of hexadecyltrimethylammonium bromide, (CTAB, Sigma Aldrich, St. Louis MO), to a final concentration of 0.1%, followed by incubation on ice for 10 min. Subsequently the sample was centrifuged at 4ºC and 8000 x g for 30 min. Proteins were precipitated by the addition of 100% trichloroacetic acid (Sigma Aldrich, St. Louis MO) to a final concentration of 10% followed by incubation on ice for 30 min and centrifugation as described above. The supernatant was decanted and the proteins were washed in absolute ethanol (Sigma Aldrich) three times and resuspended in a final volume of 5 mL of
ethanol. Proteins were divided into two-2 mL aliquots and one-1 mL aliquot. The 1 mL aliquot was pelleted by centrifugation at 4°C at 13000 rpm for 10 min, and dissolved in 10 μL 0.1M NaOH. Proteins were quantified using a commercial BCA assay according to the manufacturer’s instructions (Biorad, Hercules, CA). Proteins from a 2 mL aliquot of AAC00-1 or AAC00-1ΔgspG1:ΔgspG2 were recovered by centrifugation at 10,000 x g for 5 min at 4°C. Ethanol was aspirated and the proteins were solubilized in a 10% sodium dodecyl sulphate (SDS) buffer. Laemmli buffer (BioRad) was added to each sample at a 1:1 ratio and each sample was incubated at 100°C for 10 min. Twenty micrograms of protein were applied to a 12% Tris-HCL Criterion gel (BioRad), and subjected to 160 volts to allow proteins to move 1 cm into the gel. Proteins were visualized by staining with Biosafe coomassie brilliant blue G250 according to manufacturer’s instructions (BioRad). Each gel lane was divided into 4 sections and submitted to the Proteomics Core Facility at Emory, University, Atlanta GA. Proteins were subjected to in-gel digestion and the resulting peptides were analyzed by reverse-phase liquid chromatography coupled with tandem mass spectrometry using an LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA) (Peng and Gygi, 2001). During database search, a strategy of reverse database was used to evaluate the false discovery rate; the matched peptides were filtered according to matching scores to remove all false matches from the reverse database (Peng et. al., 2003). Finally, only proteins that were matched by at least two peptides were accepted to further improve the confidence of identification. Proteins identified in each sample were compared to determine those secreted by the T2S system. Relative amounts were determined by dividing the spectral counts observed for a protein identified in the supernatant of AAC00-1 by spectral counts of the same protein identified in AAC00-1ΔgspG1:ΔgspG2.
LITERATURE CITED


Table A.1. Proteins identified in the supernatant of wild-type *Acidovorax citrulli* strain 00-1 but not by the type II secretion system mutant AAC00-1ΔgspG1:ΔgspG2.

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<td>Proteins with signal peptides with putative intracellular localization</td>
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Proteins without signal peptides with putative intracellular localization
Table A.2. Proteins identified in greater abundance in the supernatant of wild-type *Acidovorax citrulli* strain 00-1 relative to the type II secretion system mutant, AAC00-1ΔgspG1:ΔgspG2.

<table>
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<tr>
<th>Locus Tag</th>
<th>Description</th>
<th>Mass (kDa)</th>
<th>Relative abundance ratios (AAC00-1/ AAC00-1ΔgspG1:ΔgspG2)</th>
<th>Signal peptide</th>
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