FACTORS THAT AFFECT HOST PREFERENCE AND VIRULENCE OF *ACIDOVORAX CITRULLI*, THE CAUSAL AGENT OF BACTERIAL FRUIT BLOTCH OF CUCURBITS

by

MEI ZHAO

(Under the Direction of RON WALCOTT)

ABSTRACT

Bacterial fruit blotch (BFB) is an economically important seed-borne and seed-transmitted disease of cucurbitaceous plant species, including watermelon and melon crops. *Acidovorax citrulli*, the causal agent of BFB, can be divided into two major groups, I and II. The natural association of group I and II *A. citrulli* strains with different cucurbit species strongly suggests host preference. Despite the economic importance of BFB, little is known about fundamental aspects of *A. citrulli*-host interactions. The overall goal of this study was to gain a better understanding of host preference and virulence of the two major groups of *A. citrulli*. We set up field plots with four cucurbit species and introduced group I and II *A. citrulli* strains as the inoculum sources. The results from the field trials confirmed that *A. citrulli* strains exhibited a preference for different cucurbit species, which was more pronounced in fruit tissues. Group I and II *A. citrulli* strains were inoculated on detached melon fruits by syringe- and swab-inoculation, and also on attached melon fruits by swab-inoculation. From these

artificial inoculations, we found that physical penetration, colonization, and symptom development did not account for the host preference of *A. citrulli* on melon fruits. Additionally, we did not find detectable effects of XopJ homologs on *A. citrulli* virulence on watermelon and melon cotyledons. The deletion mutant of XopJ homologs was less virulent on attached melon fruits compared to the wild-type, while their population levels on melon fruits were variable across experiments. However, the HopAF homolog may be important for *A. citrulli* colonization and symptom development on watermelon and melon cotyledons. Additionally, we developed an immature watermelon fruit assay to distinguish group I and II *A. citrulli* strains and used it to identify virulence determinants of *A. citrulli*. Only group II strains induced water-soaked lesions on immature watermelon fruit tissues. By screening an *A. citrulli* AAC00-1 transposon mutant library, we found six mutants were non-pathogenic on watermelon fruits. Three of these mutants were disrupted in type 3 secretion system associated genes. Lastly, we investigated the effect of temperature on the colonization of germinating watermelon seeds/seedlings by *A. citrulli*. Mean BFB incidence was significantly lower for seeds that were sown at 28°C and transferred to 40°C at 3 days after sowing (das), compared to seeds incubated constantly at 28°C. This means that *A. citrulli* cells associated with germinating watermelon seeds are more sensitive to elevated temperature during the first 3 das relative to the later days.

INDEX WORDS: Host preference, field study, type 3 effector, fruit infection

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MEI ZHAO

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B. Ag, China Agricultural University, China, 2015

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MEI ZHAO

| | |
|---|---|
| Major Professor: | Ron Walcott |
| Committee: | Bhabesh Dutta |
| | Brian Kvitko |
| | Saul Burdman |

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
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DEDICATION

To science and truth. To mom and dad.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW [1]

[1] Reproduced, by permission, from Zhao, M., and Walcott, R. R. 2018. *Acidovorax citrulli*: History, epidemiology, and management of bacterial fruit blotch of cucurbits. Chapter 3 in: Plant-Pathogenic *Acidovorax* Species. S. Burdman and R. R. Walcott, eds. American Phytopathological Society, St. Paul, MN. Reprinted here with permission of publisher.

## Bacterial fruit blotch (BFB) history

Bacterial fruit blotch (BFB) is an economically important seed-borne and seed-transmitted disease of cucurbitaceous plant species. *Acidovorax citrulli*, the causal agent of BFB, was first isolated by Webb and Goth in 1965 at the Regional Plant Introduction Station in Griffin, Georgia, USA (Webb and Goth 1965). The phytopathogenic bacterium, isolated from seedlings of watermelon plant introductions (PI 174103 and PI174104) collected from Turkey, was unidentified, but determined to be seed-borne (Webb and Goth 1965). Subsequently, Schaad et al. (1978) identified the causal agent as *Pseudomonas pseudoalcaligenes* subsp*. citrulli* and designated the type strain, American Type Culture Collection (ATCC) 29625. The first natural BFB outbreak occurred in the Mariana Islands in 1987 on watermelon fruits (Wall and Santos 1988). In 1989, the disease was observed in commercial watermelon fields in Florida (Somodi et al. 1991) and Indiana (Latin and Rane 1990). Subsequently, BFB outbreaks have occurred in many regions around the world, primarily on watermelon and melon crops, resulting in significant economic losses (Burdman and Walcott 2012).

## Geographical distribution of BFB

Bacterial fruit blotch has a wide geographical distribution, mainly due to the global production and trade of cucurbit seeds. However, the center of geographical origin for *A. citrulli* is currently unknown. BFB outbreaks have been reported in many watermelon and melon producing countries around the world, including Australia, Hungary, Nicaragua, Brazil, Israel, China, Thailand, Italy,

Costa Rica, Japan, Korea, Turkey, Nigeria, Greece, Serbia, Guadeloupe (France), and the USA (Amadi et al. 2009; Assis et al. 1999; Burdman et al. 2005; Cunty et al. 2019; Gemir 1996; Holeva et al. 2010; Munoz and Monterroso 2002; O'Brien and Martin 1999; Palkovics et al. 2008; Popovic and Ivanovic 2015; Shirakawa et al. 2000; Walcott et al. 2004; Zhang et al. 1998). In the USA, the disease has been reported in Georgia, Florida, Indiana, Delaware, Oklahoma, Texas, Oregon, Iowa, Illinois, South Carolina, and California (Babadoost and Pataky 2002; Black et al. 1994; Evans and Mulrooney 1991; Hamm et al. 1997; Jacobs et al. 1992; Kumagai et al. 2014; Latin and Rane 1990; Somodi et al. 1991; Walcott et al. 2000; Walcott et al. 2004).

**Economic significance of BFB**

Bacterial fruit blotch occurs sporadically but impacts the global cucurbit industry. It has the potential to affect the entire cucurbit production chain, including seed, transplant, and fruit production. Because BFB is seed-borne and seed-transmitted, some US seed producers were found legally liable for disease outbreaks in the early 1990s, and had to compensate growers for yield losses (Latin and Hopkins 1995). Consequently, this resulted in the suspension of watermelon seed sales by certain companies in some states in the USA. Because cucurbit transplant production systems are highly conducive for BFB epidemic development, transplant producers may experience up to 100% yield loss due to one or a few BFB-infected seedlings. With regards to cucurbit fruit

growers, up to 90% yield losses have been reported in the field (Latin and Rane 1990).

## BFB etiology: *Acidovorax citrulli*

### Taxonomy

In 1978, Schaad et al. isolated a bacterium from watermelon that had similar characteristics to the bacterium described by Webb and Goth (1965), and named it *Pseudomonas pseudoalcaligenes* subsp*. citrulli* (Schaad et al. 1978). Interestingly, this strain did not induce a hypersensitive response (HR) on tobacco (Schaad et al. 1978), but other strains subsequently associated with BFB outbreaks could trigger an HR on tobacco (Latin and Rane 1990; Rane and Latin 1992).

In 1992, Willems et al. transferred the BFB pathogen to the genus *Acidovorax* and renamed it *Acidovorax avenae* subsp*. citrulli* (Willems et al. 1992). In 2008, Schaad et al. elevated the BFB pathogen from subspecies to species level and renamed it *A. citrulli* based on DNA-DNA hybridization assays; sequence analysis of the 16S rRNA and the 16S-23S rRNA internally transcribed spacer region; amplified fragment length polymorphism analysis; and phenotypic assays (Schaad et al. 2008). Currently, *A. citrulli* belongs to the phylum Proteobacteria, class Betaproteobacteria, order Burkholderiales, and family *Comamonadaceae*.

## Morphological and physiological properties *of A. citrulli*

*A. citrulli* is a gram-negative, aerobic, oxidase-positive, rod-shaped bacterium with average dimensions of 0.5 x 1.7 µm (Schaad et al. 1978). The bacterium is motile by means of a ~ 5.0 µm long polar flagellum and grows at 41°C, but not at 4°C (Schaad et al. 1978). Cavalcanti et al. (2005) reported that the minimum, optimum, and maximum temperatures for *A. citrulli* growth were 1, 32, and 41°C, respectively, and the minimum, optimum, and maximum pH were 4, 7.4, and 10.8, respectively. *A. citrulli* produces non-fluorescent, round, cream-colored colonies with smooth edges after 48 h growth on King's medium B (Burdman and Walcott 2012). However, phenotypic variants of *A. citrulli* were reported to produce colonies that were translucent and larger with a fuzzy appearance on nutrient agar plates, compared to their parental strains (Shrestha et al. 2013).

## Genetic diversity of *A. citrulli*

*A. citrulli* strains can be divided into two major groups (I and II) based on carbon substrate utilization (Walcott et al. 2004), DNA fingerprint analyses by pulse-field gel electrophoresis (PFGE) (Burdman et al. 2005; Walcott et al. 2000; Yan et al. 2013), repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) (Walcott et al. 2004), multilocus sequence analysis (MLSA) (Feng et al. 2009a; Yan et al. 2013), and fatty acid methyl ester analysis (Walcott et al. 2000). The diversity of *A. citrulli* was initially reported in an Australian population by O'Brien and Martin (58). They used carbon substrate utilization profiles and pathogenicity assays to characterize strains recovered from several cucurbit

hosts, including watermelon, rockmelon, honeydew (*Cucumis melo* var. *indorus* Jacq.), and prickly paddymelon (*Cucumis myriocarpus* subsp. *myriocarpus*) in Queensland Australia. Based on carbon substrate utilization profiles, the strains were separated into two groups. One group recovered from rockmelon and honeydew failed to utilize L-leucine, but utilized 2-amino ethanol. The other group, recovered from watermelon, utilized L-leucine, but were unable to utilize 2-amino ethanol. All strains exhibited a high level of virulence on watermelon; however, the strains isolated from rockmelon and honeydew were significantly more virulent on rockmelon plants than those from watermelon.

Walcott et al. (2000) first reported two genetically distinct groups of *A. citrulli* among 120 strains collected from a broad range of geographical origins and a range of cucurbitaceous plants. These groups were established based on DNA fingerprinting by *Spe*I restriction digestion of genomic DNA followed by PFGE. *A. citrulli* strains recovered from melons in Australia and the ATCC type strain were assigned to group I in this study. In 2005, Burdman et al. (2005) reported similar findings based on an *A. citrulli* population from Israel (Burdman et al. 2005). Using MLSA, Feng et al. (2009a) showed that 64.7% (33/51) of *A. citrulli* strains from China were members of group (clonal complex) I. According to their analysis, group II strains had a wider geographical distribution than group I strains, but had a limited host range (watermelon) (Feng et al. 2009a). Similarly, Yan et al. (2013) reported the existence of two *A. citrulli* groups in China with 63.4% (71/112) of the strains tested being members of group I based on PFGE. They also reported that the Chinese group I strains were mainly collected from

Xinjiang and Inner Mongolia provinces, while group II strains were more widely distributed (Yan et al. 2013). More recently, Silva, K. M. M. et al. (2016) reported that out of 34 Brazilian *A. citrulli* strains tested, all belonged to group I based on rep-PCR and substrate utilization profile analyses. This indicated that group I strains were predominant in Brazil (Silva, K. M. M. et al. 2016). These observations were confirmed by Silva et al. (2016), who found that 97% (72/74) of the Brazilian *A. citrulli* strains, recovered predominantly from melon, were members of group I based on MLSA and PFGE (Silva, G. M. et al. 2016).

Group I and II *A. citrulli* strains can also be distinguished at the whole genome level. In 2007, the whole genome sequence of a group II *A. citrulli* strain AAC00-1 was made available (GenBank accession NC_008752). This genome is 5,352,772 bp long with 4,709 protein-coding genes and a guanine-cytosine (G+C) content of 68.5 mol%. In 2015, Wang et al. sequenced two group I Chinese strains, pslb65 and tw6 (Wang et al. 2015b; Wang et al. 2015a). Strain pslb65 was isolated from a melon seedling in Xinjiang province, China. Its genome sequence, deposited at DDBJ/EMBL/GenBank under accession number JYHM00000000, is 4,903,443 bp long with 4,532 protein-coding genes and a G+C content of 68.8 mol% (Wang et al. 2015b). Strain tw6 was isolated from a watermelon seedling in Beijing, China and was highly tolerant to copper. The genome sequence of *A. citrulli* strain tw6, deposited at DDBJ/EMBL/GenBank under accession number JXDJ00000000, is 5,080,614 bp long with 4,759 protein-coding genes and a G+C content of 68.7 mol% (Wang et al. 2015a). In 2016, the complete genome sequence of another group I strain M6 was reported

(Eckshtain-Levi et al. 2016). Strain M6 was isolated from a symptomatic melon fruit in Israel. The *A. citrulli* strain M6 genome sequence, deposited at DDBJ/EMBL/GenBank under accession number LKUW00000000, is approximately 4.85 Mb with 4,368 predicted ORFs and a G+C content of 68.9 mol% (Eckshtain-Levi et al. 2016). Recently, the M6 genome was fully assembled following sequencing with PacBio technology (chromosome GenBank accession CP029373.1) (Yang, R. Z. et al. 2019). This approach also revealed the presence of a ~53 kb plasmid, named pACM6 (plasmid GenBank accession CP029374.1) (Yang, R. Z. et al. 2019). Yang et al. showed that pACM6 is present in several group I strains, but absent in all tested group II strains of *A. citrulli.* The draft genomes of group I strains DSM17060 (German version of the ATCC type strain) and ZJU1106 are also currently available. The NCBI BioProject accession numbers for strain DSM17060 and ZJU1106 are PRJEB15996 and PRJNA175738, respectively. The complete genome of a group II strain KACC17005 was fully assembled following sequencing with PacBio technology (GenBank accession CP023687). These *A. citrulli* genome sequences provide resources for further studies of mechanisms of pathogenicity, virulence, and host preference.

Comparative analysis revealed that the genome of the group I strain, M6, is 0.5 Mb smaller than that of the group II strain, AAC00-1 (Eckshtain-Levi et al. 2016). This difference was explained primarily by the presence of eight fragments dispersed throughout the AAC00-1 genome, but absent in the M6 genome (Eckshtain-Levi et al. 2016). PCR assays confirmed that the eight

AAC00-1 fragments differentiated group II from group I strains with a few exceptions (Eckshtain-Levi et al. 2016). AAC00-1 possessed 532 open reading frames (ORFs) that were absent in strain M6, while 123 M6 ORFs were absent in AAC00-1 (Eckshtain-Levi et al. 2016). Additionally, group I and II strains can be distinguished based on differences in the arsenal and sequences of type 3-secreted (T3S) effectors (Eckshtain-Levi et al. 2014). Furthermore, A specific PCR primer set designed based on the *pilL* gene was reported to distinguish between groups I and II *A. citrulli* strains with some exceptions (Zhong et al. 2015). In 2017, a PCR assay based on the putative type III secretion effector gene, *Aave_2166*, was developed to rapidly distinguish group I and II *A. citrulli* strains (Zivanovic and Walcott 2017). Recently, Yang, Y. W. et al. (2019) designed a multiplex PCR assay based on differences in *pilA* sequences to distinguish *A. citrulli* strains into three pilus types.

In addition to genetic differences, group I and II *A. citrulli* strains vary in their physiological and phenotypic characteristics, including hosts of origin, virulence, and copper tolerance (Walcott et al. 2004). In general, strains of each group are associated with the host species from which they were isolated. Group I strains, including the ATCC type strain, were generally isolated from melon, pumpkin, squash, watermelon, and other cucurbits, while group II strains have been mainly isolated from watermelons. The two *A. citrulli* groups have been reported to differ in their virulence on various cucurbit species (Walcott et al. 2004). For example, group I strains were reported to be moderately to highly aggressive on watermelon, melon, pumpkin, and squash, while group II strains

were highly aggressive on watermelon, but weakly aggressive on other cucurbits (Walcott et al. 2004). Group I strains have also been reported to have higher copper tolerance than group II strains (Walcott et al. 2004).

### *Acidovorax citrulli* host range

BFB has a wide host range in the Cucurbitaceae family. Watermelon and melon are highly susceptible and most BFB outbreaks have been reported on these species. However, other hosts include cucumber (*Cucumis sativus* L.), squash (*Cucurbita pepo, C. maxima*), pumpkin (*Cucurbita pepo*), gramma (*Cucurbita moschata*), citron melon (*Citrullus lanatus* var. *citroides*), prickly paddymelon, and several types of gourds (Isakeit et al. 1998; Langston Jr et al. 1999; Martin and Horlock 2002; Martin et al. 1999; Schaad et al. 1978). Apart from cucurbitaceous host species, the bacterium was intercepted in eggplant and tomato seeds in Israel (Assouline et al. 1997), but natural BFB outbreaks have not been reported for solanaceous crops. There are also records of the bacterium causing bacterial leaf spot of Christ's thorn (*Paliurus spina-christi*) in Iran (Harighi 2007) and bacterial leaf blight of betelvine (*Piper betle* L.) in Taiwan (Deng et al. 2010).

### BFB symptomatology

*A. citrulli* can affect different organs of the cucurbit host plant and produce symptoms on cotyledons, hypocotyls, true leaves, and fruits. Seedling symptoms include angular water-soaked lesions delimited by veins in cotyledons and true

leaves. These lesions often become necrotic and brown on cotyledons and may produce yellow halos on the true leaves. Brown to reddish-brown lesions often progress along the midribs of true leaves.

Although leaf symptoms may become inconspicuous during the growing season, BFB fruit symptoms are distinct. Symptoms on watermelon fruits begin as irregularly- or blotch-shaped, water-soaked lesions, which may extend to cover the upper surface of the watermelon fruit. Lesions may eventually develop brown cracks that lead to fruit rot. In most cases, fruit rot is caused by secondary invading organisms. Symptoms on melon fruits include brown, sunken spots on the fruit rind and internal fruit rot with brown cavities (Burdman and Walcott 2012). Fruit symptoms render cucurbit fruits unmarketable.

### *A. citrulli* pathogenicity and virulence factors

Despite the economic importance of BFB, relatively little is known about the molecular mechanisms of *A. citrulli* pathogenesis. Studies have shown that important pathogenicity and virulence determinants of *A. citrulli* include the type II secretion system (T2SS) (Johnson 2010), type III secretion system (T3SS) (Bahar and Burdman 2010; Johnson et al. 2011; Liu et al. 2012), type VI secretion system (T6SS) (Tian et al. 2015), type IV pili (T4P) (Bahar et al. 2010; Bahar et al. 2009a), polar flagella (Bahar et al. 2011), and quorum sensing (QS) (Chen et al. 2009; Fan et al. 2011; Johnson and Walcott 2013; Wang et al. 2016).

**Type II secretion**

The *A. citrulli* AAC00-1 genome possesses one complete and one incomplete T2SS gene cluster, *gsp1* and *gsp2*. Deletion of both copies of *gspG* (*gspG1/G2*), putatively encoding a major pseudopilin, resulted in the loss of the ability of *A. citrulli* AAC00-1 to secrete endoglucanase (Johnson 2010). This mutant was also significantly reduced in its ability to colonize watermelon seed and seedling tissue, resulting in a significant reduction in BFB seed-to-seedling transmission. Among xylanase, pectate lyase, and endoglucanase mutants of AAC00-1, only the endoglucanase deletion mutant displayed a significantly reduced ability to colonize germinating watermelon seeds. These results suggested that both T2SS and endoglucanase contributed to watermelon seed colonization by *A. citrulli* (Johnson 2010).

**Type III secretion system**

The T3SS is a key virulence apparatus for many gram-negative plant pathogenic bacteria including *A. citrulli* to cause disease in their plant hosts. The AAC00-1 genome revealed the presence of a *hrp* (hypersensitive response and pathogenicity) cluster, encoding T3SS. Based on sequence similarity and cluster organization, the *A. citrulli hrp* cluster belongs to class II, which includes *Xanthomonas* species and *Ralstonia solanacearum* (Bahar and Burdman 2010). Mutagenesis analyses revealed that both group I and II *A. citrulli* strains require a functional T3SS for pathogenicity. Bahar and Burdman (2010) showed that group I strain M6 and group II strain W1 impaired in *hrcV* failed to infect melon

seedlings and to induce an HR in leaves of tomato and tobacco. Similarly, Johnson et al. (2011) showed that a *hrcC* mutant of the group II strain AAC00-1 could not induce typical BFB symptoms in watermelon seedlings nor an HR in tobacco leaves. In addition, Liu et al. (2012) screened a Tn5 mutant library of group I strain FC440 for altered pathogenicity on cucumber and found a non-pathogenic mutant that was impaired in *hrcN*. This mutant was also unable to induce an HR in tobacco leaves. More recently, Zhang et al. reported that *hrpG* and *hrpX* deletion mutants of the *A. citrulli* group II strain Aac5 were non-pathogenic on watermelon seedlings and failed to induce an HR in tobacco. They also demonstrated that HrpG activates HrpX in *A. citrulli* and the transcription and translation of the T3S effector *Aave_2166* were suppressed in *hrpG* and *hrpX* mutants (Zhang et al. 2018).

Based on sequence homology with known T3S effectors, eleven T3S effectors were identified in the genome of the group II *A. citrulli* strain AAC00-1 (Eckshtain-Levi et al. 2014). In general, group I and II strains were distinguished based on their T3S effector arsenals. Most notably, group II strains possess three putative T3S effectors (*Aave_2166*, *Aave_2708*, and *Aave_2938*) that are truncated in or absent from group I strains. The comparative analysis of T3S effector genes also revealed the presence of a third group of *A. citrulli* strains. These strains (ZUM4000 and ZUM40001) were weakly virulent on all tested cucurbits, and clustered separately from group I and II strains in terms of T3S effectors (Eckshtain-Levi et al. 2014). However to date, this third group was represented by only two strains recovered from India. Recently, Jiménez

Guerrero et al. (2019) revealed 58 putative T3Es in M6 genomes by thorough sequence similarity searches, a machine learning approach, and RNAseq data analysis. Among those 58 T3Es, ten appear to be unique to group I strains.

**Type VI secretion**

Tian et al. (2015) investigated the role of a T6SS cluster comprising of 17 genes in virulence of the *A. citrulli* group I strain XJL12. They generated 17 individual mutants and found that four of them (Δ*vasD*, Δ*impK*, Δ*impJ*, Δ*impF*) were reduced in BFB seed-to-seedling transmission, melon seed colonization, and biofilm formation. However, these mutants were not affected in virulence when infiltrated into melon seedling leaf tissues. These results suggest that T6SS plays a role in BFB seed-to-seedling transmission on melon (Tian et al. 2015).

**Type IV pili**

Screening of a random transposon library of *A. citrulli* group I strain M6 revealed that a mutant impaired in the *pilM* gene, encoding a protein required for T4P assembly, displayed significantly reduced virulence in melon seed-to-seedling transmission assays (Bahar et al. 2009a). This mutant was also impaired in twitching motility and biofilm formation ability (Bahar et al. 2010; Bahar et al. 2009a). M6 impaired in the *pilT* gene, encoding an ATPase needed for T4P retraction that drives twitching motility, was also compromised in virulence, twitching motility, and biofilm formation ability (Bahar et al. 2009a). The results demonstrated that T4P are required for virulence of *A. citrulli.*

**Polar flagella**

Screens of *A. citrulli* M6 Tn5 library also revealed a mutant impaired in *fliR*, encoding a flagellar protein involved in flagellin secretion. This mutant failed to synthesize polar flagella and swim, and showed reduced virulence in seed-to-seedling transmission and melon seedling stem inoculation assays (Bahar et al. 2011). A marker exchange mutant impaired in *fliC*, encoding flagellin, displayed a similar phenotype to the *fliR* mutant. Both *fliC* and *fliR* mutants showed reduced ability to colonize leaves and xylem vessels of melon seedlings (Bahar et al. 2011). Interestingly, the polar flagellum mutants also displayed reduced twitching motility, which might have contributed to the reduced virulence of these mutants, and suggests a relationship between swimming motility and twitching motility in *A. citrulli* (Bahar et al. 2011). In agreement with these findings, Liu et al. (2012) reported the role of the polar flagellum in *A. citrulli* virulence. They found that an *A. citrulli* FC440 mutant impaired in a gene encoding a flagellar hook-associated protein displayed reduced virulence on cucumber seedlings compared to the wild-type strain (Liu et al. 2012).

**Quorum sensing**

Chen et al. (2009) showed that *A. citrulli* possessed an N-acyl-homoserine lactones (AHLs)-type QS system. They found that *A. citrulli* strain NJF10 produced a 3-O-$C_8$-homoserine type signal molecule. They also expressed an AHL lactonase gene *aiiA* in *A. citrulli* NJF10. The transformed cells were reduced in

their ability to produce 3-O-$C_8$-homoserine and in their virulence in fresh watermelon fruits (Chen et al. 2009).

Fan et al. (2011) showed that impairment of the AHL synthase gene, *aacI* abolished the ability of group I strain XJL12 to produce AHL molecules, and caused a significant reduction in virulence in watermelon fruits and melon seedlings. The XJL12 *aacI* mutant was partially impaired in its *in vitro* growth rate and swimming motility; however, it retained the ability to form biofilm, produce exopolysaccharides, and induce an HR in tobacco leaves (Fan et al. 2011).

Johnson and Walcott (2013) demonstrated that QS plays a role in the seed-to-seedling transmission of *A. citrulli* on watermelon. They generated *aacI* and *aacR* (encoding a transcriptional regulator) mutants in *A. citrulli* AAC00-1. Interestingly, both mutants colonized germinating watermelon seed at wild-type levels (Johnson and Walcott 2013). However, BFB seed-to-seedling transmission was affected in a cell density-dependent manner. When seeds were infiltrated with $10^6$ CFU/seed, no significant differences were observed between the mutants and the wild-type in BFB seedling transmission (Johnson and Walcott 2013). In contrast, when seeds were infiltrated with $10^3$ CFU/seed, BFB seed-to-seedling transmission was significantly reduced for the *aacI* mutant (Johnson and Walcott 2013).

Wang et al. (2016) investigated the role of QS in group II strain Aac-5 by generating *aacR* and *aacI* knockout mutants. They found that the mutants were reduced in twitching motility and virulence on watermelon seedlings. In contrast, biofilm formation and seed attachment by mutant cells were significantly increased.

Their result differs from the previous study with the group I strain XJL12 (Fan et al. 2011) regarding biofilm formation, which suggests different effects of *aacI* on biofilm formation for different groups of *A. citrulli* strains.

**Host preference**

The natural association of group I and II *A. citrulli* strains with different cucurbit species strongly suggests host preference. In 1999, O'Brien and Martin observed that *A. citrulli* strains recovered from rockmelon production regions (North Queensland, Australia) were equally virulent on watermelon and rockmelon seedlings. However, strains collected from watermelon production regions (South Queensland, Australia) were more virulent on watermelon than rockmelon seedlings (O'Brien and Martin 1999). In 2000, Walcott et al. (2000) reported that, 85% (17/20) of group I *A. citrulli* strains were isolated from cantaloupe and pumpkin, while, 65% (65/100) of group II strains were isolated from watermelon, 34% from citron, and 1% from cantaloupe.

In a subsequent report using *A. citrulli* strains representing a wider geographical distribution, Walcott et al. (2004) reported a similar host preference pattern. More specifically, 93.1% (27/29) of group II strains tested were isolated from watermelon. In contrast, 85.3% (29/34) of group I strains were isolated from muskmelon, bitter gourd (*Momordica charantia*), rockmelon, pumpkin, honeydew, and melon (Walcott et al. 2004). Additionally, an MLSA study of a global *A. citrulli* strain collection supported the hypothesis of *A. citrulli* host preference (Feng et al. 2009a). Of the strains with known hosts of origin, 87.8% (36/41) from group II

were collected from watermelon; while 51.1% (24/47) of the group I strains were recovered from non-watermelon cucurbit species (Feng et al. 2009a). In agreement with these observations, Yan et al. (2013) reported that amongst a collection of 118 Chinese *A. citrulli* strains, 91.8% (67/73) determined to be group I were isolated from melon and 93.3% (42/45) of the group II strains were recovered from watermelon.

To further support the hypothesis of host preference between group I and II *A. citrulli* strains, Walcott et al. (2000) reported that in a BFB outbreak in a mixed planting of pumpkins and watermelons in Georgia USA, only pumpkin fruits displayed BFB symptoms. Interestingly, only group I strains were isolated from the symptomatic pumpkin fruits (Walcott et al. 2000).

Despite the data suggesting host preference, Walcott et al. (2004) conducted greenhouse seedling and fruit inoculation assays and reported that representative group I and II *A. citrulli* strains could infect a range of cucurbit species (i.e., no evidence for host specificity). Also, Zivanovic and Walcott (2017) reported that there were no significant differences in temporal bacterial population dynamics between M6 and AAC00-1 after seedling tissue infiltration with bacterial cell suspensions. There may be differences in host specificity between group I and II *A. citrulli* strains under natural field conditions. However, artificial inoculation techniques prevent the observation of this phenotype. Further studies using natural inoculation and pathogen spread under field conditions are needed to elucidate differences in host preference of *A. citrulli* groups.

In addition to field studies to investigate *A. citrulli* host preference, it is necessary to understand the genetic factors that differentiate group I and II strains and to determine their roles in virulence and host preference. Based on whole genome sequence comparisons between *A. citrulli* M6 (group I) and AAC00-1 (group II), Eckshtain-Levi et al. (2016) reported that there are eight fragments present in group II strains but absent in group I strains. They suggested that these fragments were gradually acquired by horizontal gene transfer events by ancestral group I strains, leading to adaptation to different cucurbit hosts. Interestingly, these fragments contain several T3S effector genes, including *xopJ* homologs. These effectors are present in group II strains, but absent in group I strains (Eckshtain-Levi et al. 2014), and we hypothesize that they are involved in host preference of group II *A. citrulli* strains to watermelon fruits.

## Epidemiology

### Disease cycle

Primary sources of *A. citrulli* inoculum include infested seeds, debris from infected fruit or foliage, volunteer cultivated or wild cucurbit seedlings, and cucurbitaceous weeds (Latin and Hopkins 1995). However, infested or infected seeds are the most important primary inoculum source for BFB outbreaks (Rane and Latin 1992). Infested seeds introduce the pathogen into transplant production facilities, whose conditions, including high plant density, overhead irrigation, and high relative humidity, are conducive for pathogen spread and BFB

development. Under these conditions, a single *A. citrulli* -infected seedling can result in multiple secondary infection cycles. Cotyledons are the main sources of secondary *A. citrulli* spread in nurseries (Chalupowicz et al. 2015). In addition, infested pumpkin seeds can transmit seedling blight to watermelons by grafting (Tian et al. 2013). In some instances, infected seedlings may not develop typical BFB symptoms under greenhouse conditions but may still harbor epiphytic populations of *A. citrulli.* As such, they may escape visual detection and introduce inoculums into production fields.

Under field conditions, *A. citrulli* may be disseminated from diseased to healthy plants and may penetrate through open stomata or wounds to facilitate infection. Multiple secondary infection cycles occur as bacteria are splash-dispersed by wind-driven rain or irrigation water throughout the field. For fruit infection, *A. citrulli* cells that are deposited onto fruit 2-3 weeks after anthesis can penetrate open stomata to initiate fruit infections. As fruits age, wax is deposited on the watermelon fruit pericarp, thereby blocking stomata and preventing further bacterial ingress (Frankle and Hopkins 1993). However, *A. citrulli* may still invade fruits through wounds in fruit rinds. Although fruit infection occurs 2-3 weeks after anthesis, BFB symptoms on watermelon usually do not develop until close to harvest maturity. This long incubation period makes BFB on watermelon difficult to manage. Little is known about how the pathogen survives latently in fruit tissues, or if fruit physiology plays a role in *A. citrulli* colonization and symptom development.

**Location of *A. citrulli* in seeds**

Rane and Latin isolated *A. citrulli* from coats and embryos of seed recovered from artificially inoculated watermelon fruit, and concluded that the bacterium might be present on the surface and within the seed (Rane and Latin 1992). However, Walcott et al. (2003) reported that fruits that developed from the stigma inoculation of female flowers with *A. citrulli* did not develop BFB symptoms, but seeds within these fruits became contaminated with the pathogen. In 2007, Lessl et al. (2007) reported that *A. citrulli* inoculated onto watermelon stigmas rapidly colonize female watermelon flowers. Recently, Dutta et al. (2015) reported that after stigma colonization, *A. citrulli* cells ingress through the stylar canal resulting in seed infestation, and that pollination was critical for pistil invasion and seed infection. They also reported that pistil invasion resulted in the deposition of *A. citrulli* cells in the embryo/endosperm of the watermelon seed (Dutta et al. 2015).

In addition to pistil invasion, watermelon seeds can be infested by *A. citrulli* through penetration of the ovary pericarp (Dutta et al. 2012a). Frankle and Hopkins (1993) reported that *A. citrulli* penetrates through open stomata in the watermelon pericarp 2-3 weeks after anthesis to induce BFB symptoms. In 2012, Dutta et al. (2012a) demonstrated that seeds within fruits infected in this manner could be infested with *A. citrulli*. However, in this case, the bacteria become localized in the seed coat and exterior to the perisperm-endosperm layer. Hence, pistil invasion of *A. citrulli* after stigma inoculation resulted in *A. citrulli* localization

deep within watermelon seeds, while ovary pericarp invasion resulted in superficial seed infestation (Dutta et al. 2012a).

**Survival**

*A. citrulli* displays remarkable longevity in stored cucurbit seeds. The bacterium has been reported to survive for 34 and 40 years on stored watermelon and melon seeds, respectively (Block and Shepherd 2009). Additionally, Dutta et al. (2014) reported that after artificial fruit inoculation, the pathogen can survive in citron melon seeds for seven years. Dutta et al. (2014) suggested that the location of *A. citrulli* inside seeds is critical for long-term *A. citrulli* survival. The authors observed that *A. citrulli* survival was significantly higher in seeds generated via stigma inoculation (pistil invasion pathway) than by pericarp inoculation (Dutta et al. 2014b). Ultimately, Dutta et al. (2016) determined that embryo/endosperm localization enhanced the survival of *A. citrulli* in watermelon seeds.

**Seed-to-seedling transmission**

Seed-to-seedling transmission is a key factor in BFB epidemiology. Dutta et al. (2012b) reported that seed inoculum loads as low as 10 *A. citrulli* CFU/seed could lead to BFB seed-to-seedling transmission under greenhouse conditions. Hence, there is a very low tolerance for seedborne *A. citrulli* inoculum, and the elimination of seedling transmission is critical for BFB management. However, little is known about factors that influence the seed-to-seedling transmission of BFB. Additionally, Johnson and Walcott demonstrated that *A. citrulli* switches from saprobic to pathogenic growth on watermelon seedlings approximately 96

hours after planting (Johnson and Walcott 2013). Johnson and Walcott also reported that quorum sensing was important for the seed-to-seedling transmission of BFB. This was supported by observations that BFB seed-to-seedling transmission declined significantly for an *aacI* mutant of AAC00-1 at low inoculum levels (Johnson and Walcott 2013). Finally, Tian et al. (2015) reported that the *A. citrulli* T6SS contributes to BFB seed-to-seedling transmission on melon.

**Management of BFB**

Considerable efforts are routinely made to mitigate the economic impact of BFB, including pathogen exclusion by quarantines, seed health testing, and seed treatment; chemical applications in the field; and plant host resistance (Walcott 2008). Unfortunately, the efficacy of these approaches has been limited and BFB still poses a serious threat to commercial watermelon and melon production (Burdman and Walcott 2012). Examples of these strategies are discussed below.

**Exclusion**

The most effective control measure for BFB is exclusion of *A. citrulli* by using pathogen-free seeds and seedlings (Walcott 2008). This requires reliable and sensitive seed testing assays (Feng et al. 2013). In most cases, there is zero tolerance for *A. citrulli* in watermelon and melon production systems. In fact, *A. citrulli* is a quarantined pest in Israel, China, and Europe Union countries (Assouline et al. 1997; Feng et al. 2013). Hence, seed health testing is an important strategy for restricting the global dissemination of *A. citrulli.*

Many techniques have been developed for *A. citrulli* detection in plants and seeds, including direct plating of seed wash onto semi-selective agar medium, seedling grow-out assays (SGO), serological methods e.g., enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR)-based assays, loop-mediated isothermal amplification (LAMP), immunomagnetic separation-PCR (IMS-PCR), membrane filtration immunostaining, and combinations of the above (Feng et al. 2013). Several *A. citrulli*-specific PCR primers have been designed with varying levels of specificity (Bahar et al. 2008; Ha et al. 2009; Minsavage et al. 1995; Schaad et al. 2000; Song et al. 2003; Walcott et al. 2000; Walcott et al. 2006). It is important to note that PCR primers and antibodies with low specificity to *A. citrulli* may yield false-positive results (Walcott and Gitaitis 2000). Also, high levels of saprophytic bacteria and PCR inhibitors co-extracted from plant tissues can yield false-negative results (Walcott and Gitaitis 2000), which can be devastating for the cucurbit industry.

**Seed treatment**

Since infested seeds are important sources of primary inoculum for BFB outbreaks, seed treatments are critical components of BFB management. These include thermotherapy, seed fermentation, and utilization of various chemicals, and biological control agents. Overall, most seed treatments can reduce seedborne inoculum significantly by eliminating bacteria from the surfaces of seed coats. However, they fail to eliminate the bacteria from seeds that are internally contaminated by *A. citrulli* (Rane and Latin 1992). Rane and Latin

reported that incubation of infested watermelon seeds at 50°C for 20 min reduced but did not eliminate the pathogen (Rane and Latin 1992). The effectiveness of thermotherapy is limited because it is difficult to ensure that all seeds receive the necessary exposure to the critical killing temperature. Kubota et al. (2012) reported that complete disinfection of seeds of melon, cucumber, and small-seeded squash was achieved with dry heat treatment at 85°C for 3–5 days without affecting seed germination. However, this practice is not routinely used for commercial seed production.

Seed fermentation at harvest is routinely employed in commercial seed production for eliminating *A. citrulli*. Hopkins et al. (1996) demonstrated that seed fermentation in watermelon juice and debris prior to washing and drying decreased the level of BFB seed transmission from 61% to less than 1%. However, fermentation cannot be utilized for certain watermelon hybrids and other cucurbits due to deleterious effects on seed physiology (Walcott 2008).

Seed treatments with NaOCl, HCl, $CaOCl_2$, and peroxyacetic acid were reported to reduce BFB seedling transmission with varying levels of success (Hopkins et al. 1996; Hopkins et al. 2003; Rane and Latin 1992). Rane and Latin reported that seed treatment with 0.53% NaOCl for 20 min, or 1.8% HCl for 5 min significantly reduced BFB seedling transmission (Rane and Latin 1992). However, the HCl seed treatment negatively affected seedling growth (Rane and Latin 1992). Hopkins et al. (1996) reported that seed treatment with 1% $CaOCl_2$ for 15 min was relatively ineffective in reducing seed transmission, but 1% HCl for 15 min was as effective as fermentation. Hopkins et al. (2003) also reported

that the treatment of contaminated seeds with 1600 µg/ml of peroxyacetic acid for 30 min followed by drying at 40°C and low humidity for 48 h eliminated *A. citrulli* and *Didymella bryoniae* from watermelon and melon seeds. Currently, wet seed treatment with peroxyacetic acid is a standard practice employed by cucurbit seed producers.

Feng et al. (2009b) reported that treatment with acidic electrolyzed water for 30 min eradicated *A. citrulli* from infested seeds without decreasing seed germination or seedling establishment. Treatment with chlorine gas exposure for 9 h was also reported to be effective (Stephens et al. 2008); however, this is difficult to implement on a commercial scale due to the toxic and corrosive nature of chlorine gas. Finally, Li et al. (2013) reported that Chitosan A at 0.40 mg/mL significantly reduced disease development for watermelon planted in soil or perlite when used as a seed treatment.

Biological control agents have been explored as a seed treatment in multiple studies. Several plant beneficial bacteria including *Bacillus* sp. RAB9 reduced BFB development and increased the incubation period when applied to infected melon seeds (Medeiros et al. 2009). Wang et al. (2009) also demonstrated that the treatment of Hami melon seeds with cell-free culture filtrates of *Pichia anomala* Kurtzman strain 0732-1 significantly reduced BFB severity. Johnson et al. (2011) reported that a non-pathogenic *A. citrulli* strain effectively reduced seedling transmission of BFB when applied to watermelon seeds.

**Avoidance**

Since cucurbit seeds are the most important source of primary inoculum for BFB epidemics, the production of *A. citrulli*-free seed is important for BFB management. Seed production fields should be located in areas with no history of cucurbit seed or fruit production, with cool and dry climates (or during cool dry seasons), and with no BFB history (Walcott 2008). Additionally, seed fields should be isolated from commercial cucurbit production fields that may be sources of *A. citrulli* (Walcott 2008).

**Eradication**

Eradication methods including sanitation, host eradication, and crop rotation can significantly reduce the risk of BFB outbreaks. Sanitation is essential when working in seed or seedling and fruit production systems. Plants should be handled minimally, and gloves should be used. Additionally, workers should decontaminate tools and equipment as they move between transplant houses. Planting trays and bench surfaces should be disinfested with appropriate disinfectants.

To prevent *A. citrulli* survival in the field, weed hosts and infested plant debris should be rogued. In general, three-year rotations to non-cucurbit crops should be practiced. Additionally, overhead irrigation should be avoided and drip irrigation should be used in greenhouses and fields to reduce pathogen dissemination.

## Protection

### Chemical control

Protective applications of copper-containing chemicals have been widely used to manage BFB. Cupric hydroxide was reported to be effective in reducing BFB losses by controlling *A. citrulli* spread, however, phytotoxicity was observed (Hopkins 1991). Also, under conditions of excessive rainfall, the efficacy of protectant chemicals is reduced. Additionally, there is a risk of copper tolerance development in *A. citrulli* populations (Walcott et al. 2004). There have been limited attempts to employ antibiotics for BFB management. Hopkins reported that streptomycin was not significantly better than the unsprayed control for BFB management in the field (Hopkins 1991). With regards to chemical control in the transplant house condition, Hopkins et al. (2009) reported that combining ionized copper or peroxyacetic acid in irrigation water with weekly foliar application of acibenzolar-S-methyl was most effective in reducing BFB spread.

### Biological control

Biological control has been explored to protect watermelon flowers and thereby limit seed infection by *A. citrulli*. Fessehaie and Walcott (2005) demonstrated that when *A. avenae* strain 99-2 was applied to watermelon stigmas 5 h prior to inoculation with *A. citrulli*, BFB seedling transmission was reduced significantly in the resulting seeds. Stigma treatment with *A. avenae* strain 99-2 also significantly improved germination percentages of the resulting seedlots (Fessehaie and Walcott 2005). However, since *A. avenae* strain 99-2 is

pathogenic on maize, it is not suitable for BFB control. Using a non-pathogenic T3SS mutant of *A. citrulli* (AAC00-1Δ*hrcC*), Johnson et al. demonstrated the efficacy of biocontrol seed treatment and blossom protection for BFB. When used as a blossom protectant prior to challenge inoculation with AAC00-1, AAC00-1Δ*hrcC* significantly reduced BFB seedling transmission in the resulting seedlots (Johnson et al. 2011). These observations provided evidence for the potential of biocontrol for reducing seed infection and seedling transmission of BFB.

**Resistance**

One of the most effective strategies for managing plant diseases is the use of disease-resistant varieties. However, to date, there are no commercial BFB resistant cultivars available. Studies to identify sources of BFB resistance in cucurbit germplasm have yielded inconsistent results (Hopkins et al. 1993; Sowell Jr and Schaad 1979). These discrepancies may be due to differences in experimental conditions, or differences among the *A. citrulli* strains used for the resistance screens.

It has been reported that fruits of some watermelon cultivars are more susceptible to BFB than others and differences in rind color affect the degree of fruit susceptibility (Hopkins et al. 1993). The most susceptible watermelon fruits are those with a light green rind, while moderately susceptible fruits have light and dark-green stripes and the least susceptible fruits have a solid dark-green rind (Hopkins et al. 1993). Additionally, Hopkins and Thompson reported two PIs out of 1344 accessions (PI 482279 from Zimbabwe and PI 494817 from Zambia)

exhibited resistance to BFB under field and greenhouse conditions (Hopkins and Thompson 2002a). However, incorporating BFB resistance from those two PIs into watermelon cv. 'Crimson Sweet' was unsuccessful (Hopkins and Levi 2008).

Carvalho et al. (2013) evaluated 74 watermelon genotypes from the Northeast Brazil Cucurbit Germplasm Active Bank and reported that no watermelon genotypes were immune to BFB. However, they reported that the genotypes BGCIA 979, BGCIA 34, and Sugar Baby showed high levels of resistance at most plant development stages tested (Carvalho et al. 2013). In 2015, Ma and Wehner (2015) evaluated 1699 watermelon PI accessions from the USDA germplasm collection for BFB resistance based on foliar disease symptoms at the flowering stage. They identified 23 BFB resistant cultigens from Africa.

With regards to melon, Bahar et al. (2009b) reported that all 15 commercial cultivars/breeding lines and 20 wild melons tested were susceptible to *A. citrulli* strain M6. Additionally, Wechter et al. reported that most of the 332 *Cucumis* spp. PIs tested were susceptible to BFB, while four PIs exhibited lower levels of resistance. These PIs may be useful in melon breeding programs (Wechter et al. 2011).

**Research objectives**

The overall goal of this study is to gain a better understanding of host preference and virulence of the two major groups of *A. citrulli*. Findings from these studies will provide basic knowledge about interactions between two *A. citrulli* groups

and their cucurbit hosts, and might lead to the development of new strategies to manage BFB. Specific objectives include:

1. To assess differences in cucurbit host preference between group I and II *A. citrulli* strains under field conditions;

2. To characterize melon fruit infection by *A. citrulli*;

3. To characterize the role of the putative type 3 secreted effector XopJ and HopAF in *A. citrulli* virulence;

4. To identify unique virulence determinants of *A. citrulli* group II strains using an immature watermelon fruit assay;

5. To investigate the effect of elevated temperature on *A. citrulli* colonization of germinating watermelon seeds.

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# CHAPTER 2

# GENETICALLY DISTINCT *ACIDOVORAX CITRULLI* STRAINS DISPLAY CUCURBIT FRUIT PREFERENCE UNDER FIELD CONDITIONS [1]

[1] Zhao, M., Dutta, B., Luo, X., Burdman, S., and Walcott R. R. Submitted to *Phytopathology*, 10/19/2019.

## ABSTRACT

Strains of *Acidovorax citrulli*, the causal agent of bacterial fruit blotch (BFB) of cucurbits, can be assigned to two groups, I and II. The natural association of group I and II strains with different cucurbit species suggests host preference; however, there are no direct data to support this hypothesis under field conditions. Hence, the objective of this study was to assess differences in the prevalence of group I and II *A. citrulli* strains on cucurbit species in the field. From 2017-2019, we used group I and II strains to initiate BFB outbreaks in field plots planted with four cucurbit species. At different times, we collected symptomatic tissues and assayed them for group I and II strains using a group-specific PCR assay. Binary distribution data analysis revealed that the odds of melon, pumpkin, and squash foliage infection by group I strains were 21.7, 11.5, and 22.1 times greater, respectively, than the odds of watermelon foliage infection by the group I strain ($p < 0.0001$). More strikingly, the odds of melon fruit infection by the group I strain were 97.5 times greater than watermelon fruit infection by the same strain ($p < 0.0001$). Unexpectedly, some of the group II isolates recovered from the 2017 and 2019 studies were different from the group II strains used as inocula. These data confirmed that *A. citrulli* strains exhibited a preference for different cucurbit species, which was more pronounced in fruit tissues.

## KEYWORDS

Host preference, *Acidovorax citrulli*, watermelon fruit blotch

## INTRODUCTION

Bacterial fruit blotch (BFB) is an economically important seed-borne and seed-transmitted disease of cucurbitaceous plant species including watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) and melon *(Cucumis melo* L.). *Acidovorax citrulli*, the causal agent of BFB, was first isolated by Webb and Goth in 1965 at the Regional Plant Introduction Station in Griffin, Georgia, USA (Webb and Goth 1965). However, it was not until 1987 that the first natural BFB outbreak occurred in the Mariana Islands on watermelon (Wall and Santos 1988). In 1989, the disease was observed in commercial watermelon fields in Florida (Somodi et al. 1991) and Indiana (Latin and Rane 1990). Subsequently, BFB outbreaks have occurred in many regions around the world, primarily on watermelon and melon crops, resulting in significant economic losses (Burdman and Walcott 2012).

BFB has a wide host range in the Cucurbitaceae family (Zhao and Walcott 2018). Watermelon and melon are highly susceptible, and as mentioned above, most BFB outbreaks have been reported on these species. However, other hosts include cucumber (*Cucumis sativu*s L.) (Martin et al. 1999), gramma (*Cucurbita moschata*) (Martin and Horlock 2002), squash and pumpkin (*Cucurbita pepo*, *C. maxima*, and *C. moschata*) (Langston Jr et al. 1999), citron melon (*Citrullus lanatus* var. *citroides*) (Isakeit et al. 1998), prickly paddymelon (*Cucumis myriocarpus* subsp. *myriocarpus*) (O'Brien and Martin 1999), and several types of gourds.

*Acidovorax citrulli* strains can be divided into two major groups, I and II, based on carbon substrate utilization (Walcott et al. 2004), DNA fingerprint analyses by pulse field gel electrophoresis (PFGE) (Burdman et al. 2005; Walcott, R. R. et al. 2000; Yan et al. 2013), repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) (Walcott et al. 2004), multi-locus sequence analysis (MLSA) (Feng et al. 2009a; Yan et al. 2013), and fatty acid methyl ester analysis (Walcott, R. R. et al. 2000). The diversity of *A. citrulli* was initially reported in an Australian population by O'Brien and Martin (1999). Carbon substrate utilization profiles and pathogenicity assays were employed to characterize strains recovered from several cucurbit hosts including watermelon, rockmelon, honeydew (*Cucumis melo* var. *indorus Jacq*.), and prickly paddymelon. Carbon substrate utilization profile analysis separated the strains into two groups. One group recovered from rockmelon and honeydew failed to utilize L-leucine, but utilized 2-amino ethanol. The other group, recovered from watermelon, utilized L-leucine, but were unable to utilize 2-amino ethanol.

Walcott et al. (2000) first reported two genetically distinct groups of *A. citrulli* among 120 strains collected from a broad range of geographical origins and a range of cucurbitaceous plants. In 2005, a study on an *A. citrulli* population from Israel yielded similar results (Burdman et al. 2005). Using MLSA, Feng et al. (2009a) showed that 65% (33/51) of the *A. citrulli* strains from China belonged to group I. Similarly, Yan et al. (2013) reported two *A. citrulli* groups in China with 63% (71/112) of the tested strains being members of group I. More recently, 97% (72/74) of a Brazilian population of *A. citrulli* strains, recovered predominantly

from melon, were found to be members of group I (Silva, G. M. et al. 2016). This indicated a predominance of group I strains in Brazil.

The natural association of group I and II *A. citrulli* strains with different cucurbit species strongly suggests host preference. In 1999, O'Brien and Martin observed that *A. citrulli* strains recovered from rockmelon production regions (North Queensland, Australia) were equally virulent on watermelon and rockmelon seedlings. However, strains collected from watermelon production regions (South Queensland, Australia) were more virulent on watermelon than rockmelon seedlings (O'Brien and Martin 1999). In 2000, Walcott et al. reported that 85% (17/20) of group I *A. citrulli* strains were isolated from cantaloupe and pumpkin, while 65% (65/100) of group II strains were isolated from watermelon, 34% from citron, and 1% from cantaloupe (Walcott, R. R. et al. 2000).

In a subsequent report using *A. citrulli* strains representing a wider geographical distribution, Walcott et al. (2004) reported a similar host preference pattern. More specifically, 93% (27/29) of group II strains were isolated from watermelon, and in contrast, 85% (29/34) of group I strains were isolated from muskmelon, bitter gourd (*Momordica charantia*), rockmelon, pumpkin, honeydew, and melon (Walcott et al. 2004). Additionally, an MLSA study of a global *A. citrulli* strain collection supported the hypothesis of *A. citrulli* host preference (Feng et al. 2009a). Of the strains with known hosts of origin, 88% (36/41) from group II were collected from watermelon, while 51% (24/47) of the group I strains were recovered from non-watermelon cucurbit species (Feng et al. 2009a). In agreement with these observations, Yan et al. (2013) reported that amongst a

collection of 118 Chinese *A. citrulli* strains, 92% (67/73) of the strains belonging to group I were isolated from melon, while 93% (42/45) of the group II strains were recovered from watermelon. To further support the hypothesis of host preference between group I and II *A. citrulli* strains, Walcott et al. (2000) reported that in a BFB outbreak in a mixed planting of pumpkins and watermelons in Georgia, USA, only pumpkin fruits displayed BFB symptoms. In this study, only group I strains were isolated from the symptomatic pumpkin fruits.

Despite the data suggesting host preference, Walcott et al. (2004) conducted greenhouse seedling and fruit inoculation assays and reported that representative group I and II *A. citrulli* strains could infect a range of cucurbit species, with no clear evidence for strict host specificity. In agreement with this study, Zivanovic and Walcott (2017) reported that there were no significant differences in temporal bacterial population dynamics between *A. citrulli* group I strain M6 and group II strain AAC00-1 after cotyledon tissue infiltration with bacterial cell suspensions at $10^4$ colony forming units (CFU)/ml. It is possible that there are differences in host preference between group I and II *A. citrulli* strains under natural field conditions, but artificial inoculation techniques may prevent the observation of this phenotype. Therefore, further studies using natural inoculation and pathogen spread under field conditions are needed to elucidate differences in host preference of *A. citrulli* groups.

Yan et al. (2017) recently developed a detached melon fruit assay that clearly distinguished representative group I and II *A. citrulli* strains based on symptom development. Using this assay, they observed a consistent difference

in pathogenicity between group I and II *A. citrulli* strains in the fruit (Yan et al. 2017). Based on these observations, we hypothesized that *A. citrulli* host preference manifests more strongly in cucurbit fruits than in foliar tissues. Consequently, the objective of this study was to assess differences in cucurbit host preference in foliar and fruit tissues between group I and II *A. citrulli* strains under field conditions.

## MATERIALS AND METHODS

### Bacterial strains and growth condition

Naturally occurring, antibiotic-resistant strains of representative group I (AAC203-1RR1, resistant to rifampicin) and II (AAC94-21SR1, resistant to streptomycin) *A. citrulli* were selected as inocula. These strains represent the most prevalent haplotypes of group I (B5) and II (A3) strains that were recovered from China, and Georgia USA, respectively (Silva, G. M. et al. 2016). The *A. citrulli* group II strain 17-6 was isolated from a field trial that we conducted in 2017, and it was used as an additional group II inoculum strain in 2019. For plant inoculation, strains were cultured overnight in nutrient broth (Becton Dickinson, Sparks, MD) at 28°C on a rotary shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm, adjusted to an $OD_{600}$ of 0.3 (~$10^8$ CFU/ml) spectrophotometrically (Spectronic 20; Bausch and Lomb, Rochester, NY), and diluted to the desired concentration using sterilized distilled water (sd$H_2O$). When required, media were supplemented with the following antibiotics: rifampicin (100 µg/ml) and streptomycin (100 µg/ml).

**Field experiment design**

Watermelon (cv. 'Crimson Sweet') (Johnny's Selected Seeds, Winslow, ME), melon (cv. 'Joaquin Gold') (Rogers Brand, Syngenta, Greensboro, NC), squash (cv. 'Golden Summer Crookneck') (Wild West Seed, Albany, OR), and pumpkin (cv. 'Jack O' Lantern') (Dorsing Seeds Inc, Nyssa, OR) seedlings were grown in 36-cell plastic trays (Grower's Solution, LLC, Cookeville, TN) in a commercial potting mix (Sungro Horticulture, Agawam, MA) under standard greenhouse conditions for four weeks and transplanted to a field plot on raised beds in Tifton, GA. Each cucurbit plot consisted of five rows of plants, each 6.1 m long with 0.6 m plant spacing. The distance between plots was approximately 1.8 m and the distance between rows was approximately 2.4 m. Concurrently, two-week-old melon and watermelon seedlings that were spray-inoculated with $10^6$ CFU/ml of *A. citrulli* strains AAC203-1-RR1 and AAC94-21SR1, respectively, were transplanted into the field plots. Inoculated seedlings that displayed typical BFB symptoms were placed at the middles and edges of each plot of each cucurbit species. These inoculated seedlings served as inoculum sources for natural BFB epidemics. Irrigation was applied through three automated overhead sprinkler systems (Strongway Tripod Sprinkler, Northern Tool + Equipment, Burnsville, MN) twice a week for 1 h each time. Leaves with BFB symptoms were collected from each plot at three sampling times over a 3 to 4-week period. Additionally, all cucurbit fruits that developed BFB symptoms were collected. This study was conducted three times from 2017-2019, and in the 2019 experiment, watermelon seedlings were spray-inoculated with *A. citrulli* 94-21SR1 and the group II field

isolate 17-6, recovered from the 2017 trial. Total DNA was extracted from the BFB lesion of each sample using the Synergy™ 2.0 Plant DNA Extraction Kit (OPS Diagnostics LLC, Lebanon, NJ). DNA samples were used as templates to detect *A. citrulli* and to determine whether the strain belonged to group I or II by an *A. citrulli* group-specific polymerase chain reaction (PCR) assay. To efficiently accomplish this task, we developed a duplex PCR assay with primer sets that were specific for group I and II *A. citrulli* strains (described below). To ensure that PCR-negative results were not due to inhibitory compounds coextracted with DNA, DNA samples were 10-fold diluted and subjected to a second round of PCR. If the results were still negative, DNA samples were further diluted 100-fold and subjected to a third round of PCR. Field studies were conducted at the University of Georgia Tifton Vegetable Park farm in Tifton, GA.

**Development of a duplex PCR assay to rapidly distinguish group I and II *A. citrulli* strains.**

To rapidly detect the presence of *A. citrulli* in symptomatic plant tissues and to determine the group to which each strain belonged, we developed a duplex PCR assay with group-specific primer sets (Table 2.1). Primers for this assay were designed using the Primer 3 program (Kõressaar et al. 2018) based on genes *APS58_3303* (gene ID according to the M6 annotation; GenBank accession CP029373.1 (Yang, R. Z. et al. 2019)) and *Aave_2708* (gene ID according to the AAC00-1 annotation; GenBank accession CP000512.1), which were present only in group I and II strains, respectively (Eckshtain-Levi et al. 2014; Jiménez-Guerrero et al. 2019). PCR was conducted using One*Taq* DNA polymerase

according to the manufacturer's instructions (New England Biolabs Inc., Ipswich, MA). The sensitivity of the duplex PCR assay was evaluated using DNA from 10-fold serial dilutions of boiled cells of *A. citrulli* group I strain AAC203-1RR1 and group II strain AAC94-21SR1. The specificity of the PCR assay was evaluated using DNA from boiled cells ($10^8$ CFU/ml) of group I (n=9) and group II (n=10) *A. citrulli* strains (Table 2.2). Cell suspensions of each strain were generated by suspending bacterial cells from overnight cultures in $sdH_2O$. The concentration of each cell suspension was adjusted to $OD_{600}$=0.3 (~$10^8$ CFU/ml) and a 1 ml aliquot was lysed by incubating at 100°C for 10 min to release genomic DNA. Samples of 2.5 µl of lysed cells were used as templates for PCR assays with the following thermal profile: 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s; then a final extension of 68°C for 5 min. Products were separated by electrophoresis on 1% agarose gels stained with ethidium bromide (0.5 µl /ml) and in 1x Tris-acetate-EDTA buffer for 30 min at 120 V.

**DNA fingerprinting of representative field isolates**

Bacterial isolation was performed from selected leaf and fruit tissues on King's B medium (King et al. 1954) to confirm the presence and viability of the *A. citrulli* isolates detected by PCR. To test if the group I and II *A. citrulli* isolates causing BFB symptoms in the field were identical to those used for inoculation, DNA restriction digestion with *SpeI* (NEB) followed by PFGE was conducted on representative field isolates (Table 2.3) as previously described (Walcott, R. R. et al. 2000).

### Statistical analysis

For each cucurbit species, the data were expressed as the presence of group I and II *A. citrulli* members detected from symptomatic foliar and fruit lesions. We used the GLIMMIX (SAS V9.4; SAS Institute, Cary, NC) procedure on the leaf and fruit tissue types for binary distribution data of five replicates, and the odds ratios were calculated using a logit link function. As BFB-infected squash fruits were detected only in one year, data from watermelon and melon fruits were used for comparisons at the fruit tissue type level.

## RESULTS

### Development of a duplex PCR assay to distinguish group I and II *A. citrulli* strains

We developed a new duplex PCR assay to distinguish the two groups of *A. citrulli* in a single reaction. The detection thresholds of the group I- and II-specific PCR assays based on primer sets, 550-34F/550-626R and 2708-225F/2708-433R, were $10^7$ and $10^6$ CFU/mL, respectively (Fig 2.1). With regards to specificity, the group I primer set amplified DNA from all nine group I strains tested, but yielded no amplicons with DNA from boiled cells from ten group II strains (Table 2.2). In contrast, the group II primer set amplified DNA from all ten group II strains tested, but yielded no amplicon with DNA from the nine group I strains (Table 2.2).

### Field experiments to investigate *A. citrulli* cucurbit host preference

**2017 field study**: A field plot planted with four cucurbit species (watermelon, melon, pumpkin, and squash) was established in Tifton, GA in the summer of 2017 and exposed to group I and II *A. citrulli* strains. During the field study, total

precipitation was 20.85 cm and average rainfall per day was 0.41 cm. The average temperature during the field study was 26.1°C (Table 2.4). In total, 37% (11/30) of watermelon leaf samples that exhibited visible BFB symptoms were infected with the group I strain, while 60% (15/25) of symptomatic melon leaf samples were infected with the group I strain. Additionally, 67% (4/6) of symptomatic pumpkin and 71% (10/14) of symptomatic squash leaf samples were infected with the group I strain (Fig 2.2A). For fruits, none of the pumpkin or squash fruit samples with putative BFB lesions were determined to be positive for *A. citrulli* by PCR assay. On the other hand, 100% (9/9) of the symptomatic watermelon fruit samples were determined to be infected with the group II strain. With regards to symptomatic melon fruits, 86% (6/7) were determined to be infected with the group I strain (Fig 2.2B).

**2018 field study**: Two field plots, each with four cucurbit species, were established in Tifton, GA in summer 2018. During the field study, total precipitation was 26.59 cm and average rainfall per day was 0.44 cm. The average temperature during the field study was 25.6°C (Table 2.4). In total, 42% (28/67) of the watermelon leaf samples that exhibited BFB symptoms were infected with the group II strain, while 100% of symptomatic melon (n=80), pumpkin (n=2), and squash (n=19) leaf samples were infected with the group I strain (Fig 2.3A). For fruit samples, no putative pumpkin or squash fruit lesions were determined to be positive for *A. citrulli* by PCR assays. On the other hand, 100% (n=20) of the symptomatic melon fruit samples were infected with the

group I strain. Interestingly, in 2018, only two infected watermelon fruits were observed and both were infected with the group I strain (Fig 2.3B).

**2019 field study**: Two field plots, each with four cucurbit species, were established in Tifton GA in the summer of 2019. During the field study, total precipitation was 18.62 cm and average rainfall per day was 0.31 cm. The average temperature during the field study was 25.4°C (Table 2.4). Seven percent (5/69) of infected watermelon leaf samples were determined to be infected with the group I strain, while 82% (75/92) of symptomatic melon leaf samples were infected with the group I strain. Additionally, 100% (5/5) of the BFB-infected squash leaf samples were infected with the group I strain (Fig 2.4A). No pumpkin leaves or fruit samples tested positive for *A. citrulli* by PCR assay. In contrast, 95% (36/38) of the symptomatic watermelon fruit samples were determined to be infected with group II by PCR assay. With regards to symptomatic melon fruits, 78% (7/9) were infected with the group I strain (Fig 2.4B). For the first time in 2019, we observed squash fruits with BFB symptoms and 100% (5/5) of these fruits were infected with the group I strain.

**Genetic analysis of *A. citrulli* isolates recovered from field plots**

To determine if the group I and II *A. citrulli* isolates that caused BFB symptoms in the field were identical to those used for inoculation, we conducted *Spe*I digestion and PFGE on representative field isolates (Table 2.3) from 2017 (n=8), 2018 (n=6), and 2019 (n=8). All group I *A. citrulli* field isolates had *Spe*I-PFGE profiles that were identical to the strain used for inoculation (AAC203-1RR1) (Fig 2.5). Additionally, the recovered isolates were resistant to rifampicin as expected.

In contrast, the 2017 group II field isolates had two *Spe*I-PFGE profiles that were both different from that of the group II strain used for inoculation (AAC94-21SR1) (Fig 2.5). Moreover, these isolates were sensitive to streptomycin, indicating that they occurred naturally in the environment. The 2018 group II field isolates recovered were genetically identical to the strain used for inoculation (AAC94-21SR1) based on *Spe*I-PFGE analysis (Fig 2.5). The 2019 group II field isolates (n=4) were identical to the strain used for inoculation (17-6), with one exception (AAC strain 19-5) (Fig 2.5).

**Statistical analysis**

Statistical analysis of binary distribution data from five replicates over the three year field study showed that the odds of melon, pumpkin, and squash foliage being infected by the *A. citrulli* group I strain were 21.7, 11.5, and 22.1 times greater than the odds of watermelon foliage being infected by the group I strain, respectively ($p < 0.0001$). However, the odds ratio of melon, pumpkin, and squash foliage being infected by *A. citrulli* group I strain were not significantly different. More strikingly, the odds of melon fruits being infected by the group I *A. citrulli* strain were 97.5 times greater than watermelon fruits being infected by the group I strain ($p < 0.0001$).

**DISCUSSION**

To rapidly determine if a BFB lesion from a plant sample was caused by a group I or II *A. citrulli* strain, we developed a group-specific duplex PCR-based assay. Primers for this assay were designed to amplify group-specific type III-secreted effectors (T3Es) (Eckshtain-Levi et al. 2014; Jiménez-Guerrero et al.

2019). As expected, the two primer sets successfully discriminated group I and II strains. The detection sensitivity of this assay was low ($10^6$-$10^7$ CFU/ml) relative to previously developed PCR-based assays for *A. citrulli* (Ha et al. 2009; Yang, Y. W. et al. 2019). However, since our goal was to detect the presence of *A. citrulli* in well-established leaf and fruit lesions, and we expected pathogen populations in lesions to be relatively high (Yan et al. 2017; Zivanovic and Walcott 2017), we determined that the PCR assays were suitable for this study.

In 2017, we observed no substantial difference in the proportion of group I and II *A. citrulli* detected on the foliage of the four cucurbit species. Foliage from non-watermelon hosts (melon, pumpkin, and squash) showed slightly higher percentages (60%, 67%, and 71%, respectively) of samples infected with group I strains, while watermelon foliage showed a slightly higher percentage (63%) of samples infected with group II strains. On the other hand, there appeared to be a difference in susceptibility of cucurbit fruits to different *A. citrulli* strains. More specifically, all watermelon fruit samples were infected by the group II strain, while all melon fruits, except one, were infected by the group I strain. The fruits of the pumpkin and squash cultivars tested did not appear to be susceptible to infection by the *A. citrulli* strains used under the tested conditions.

Interestingly, the group II *A. citrulli* isolates recovered from the 2017 field samples differed from the inoculated strain, AAC94-21SR1, in that they were sensitive to streptomycin. *Spe*I-PFGE analysis of the 2017 group II field isolates revealed two distinguishable haplotypes, that were both different to the profile of AAC94-21SR1. Based on this observation, we conclude that the group II isolates

responsible for the 2017 BFB epidemic originated from the local environment. On the other hand, the group I isolates recovered from the 2017 study were rifampicin-resistant and yielded identical PFGE profiles as the inoculum strain, AAC203-1RR1. These results suggest that group II isolates may be prevalent at the Tifton Vegetable Park farm, while group I isolates likely do not have localized sources of inoculum.

In 2018, group II *A. citrulli* strains were only observed on infected, symptomatic watermelon foliage. In contrast, we detected group I *A. citrulli* strains in leaf lesions of all cucurbit hosts, including watermelon. With regards to fruit samples, we only detected group I *A. citrulli* strains in BFB symptomatic melon and watermelon fruits. These data suggest that while the group II strain tested was specific to watermelon foliage, the group I strain could infect the foliage of all cucurbits tested under natural field conditions. As expected, the 2018 field isolates that were recovered had *Spe*I-PFGE profiles that were identical to their respective inocula. However, since we did not recover AAC94-21SR1 from infected watermelon fruits, we suspected this group II strain was weakly virulent under tested conditions. We also suspected that the local field isolate from the 2017 field trial was more virulent than AAC94-21SR1 under field conditions. Since this strain (called 17-6) occurred naturally, we considered that it might better allow us to test our hypothesis regarding cucurbit host preference of *A. citrulli* groups under field conditions. Hence, for the 2019 field study, we included both AAC94-21SR1 and the field isolate 17-6 as group II inocula.

For the 2019 field study, we observed a substantial difference in the proportion of group I and II *A. citrulli* strains detected on the foliage of the four cucurbit species. While all pumpkin leaf samples were negative for *A. citrulli,* symptomatic melon and squash leaf samples were infected with high percentages (82% and 100%, respectively) of group I strains. Additionally, 93% of the infected watermelon leaf samples were infected with group II strains. Unlike data from the 2017 and 2018 studies, these data strongly suggested *A. citrulli* host preference on cucurbit foliage, with the group II strains showing a strong preference for watermelon and the group I strain showing a strong preference for melon and squash foliage.

In 2019, the *A. citrulli* strains showed strong host preference on the fruits of different cucurbits. More specifically, 95% of the symptomatic watermelon fruits were infected with the group II strains, while 78% of melon fruits were infected with the group I strain. Interestingly, for the first time in 2019, we observed natural BFB squash fruit infection and 100% (5/5) of these fruits were infected with the group I strain. Remarkably, lesions of all five infected squash fruits were associated with injury caused by pickleworms (*Diaphania nitidalis*). It is possible that wounds created by insects facilitated the entry of *A. citrulli* into fruit tissues. We observed pickleworm damage on melon fruits in 2017 and 2018 trials, and on melon, squash, and pumpkin fruits in 2019 trials. Despite this, throughout this study, pumpkin fruits did not develop BFB symptoms. Hence, it is likely that insect damage increases the likelihood of BFB fruit infection for some cucurbit fruits.

Hopkins and Thompson (2002b) reported that in a field study using a single *A. citrulli* group II strain, fruit symptoms were not observed on the fruits of acorn squash cv. 'Tay Belle', pumpkin cv. 'Ichabod', or butternut squash cv. 'Butterboy'. However, they observed a few small lesions on zucchini squash cv. 'Elite 828' and yellow squash cv. 'Crescent' in one of two years (Hopkins and Thompson 2002b). Based on these results and our direct observations, we conclude that under natural field conditions, pumpkin and squash fruits are at low risk for *A. citrulli* infection relative to watermelon and melon fruits. However, *A. citrulli* infection of pumpkin fruits was reported to occur in a commercial field in Georgia in 1998 (Langston Jr et al. 1999). To date, only watermelon and melon cultivars and germplasm collections have been extensively screened for BFB resistance (Bahar et al. 2009b; Carvalho et al. 2013; Hopkins et al. 1993; Hopkins and Thompson 2002a; Wechter et al. 2011). It would be interesting to screen pumpkin germplasm for BFB-resistance.

Host preference within a pathogen species has been demonstrated in other pathosystems. For example, *Erwinia tracheiphila*, the causal agent of bacterial wilt of cucurbits, infects two genera of cucurbit crop plants, *Cucurbita* spp. (pumpkin and squash) and *Cucumis* spp. (muskmelon and cucumber) (Rojas et al. 2015). Based on whole genome sequence analysis, Shapiro et al. (2018) found that 88 *E. tracheiphila* strains from different geographic ranges were divided into three clusters, Et-melon, Et-C1, and Et-C2. Strains from the three clusters were detected in field-infected cucumber plants, while muskmelon was infected only by the Et-melon strains, and squash was only infected by the

Et-C1 and Et-C2 strains (Shapiro et al. 2018). *E. tracheiphila* strains also varied in their virulence in a host-dependent pattern. The authors suggested that the introduced and highly susceptible cucumber cultivars were driving *E. tracheiphila* lineage diversification.

Determining the host preference of a pathogen is important, as it helps to estimate the risk of disease outbreaks. While previous anecdotal observations suggested differences in cucurbit host preference of group I and II *A. citrulli* strains, no empirical data were generated to directly test this hypothesis under field conditions. In the current study, we demonstrated that the two *A. citrulli* groups differed in their ability to infect the foliage of different cucurbit species. More importantly, we demonstrated that this host preference manifested itself more strongly in cucurbit fruit tissues. We observed differential susceptibility of host tissue types (foliage vs. fruit) to *A. citrulli* strains. Importantly, most of the fundamental investigations on model plant pathogenic bacterial species, such as *Pseudomonas syringae* pv. tomato, have involved foliar tissue inoculations (Xin et al. 2018). Fruit tissue represents a unique niche for plant and human pathogens that may require different adaptations compared to other host tissue types. Therefore, exploring pathogen-fruit interactions has biological and economic significance for improving disease management. From an economic standpoint, fruit infection is the most important phase of BFB epidemiology. Studying the mechanisms of fruit infection is also important for improving BFB management. *Acidovorax citrulli* cells have been shown to penetrate watermelon (Frankle and Hopkins 1993) and melon (Silva Neto et al. 2006) fruits via stomata.

It would be interesting to test the factors that permit or promote entry of *A. citrulli* into plant tissues to initiate infection, and to investigate differences in the mechanisms of fruit infection by different *A. citrulli* strains in combination with fruits from distinct cucurbit crops.

Elucidating variations in virulence and host preference determinants among *A. citrulli* strains could set the stage for detection of genetic markers of host resistance. Currently, no BFB-resistant cucurbit cultivars are commercially available. Thus, in addition to field studies to investigate *A. citrulli* host preference, it is necessary to understand the genetic factors that differentiate group I and II strains and to determine their roles in virulence and host preference. Eckshtain-Levi et al. (2014) reported that three effectors in the XopJ family were present in group II strains, but were absent or non-functional in group I strains. In addition, Jiménez Guerrero et al. (2019) identified ten unique T3Es that were present in the *A. citrulli* group I strain M6, but absent in the group II strain AAC00-1. The variability in T3E arsenals between *A. citrulli* group I and II strains may contribute to the differences in the host-preferential association between the groups. Despite this, more research is needed to test this hypothesis and to understand the mechanisms of host preference of *A. citrulli*. The current study demonstrates host preference of *A. citrulli* under natural field conditions, but additional research is critical for the development of more effective strategies for BFB management.

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Table 2.1 List of primers used in this study.

| Primer designation | Sequence (5' – 3') | Group | Target gene | Amplicon size (bp) |
|---|---|---|---|---|
| 550-34F | TCATCGCCAACCGTTACACA | I | *APS58_3303*[a] | 593 |
| 550-626R | CTCCACGAGTCGAGAACCAC | | | |
| 2708-225F | GCTGTTAGCCAACTCCCTGT | II | *Aave_2708*[b] | 209 |
| 2708-433R | TAGGCGAGTCAAAGTGCTGG | | | |

[a] Based on the annotation of the *Acidovorax citrulli* M6 chromosome (GenBank accession CP029373.1).
[b] Based on the annotation of the *Acidovorax citrulli* AAC00-1 chromosome (GenBank accession CP000512.1).

Table 2.2 Specificity of *Acidovorax citrulli* group I and II-specific oligonucleotide primers as determined by PCR assay.

| Strain[a] | Group | *Spe*I PFGE haplotype | PCR assay results with: | |
|---|---|---|---|---|
| | | | 550-34F/550-626R | 2708-225F/2708-433R |
| 92-300 | I | B3 | + | - |
| 92-301 | I | B1 | + | - |
| 201-14 | I | B11 | + | - |
| 203-16 | I | B13 | + | - |
| 208-1 | I | B14 | + | - |
| 209-108 | I | B5 | + | - |
| 209-128 | I | B21 | + | - |
| IACANT58-1 | I | B8 | + | - |
| AU-2 | I | B4 | + | - |
| 92-2 | II | A1 | - | + |
| 92-3 | II | A4 | - | + |
| 92-303 | II | A2 | - | + |
| 94-39 | II | A7 | - | + |
| 96-6 | II | A6 | - | + |
| 209-118 | II | A30 | - | + |
| 211-2 | II | A5 | - | + |
| 211-8 | II | A14 | - | + |
| 211-29 | II | A24 | - | + |
| 211-36 | II | A17 | - | + |

+ and - indicate the presence or absence of a 593-bp band or a 209-bp band resulting from conventional PCR using primer sets 550-34F/550-626R and 2708-225F/2708-433R, respectively.

[a] Strain source: R. Walcott collection.

Table 2.3 List of *Acidovorax citrulli* isolates analyzed by *Spe*I-digested pulse field gel electrophoresis (PFGE).

| Isolate | Sample date | Host | Tissue | PFGE group | PFGE profile | Rifiampicin resistance | Streptomycin resistance |
|---|---|---|---|---|---|---|---|
| 17-1 | 7/4/2017 | Watermelon | Fruit | II | 2 | + | - |
| 17-2 | 7/4/2017 | Melon | Fruit | II | 2 | + | - |
| 17-3 | 6/19/2017 | Melon | Leaf | I | 1 | + | - |
| 17-4 | 6/19/2017 | Watermelon | Leaf | I | 1 | + | - |
| 17-5 | 7/12/2017 | Watermelon | Fruit | II | 3 | + | - |
| 17-6 | 7/12/2017 | Watermelon | Fruit | II | 2 | + | - |
| 17-7 | 7/12/2017 | Watermelon | Fruit | II | 3 | + | - |
| 17-8 | 7/20/2017 | Melon | Fruit | I | 1 | + | - |
| 18-1 | 5/22/2018 | Melon | Leaf | I | 1 | + | - |
| 18-3 | 5/22/2018 | Watermelon | Leaf | I | 1 | + | - |
| 18-8 | 5/22/2018 | Watermelon | Leaf | II | 4 | + | + |
| 18-18 | 6/1/2018 | Watermelon | Leaf | II | 4 | + | + |
| 18-19 | 6/22/2018 | Watermelon | Fruit | I | 1 | + | - |
| 18-20 | 6/22/2018 | Melon | Fruit | I | 1 | + | - |
| 19-2 | 6/1/2019 | Watermelon | Fruit | II | 2 | + | - |
| 19-3 | 6/1/2019 | Melon | Fruit | I | 1 | + | - |
| 19-4 | 6/1/2019 | Melon | Leaf | II | 2 | + | - |
| 19-5 | 6/1/2019 | Watermelon | Leaf | II | 5 | + | - |
| 19-7 | 6/1/2019 | Melon | Leaf | I | 1 | + | - |
| 19-8 | 6/1/2019 | Melon | Leaf | I | 1 | + | - |
| 19-10 | 6/13/2019 | Melon | Fruit | I | 1 | + | - |
| 19-13 | 6/21/2019 | Watermelon | Fruit | II | 2 | + | - |

Table 2.4 Temperature and precipitation for the Coastal Plain Experiment Station in Tifton GA during the field trials in 2017-2019.

| Year | Mean maximum temperature (°C) | Mean minimum temperature (°C) | Mean average temperature (°C) | Total precipitation (cm) | Mean precipitation (cm)/day |
|---|---|---|---|---|---|
| 2017 | 31.0 | 21.2 | 26.1 | 20.85 | 0.41 |
| 2018 | 31.0 | 20.1 | 25.6 | 26.59 | 0.44 |
| 2019 | 31.5 | 19.4 | 25.4 | 18.62 | 0.31 |

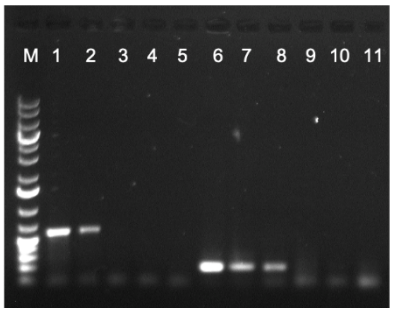


Fig 2.1 Detection sensitivity of group I (550-34F/550-626R)- and II (2708-225F/2708-433R)-specific PCR assays for *Acidovorax citrulli* strains based on 10-fold serial dilutions of bacterial cell suspensions. Lane: M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; 1: AAC203-1RR1, $10^8$ CFU/ml; 2: AAC203-1RR1, $10^7$ CFU/ml; 3: AAC203-1RR1, $10^6$ CFU/ml; 4: AAC203-1RR1, $10^5$ CFU/ml; 5: AAC203-1RR1, $10^4$ CFU/ml; 6: AAC94-21SR1, $10^8$ CFU/ml; 7: AAC94-21SR1, $10^7$ CFU/ml; 8: AAC94-21SR1, $10^6$ CFU/ml; 9: AAC94-21SR1, $10^5$ CFU/ml; 10: AAC94-21SR1, $10^4$ CFU/ml; 11: $H_2O$ negative control.

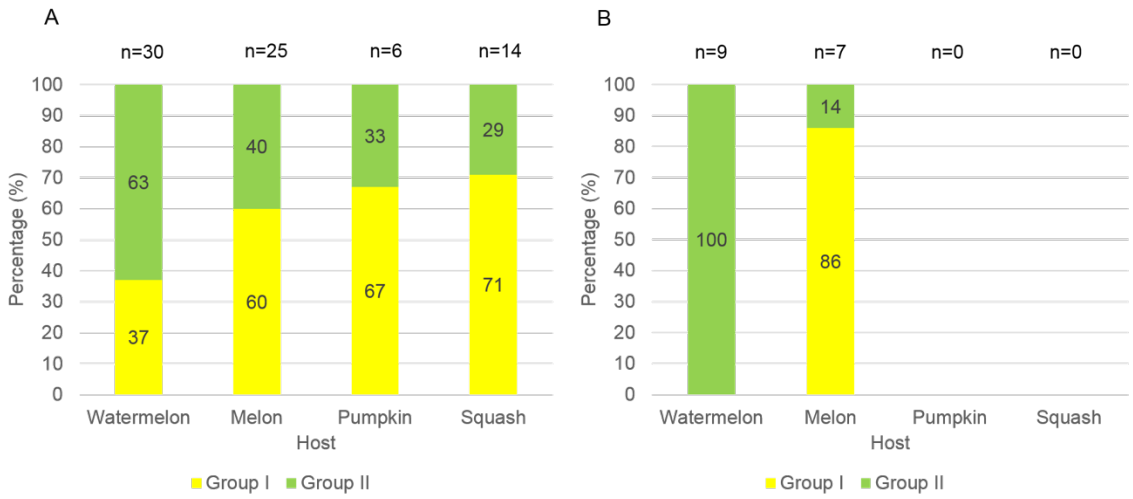


Fig 2.2 The proportion of group I and II *Acidovorax citrulli* PCR-positives detected in symptomatic cucurbit foliage (A) and fruit (B) samples from the 2017 field trial to investigate cucurbit host preference. The percentages of group I (yellow) and II (green) *A. citrulli* isolates are shown in the bar charts.

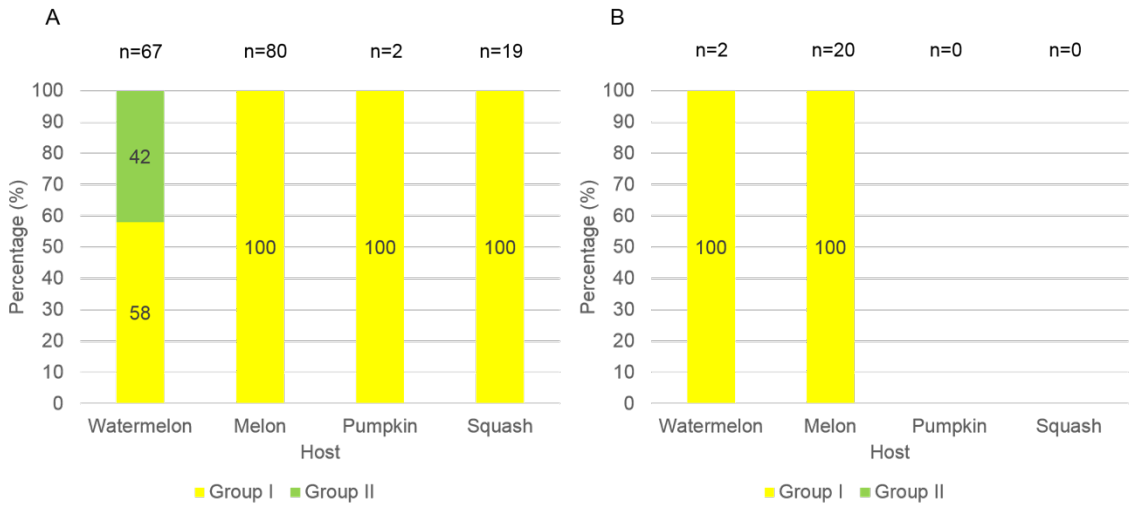


Fig 2.3 The proportion of group I and II *Acidovorax citrulli* PCR-positives detected in symptomatic cucurbit foliage (A) and fruit (B) samples from the 2018 field trial to investigate cucurbit host preference. The percentages of group I (yellow) and II (green) *A. citrulli* isolates are shown in the bar charts.

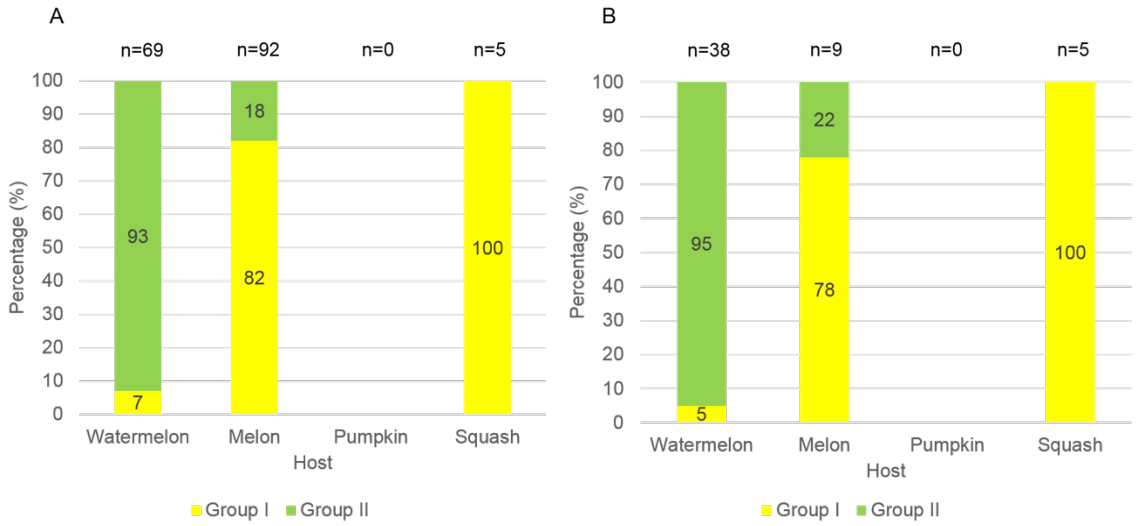


Fig 2.4 The proportion of group I and II *Acidovorax citrulli* PCR-positives detected in symptomatic cucurbit foliage (A) and fruit (B) samples from the 2019 field trial to investigate cucurbit host preference. The percentages of group I (yellow) and II (green) *A. citrulli* isolates are shown in the bar charts.

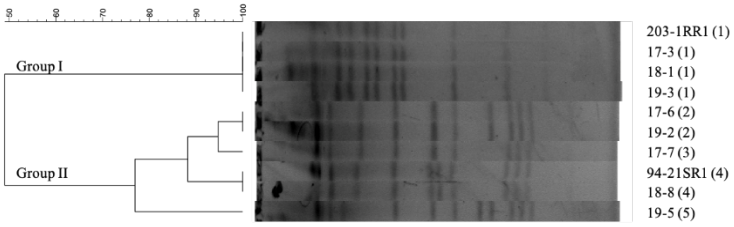


Fig 2.5 Dendrogram indicating the relationship among representative field isolates of *Acidovorax citrulli* based on polymorphisms among restriction fragments obtained following *Spe*I digestion of whole-cell genomic DNA and separation of fragments by pulse field gel electrophoresis (PFGE). The distance matrix for the dendrogram was generated by Dice's coefficient of similarity and the dendrogram was generated based on the unweighted pairwise group method with arithmetic mean algorithm. The numbers in parentheses represent the PFGE types recovered from the field trials.

# CHAPTER 3

# CHARACTERIZATION OF *ACIDOVORAX CITRULLI* MELON FRUIT COLONIZATION [1]

[1] Zhao, M., and Walcott R. R. To be submitted to *Phytopathology*.

## ABSTRACT

*Acidovorax citrulli* causes bacterial fruit blotch of cucurbits (BFB) and can be assigned to two genetically distinct groups: I and II. The natural association of group I and II *A. citrulli* strains with different cucurbit hosts suggests host preference. Additionally, in field experiments, we observed significant differences in BFB incidence between representative group I and II *A. citrulli* strains on melon and watermelon fruits. The objective of this study was to further characterize differences in the abilities of group I and II *A. citrulli* strains to infect melon fruit tissues. Group I and II *A. citrulli* strains were inoculated onto detached melon fruits by syringe- and swab-inoculation, and onto attached melon fruits by swab-inoculation. Both group I and II *A. citrulli* strains penetrated melon fruit mesocarp tissues at similar levels 3 h after swab-inoculation, and colonized melon fruit mesocarp tissues at similar population levels 4 days after syringe inoculation. By swab-inoculation of attached melon fruits, group I strains produced more severe symptoms and earlier than group II strains. We conclude that the elements of physical penetration, colonization, and symptom development do not account for the host preference of *A. citrulli.*

## INTRODUCTION

*Acidovorax citrulli* (Schaad et al. 2008; Willems et al. 1992) causes bacterial fruit blotch (BFB), a devastating disease of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) and other cucurbits, including muskmelon and honeydew melon (*Cucumis melo* L.) (Isakeit et al. 1997; Latin and Hopkins

1995). BFB symptoms on watermelon fruits develop into large, dark-green, water-soaked lesions with irregular margins, which do not initially extend into the fruit flesh. As the lesions age, the fruit surface may crack, leading to fruit rot (Hopkins 1991). Symptoms on melon fruits include water-soaked pits on the fruit surface (Latin and Hopkins 1995). O'Brien and Martin (1999) reported that BFB symptoms on melon fruits at later stages of development include small, depressed infection points surrounded by a water-soaked area, which later failed to develop netting. Lesions often extend into the flesh of the fruit and cavities develop in the affected melon fruit tissue (O'Brien and Martin 1999).

*Acidovorax citrulli* strains can be divided into two major groups, I and II, based on carbon substrate utilization (Walcott et al. 2004), DNA fingerprint analyses by pulse field gel electrophoresis (PFGE) (Burdman et al. 2005; Walcott et al. 2000; Yan et al. 2013), repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) (Walcott et al. 2004), multi-locus sequence analysis (MLSA) (Feng et al. 2009a; Yan et al. 2013), and fatty acid methyl ester analysis (Walcott et al. 2000). We recently demonstrated that representative strains of the two *A. citrulli* groups differed in their ability to infect different cucurbit species. More importantly, we demonstrated that this host preference manifested itself more strongly in cucurbit fruit tissues. From an economic standpoint, fruit infection is the most important phase of BFB epidemiology. However, most of the fundamental investigations on model plant pathogenic bacterial species, such as *Pseudomonas syringae* pv. tomato, have involved foliar tissue inoculations (Xin et al. 2018). Fruit represents a unique niche for

plant and human pathogens that may require different adaptations compared to other host tissue types. Therefore, exploring pathogen-fruit interactions has economic and biological significance for improving disease management.

Despite the economic importance of BFB, little is known about fundamental aspects of *A. citrulli*-host interactions. Major losses (up to 90%) caused by BFB were due to fruit symptoms that rendered mature fruits unmarketable (Hopkins 1991; Rane and Latin 1992). Thus, it is important to characterize the mechanisms and pathways by which different *A. citrulli* strains infect fruit tissues. *Acidovorax citrulli* cells have been shown to penetrate watermelon (Frankle and Hopkins 1993) and melon (Silva Neto et al. 2006) fruits via stomata. However, the factors that promote entry of *A. citrulli* into fruit tissues and infection are largely unknown. The objective of this study was to characterize differences in the abilities of group I and II *A. citrulli* strains to infect and colonize melon fruit tissues.

## MATERIALS AND METHODS

### Bacterial strain and inoculum preparation.

Bacterial strains and plasmids used in this study are listed in Table 3.1. *Acidovorax citrulli* strains were routinely cultured on nutrient agar (Becton Dickinson, Sparks, MD) at 28°C for 2 days and *Escherichia coli* strains on Luria-Bertani broth (LB, VWR chemicals, Solon, Ohio) or agar at 37°C for 1 day. When required, media were supplemented with kanamycin at 50 µg/ml and diaminopimelic acid (DAP) for *E. coli* Rho5 at 200 µg/ml. Green fluorescent protein (GFP)-tagged mutants of *A. citrulli* were generated by transposon

mutagenesis as follows. The donor strain was *E. coli* Rho5 carrying the plasmid pAG408 with a mini-Tn5 transposon containing a promoterless gfp gene. The recipients were *A. citrulli* group I strains M6 and AAC213-60, and group II strains AAC00-1 and AAC94-21. Transconjugants were selected on LB amended with kanamycin (50 µg/ml) and visually screened for the green fluorescent phenotype under ultraviolet (UV) light.

To prepare *A. citrulli* inocula, strains were cultured in nutrient broth at 28°C in a rotary shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm for about 16 h. Subsequently, the cultures were centrifuged at 16,100 x g for 1 min and the supernatants were decanted. The resulting pellets were resuspended in sterilized distilled water (sd$H_2O$). The bacterial concentrations were then adjusted to an optical density of 0.3 at 600 (~ $10^8$ colony forming units (CFU/ml) spectrophotometrically (Spectronic 20; Bausch and Lomb, Rochester, NY), and adjusted to the desired inoculum level by 10-fold serial dilutions.

**Characterization of the GFP mutants of *A. citrulli*.**

GFP mutants of *A. citrulli* were confirmed as members of group I or II by polymerase chain reaction (PCR) using primer sets 550-34F/550-626R, 2708-225F/2708-433R (Table 2.1), and BX-S (Bahar et al. 2008). To confirm the pathogenicity of GFP mutants of *A. citrulli*, two-week-old watermelon (cv. 'Crimson Sweet') (Johnny's Selected Seeds, Winslow, ME) and melon (cv. 'Joaquin Gold') (Rogers Brand, Syngenta, Greensboro, NC) cotyledons were syringe infiltrated with the following strains at $10^3$ CFU/ml: group I strains M6GFP and 213-60-GFP, and their wild-type strains M6 and AAC213-60; group II strains

00-1-GFP and 94-21-GFP, and their wild-type strains AAC00-1 and AAC94-21. Four seedlings were inoculated with each strain. Seedlings inoculated with sd$H_2O$ served as negative controls. All plants were maintained in a plastic box (45 cm x 59.8 cm x 28.4 cm, IRIS USA, Inc, Pleasant Prairie, WI) in an incubator (Percival, Perry, IA) at 28°C and 100% relative humidity (RH). Pictures of melon and watermelon cotyledons were taken 4 days after infiltration. Pathogenicity tests on watermelon and melon seedlings were conducted twice.

**Plant growth for attached and detached fruit infection assays.**

To characterize differences in the abilities of group I and II *A. citrulli* strains to infect and colonize melon fruit tissues, attached and detached fruit infection assays were used. For attached fruit assays, melon (cv. Joaquin Gold) seeds were planted in pots filled with plant growth media (Sun gro® Horticulture, Agawam, MA) under greenhouse conditions. In greenhouses, the conditions were set to maintain a day temperature of 28°C and a night temperature of 22°C. The daytime temperatures reached a maximum of 38°C during the summer months. The relative humidity was approximately 80% and plants were watered daily. At anthesis, open female flowers were pollinated by rubbing pollen from the anthers of male flowers onto the stigmas of female flowers. Fruits were inoculated at 5-8 days after pollination and allowed to develop until 10 or 20 days after inoculation.

For the detached fruit assay, melon seeds were planted in a commercially available potting mix (Sun gro® Horticulture) in the greenhouse. Three to four weeks after planting, melon seedlings were transplanted to field plots at the

University of Georgia Durham Horticulture Farm, Watkinsville GA in the summer of 2019 (from April to July). Melon plants were grown on raised beds covered with plastic and immature melon fruits (diameter < 7 cm) were harvested every other day.

**Factors that influence *A. citrulli* ingress into melon fruits.**

To investigate if the representative group I and II *A. citrulli* strains differed in their ability to enter the fruit tissue by swab-inoculation on detached melon fruits, and if certain virulence factors contributed to bacterial penetration, selected wild-type and mutant strains of *A. citrulli* were tested. Immature melon fruits (diameter < 7 cm) were washed with tap water and surface-disinfected with 70% ethanol. Each melon fruit was cut into halves longitudinally and each half was placed on a 1-cm-high sterilized plastic block. A 1 x 1 $cm^2$ square was marked on the fruit rind surface and bacterial suspensions ($10^8$ CFU/ml) of strains 213-60-GFP, 94-21-GFP, M6, M6Δ*hrcV*, and M6Δ*pilA* were gently deposited on the marked area using sterilized cotton tip applicators (Puritan, Guilford, ME). The fruit halves were air-dried and incubated at room temperature for 3 h. Fruits were then surface disinfected with 0.5% NaOCl for 1 min, and rinsed with water for 1 min. The rind tissue over the sampling region was removed using a sterilized scalpel. Then, tissue samples within the marked area were excised using a sterilized cork borer (diameter 0.7 cm) (Carolina Biological Supply Company, Burlington, NC) and removed from the cork borer using a sterilized wooden stick. The fruit mesocarp tissue cores were cut 0.2 cm long from the rind end using a sterilized scalpel. For 213-60-GFP and 94-21-GFP-inoculated fruit samples,

tissues were crushed in 100 µl sd$H_2O$ and 10-fold serial dilutions of the tissue extracts were plated on LB agar plates with kanamycin. Plates were incubated at 28°C for 48 h and colony counts were converted to $Log_{10}$ CFU/fruit sample.

For M6, M6Δ*hrcV*, and M6Δ*pilA* inoculated fruit samples, tissue samples were subjected to DNA extraction using the Synergy™ 2.0 Plant DNA Extraction Kit (OPS Diagnostics LLC, Lebanon, NJ) according to manufacturer's instructions. Total DNA from each sample was concentrated into 50 µl of sd$H_2O$ and 5 µl of each sample was used for quantitative real-time PCR using the *A. citrulli*-specific primers 3979F/3979R and 3979 probe as described (Zivanovic and Walcott 2017). Cycle threshold (Ct) values less than 35 were considered positive for *A. citrulli* and were converted to $Log_{10}$ CFU/fruit sample based on a standard curve (Zivanovic and Walcott 2017).

For each experiment, each strain was inoculated onto six melon fruit halves. The experiment was conducted twice, and analysis of variance (ANOVA) was conducted on $Log_{10}$ CFU/fruit sample data using JMP statistical analysis software (version Pro 14; SAS Institute Inc., Cary, NC). The effect of strain on the $Log_{10}$ CFU/fruit sample data was compared using the Tukey-Kramer's honestly significant difference (HSD) test.

**Pathogenicity of group I and II *A. citrulli* strains on detached melon fruits after syringe-injection.**

To investigate if group I and II *A. citrulli* strains could infect melon fruit mesocarp tissues, immature (diameter < 7 cm) melon fruits were harvested from the field plots. Fruits were washed with tap water, surface-disinfected with 70%

ethanol, and placed on 1-cm-high sterilized plastic blocks. Then, fruits were injected with 10 µl bacterial suspensions ($10^8$ CFU/ml) of *A. citrulli* group I strains 213-60GFP and M6GFP4 and group II strains 94-21GFP and 00-1-GFP using a 1 ml sterilized syringe (Exelint International Co, Redondo Beach, CA) attached to a 34 gauge sterilized needle (Hems Design, Los Angeles, CA). To ensure equal injection depth, the needles were injected 1.2 cm into the fruits, and the inocula were delivered. After injection, fruits were incubated in a transparent clamshell box (Solo Container Corporation, Mason, MI) under conditions of 28°C and 100% relative humidity (RH) with continuous fluorescent light in an incubator (Percival, Perry, IA). For one experiment, each strain was inoculated into three melon fruits. At 8 days post inoculation (dpi), fruits were cut open at the inoculation point and images were captured under fluorescent light and UV light. The experiment was conducted twice.

**Differences in temporal population dynamics between group I and II *A. citrulli* strains in detached melon fruit tissues after inoculation by syringe-injection.**

To determine if *A. citrulli* group I and II strains colonized melon fruit mesocarp tissues at the same level and if the type 3 secretion system (T3SS) was important for melon fruit mesocarp colonization, detached immature melon fruits (diameter < 7 cm) were injected, as described above with 10 µl bacterial suspensions ($10^6$ CFU/ml) of *A. citrulli* strains M6, M6Δ*hrcV* (non-pathogenic T3SS mutant) (Bahar and Burdman 2010), AAC00-1, and AAC00-1Δ*hrcC* (non-pathogenic T3SS mutant) (Johnson et al. 2011). At 0 and 4 dpi, melon fruit tissue

cores were sampled (n = 3 fruits/time point/strain) using a sterilized cork borer (diameter 0.7 cm) and cut 1.2 cm long. DNA was extracted from each sample as described above and subjected to quantitative real-time PCR using *A. citrulli* specific primers, 3979F/3979R and 3979 probe as described (Zivanovic and Walcott 2017). Ct values less than 35 were considered positive for *A. citrulli* and were converted to $Log_{10}$ CFU/fruit sample based on a standard curve (Zivanovic and Walcott 2017). The experiments were conducted twice. ANOVA was conducted on $Log_{10}$ CFU/fruit sample data for each time point using JMP software. The effect of strain on $Log_{10}$ CFU/fruit sample data was compared using the Tukey-Kramer's HSD test.

**Differences in pathogenicity between group I and II *A. citrulli* strains on attached melon fruit tissues after swab-inoculation.**

To determine if there were differences in the abilities of group I and II *A. citrulli* strains to naturally penetrate and induce BFB symptoms on melon fruits, immature melon fruits (5-8 days after pollination) were inoculated with 213-60-GFP and M6-GFP (group I) and 94-21-GFP and 00-1-GFP (group II) by applying bacterial cell suspension ($10^8$ CFU/ml) to the entire fruit surface using sterilized cotton tip applicators. After inoculation, each fruit was incubated in a plastic bag for approximately 60 h to maintain high humidity followed by removal of the plastic bag and incubation under greenhouse conditions, including daily temperatures ranging from 22 to 28°C, RH of 80%, and with natural diurnal light cycles. Fruits were harvested at 10 and 20 dpi, sectioned into quarters, and visually observed under UV. Each strain was inoculated onto at least three fruits

for each time point. Fruits inoculated with sd$H_2O$ served as negative controls. The percentages of inoculated fruit that developed BFB symptoms were determined at harvest. The infection status of fruits was confirmed by bacterial isolation followed by visual observation of the GFP phenotype.

**Differences in population levels between group I and II *A. citrulli* strains after swab inoculation of attached melon fruit tissues.**

To determine if *A. citrulli* group I and II strains differed in colonizing melon fruits by the natural infection process, immature melon (cv. 'Joaquin Gold') fruits at 5-8 days after pollination were inoculated with *A. citrulli* strain M6 and strain AAC00-1 by applying bacterial cell suspension ($10^8$ CFU/ml) to the entire fruit surface using a sterilized cotton tip applicator. For experiments 1 and 2, each fruit was incubated in a plastic bag for 60 h to maintain high humidity, followed by removal of the plastic bag and incubation in the greenhouse for 20 days with natural diurnal light cycles. For experiments 3 and 4, fruits were not bagged after inoculation. Fruits were harvested at 20 dpi, and surface disinfected as described above. Fruits were cut longitudinally into halves and the halves were cut vertically into quarters and photographed. One tissue sample from each quarter was excised using a sterilized cork borer (diameter 0.7 cm) and removed from the cork borer using a sterilized wooden stick. The fruit tissue cores were cut to 1.0 cm long from the rind end using a sterilized scalpel. DNA was extracted from the four fruit tissue core samples from three fruits per strain as described above and used for real-time PCR using *A. citrulli* specific primers 3979F/3979R and 3979 probe (Zivanovic and Walcott 2017) to determine bacterial population. The percentages of fruits that

exhibited symptoms of water-soaked lesions, yellow halos, and internal lesions were also recorded. The experiment was conducted four times. The student t-test was conducted on *A. citrulli* population data using JMP.

## RESULTS

### Characterization of the GFP mutants of *A. citrulli*.

All four GFP-expressing mutants generated for this study were confirmed as *A. citrulli* by PCR assay using the BX-S primer set. When DNA from these strains were subjected to PCR assay using primer sets 550-34F/550-626R, and 2708-225F/2708-433R, the group I strains M6-GFP and 213-60-GFP yielded the expected 593-bp fragment, and group II strains 00-1-GFP and 94-21-GFP yielded the expected 209-bp fragment. The GFP strains were pathogenic on watermelon and melon cotyledons (Fig 3.1). No symptoms developed on seedlings inoculated with $sdH_2O$.

### Factors that influence *A. citrulli* ingress into melon fruits.

The ability of representative group I and II *A. citrulli* mutants to penetrate melon fruit rinds was examined. *A. citrulli* strains 213-60-GFP and 94-21-GFP did not differ in their ability to penetrate melon rinds by 3 h after swab-inoculation (Fig 3.2A). The average $Log_{10}$ *A. citrulli* populations per fruit sample for 213-60-GFP and 94-21-GFP were 3.28 and 1.94, respectively.

The ability of several previously characterized *A. citrulli* mutants to penetrate melon fruit rinds was also examined. M6Δ*hrcV* and M6Δ*pilA* entered fruits at levels similar to that of the wild-type M6 strain (Fig 3.2B). The average *A.*

*citrulli* population levels per fruit sample for M6, M6Δ*hrcV*, and M6Δ*pilA* were 3.40, 4.43, and 2.57, respectively.

**Pathogenicity of group I and II *A. citrulli* strains on detached melon fruits after syringe-injection.**

Both representative group I and II strains colonized melon fruit mesocarp tissues after syringe-inoculation. More specifically, the GFP signal was visible in the melon mesocarp as early as 1 dpi, even though BFB symptoms were not visible. All four tested strains produced fruit tissue lesions and exhibited GFP in the melon mesocarp tissue at 8 dpi (Fig 3.3A). In addition, the T3SS mutants M6Δ*hrcV* and 00-1Δ*hrcC* did not produce BFB symptoms while their wild-type strains M6 and AAC00-1 did (Fig 3.3B).

**Differences in temporal population dynamics between group I and II *A. citrulli* strains in detached melon fruit tissues after inoculation by syringe-injection.**

To determine if there were differences in the abilities of group I and II *A. citrulli* strains to colonize melon fruit mesocarp tissues, and if the T3SS is important for the fruit mesocarp tissue colonization, bacterial populations in inoculated fruits were measured at 0 and 4 dpi. At 0 dpi (right after inoculation), all strains were at similar population levels. The average $Log_{10}$ CFU per fruit sample for 00-1Δ*hrcC*, AAC00-1, M6Δ*hrcV*, M6 were 4.79, 5.16, 5.07, and 4.56, respectively. By 4 dpi, *A. citrulli* strains M6 and AAC00-1 reached similar population levels. The average $Log_{10}$ CFU per fruit sample for M6 and AAC00-1 was 8.91 and 8.79, respectively. However, populations of the T3SS mutants

M6Δ*hrcV* and 00-1Δ*hrcC* were significantly lower compared to their respective wild-type strains (Fig 3.4). The average $Log_{10}$ CFU per fruit sample for M6Δ*hrcV* and 00-1Δ*hrcC* was 5.81 and 6.91, respectively.

**Differences in pathogenicity between group I and II *A. citrulli* strains on attached melon fruit tissues after swab inoculation.**

The group I GFP strains penetrated and caused BFB symptoms on melon fruits as early as 7 dpi. In total, 33.3% (2/6) and 77.8% (7/9) of fruit samples inoculated with the group I strains were symptomatic at 10 and 20 dpi, respectively. In general, GFP signals were associated with distinctive BFB lesions, and lesions were most often associated with a rind crack on melon fruits. In contrast, only 16.7% (1/6) and 37.5% (3/8) of the melon fruit samples inoculated with the group II GFP strains were symptomatic at 10 and 20 dpi, respectively.

**Differences in population levels between group I and II *A. citrulli* strains after swab inoculation of attached melon fruit tissues.**

In experiments 2 and 3, the population levels of M6 and AAC00-1 were not significantly different. In contrast, in experiments 1 and 4, M6 grew significantly higher than AAC00-1 (Fig 3.5). For each experiment, the average $Log_{10}$ CFU per fruit sample for M6 and AAC00-1 were approximately 8 and 7, respectively. In total, all 12 M6 inoculated fruits showed surface lesions and internal lesions, and 10 of them showed yellow halos on the rind surfaces. For AAC00-1 inoculated melon fruits (n=12), all 100% showed surface lesions, 11 showed internal lesions, and 1 showed yellow halos.

## DISCUSSION

The natural association of group I and II *A. citrulli* strains with melon and watermelon fruits strongly suggests host preference and tissue specificity. Additionally, in field experiments, we observed significant differences in BFB incidence between representative group I and II *A. citrulli* strains on melon and watermelon fruits. However, to date, no studies have explored the mechanisms of *A. citrulli* cucurbit host preference. Hence, we aimed to elucidate the mechanisms of host preference among *A. citrulli* groups, with an emphasis on melon fruits.

To cause BFB symptoms on susceptible melon fruits, *A. citrulli* must complete the following three steps: 1) land on the melon fruit rind and penetrate the mesocarp through openings, 2) colonize melon fruit tissues, and 3) induce BFB symptoms. Hence, we compared the abilities of group I and II *A. citrulli* strains to penetrate, colonize, and induce symptom development in melon fruit tissues.

Most plant pathogenic bacteria penetrate their hosts through stomata. Silva Neto et al. (2006) suggested that *A. citrulli* penetrated melon fruits through stomata and lenticels. Additionally, *A. citrulli* may enter the melon fruits through wounds. In the current study, we observed that group I and II *A. citrulli* strains did not differ in their ability to penetrate melon fruit tissues with swab inoculation. Three-hour after swab-inoculation of detached immature melon fruits, the representative group I and II strains showed similar population levels in the fruit mesocarp tissues (0.2 cm depth). This indicates that group I and II *A. citrulli*

strains did not differ in their ability to penetrate melon fruit rinds and enter the mesocarp tissues.

With regards to the ability to colonize melon mesocarp tissues, both group I and II *A. citrulli* strains reached similar population levels at 4 dpi and induced BFB symptoms after syringe inoculation. We also found that the T3SS was required for both groups to colonize melon mesocarp tissues after syringe injection. The syringe-injection method introduced the bacterial cells directly into fruit mesocarp, which bypassed the natural infection process. This might explain why we did not see a difference between M6 and AAC00-1 in fruit mesocarp colonization.

After swab-inoculation on attached melon fruits, AAC00-1 always reached relatively lower populations compared to M6 at 20 dpi. In addition, we swabbed high inoculum concentration ($10^8$ CFU/ml) over the entire fruit surfaces. We may see a more dramatic difference between group I and II colonization ability using a lower concentration. We speculate that during the natural fruit infection process, group II *A. citrulli* strains may not have the capacity to grow to high concentrations to induce distinctive BFB symptoms.

We explored the abilities of group I and II strains to induce symptom development on melon fruit tissues. After swab-inoculation of attached melon fruits, group I strains produced more severe symptoms and sooner. Group II strains also induced BFB symptoms at 10 and 20 dpi, but at a lower frequency. This indicated that the group I strains were more aggressive than group II regarding symptom development on melon fruit rind. Yan et al. (2017) reported

that by pin-prick inoculation of detached immature melon (cv. Joaquin Gold) fruits, only group I strains induced water-soaked symptoms. The discrepancy between that study and the results from this study could be due to the fact that melon fruit symptoms were recorded at 8 dpi in the detached fruit assay. It is possible that group II *A. citrulli* strains can cause disease after swab inoculation of melon fruits, but it may require an incubation period of more than eight days.

To elucidate the mechanisms of *A. citrulli* host preference, we investigated the factors that affect bacterial entry into melon fruit tissues. In preliminary experiments, we found that M6Δ*pilA* entered melon fruits significantly less than wild-type M6, by serial dilutions and agar plating. However, when we repeated the experiment with a qPCR assay 3 h after swab-inoculation, the populations were not significantly different between M6Δ*pilA* and M6. The counts from agar plating for M6Δ*pilA* ranged from 0-5 CFU/sample, compared to 0-250 CFU/sample for M6. This means qPCR may be more sensitive than agar plating when the concentrations were relatively low.

In the current study, we observed that a non-pathogenic group I *A. citrulli* strain (M6Δ*hrcV*) entered melon fruits at levels similar to those of the wild-type M6 strain. Similarly, the *Xanthomonas campestris* pv. *campestris* (*Xcc*) *hrp* mutant that carries an approximately 20 kb deletion of its *hrp* gene cluster, was unaffected in hydathode colonization. This indicated that entry into plants could be dissociated from other pathogenicity attributes (Hugouvieux et al. 1998). However, *rpf* (regulation of pathogenicity factors) mutant of *Xcc* showed reduced entry ability, suggesting members of the *rpf* regulon contributed to *Xcc* plant

tissue entry. The *rpf* mutants are characterized by the down-regulation of the synthesis of extracellular enzymes and polysaccharide (Hugouvieux et al. 1998). In addition, the *rfaX* mutant of *Xcc*, which has defects in lipopolysaccharide (LPS) synthesis, was severely impaired in hydathode colonization (Hugouvieux et al. 1998). It would be interesting to test whether there are differences among *A. citrulli* strains regarding LPS composition and whether LPS plays a role in *A. citrulli* strains interactions with melon fruit tissues during initial penetration and colonization.

Based on the results of this study, we conclude that the abilities to penetrate, colonize, and induce symptom development do not account for differences in host preference of group I and II *A. citrulli* strains on melon fruits. Further studies are needed to uncover the mechanisms of host preference of *A. citrulli.*

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Table 3.1 Strains and plasmids used in this study.

| Strains and plasmids | Relevant characteristics | Reference or source |
|---|---|---|
| *Acidovorax citrulli* (AAC) | | |
| AAC00-1 | Wild-type, group II | R. Walcott collection |
| 00-1-GFP | Random GFP insertion mutant, $Km^R$, $Gm^R$ | R. Walcott collection |
| 00-1Δ*hrcC* | *hrcC* deletion mutant | (Johnson et al. 2011) |
| 94-21 | Wild-type, group II | R. Walcott collection |
| 94-21-GFP | Random GFP insertion mutant, $Km^R$, $Gm^R$ | This study |
| M6 | Wild-type, group I | (Burdman et al. 2005) |
| M6-GFP | Random GFP insertion mutant, $Km^R$, $Gm^R$ | This study |
| M6Δ*hrcV* | *hrcV* deletion mutant | (Bahar and Burdman 2010) |
| M6Δ*pilA* | Mutant defected in the gene encoding type 4 pilin, impaired in twitching motility; $Km^R$ | (Rosenberg et al. 2018) |
| 213-60 | Wild-type, group I | R. Walcott collection |
| 213-60-GFP | Random GFP insertion mutant, $Km^R$, $Gm^R$ | This study |
| *Escherichia coli* | | |
| Rho5 | attTn7::*pir116*$^+$ *thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA* integrated RP4-2 $Tc^r$::Mu (λ*pir*$^+$)Δ*asd*::*FRT* Δ*aphA*::*FRT* | (Kvitko et al. 2012) |
| Plasmid | | |
| pAUG408 | mini-Tn5 transposon containing a promoter-less gfp gene | (Walcott et al. 2003) |

$Km^R$ and $Gm^R$ indicate resistant to kanamycin and gentamycin, respectively.

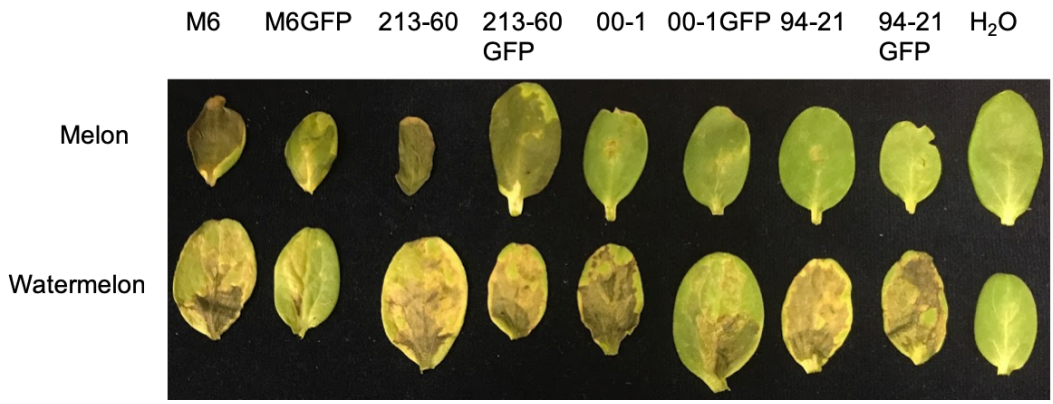


Fig 3.1 The virulence of *Acidovorax citrulli* wild-type and GFP mutants on two-week-old watermelon (cv. 'Crimson Sweet') and melon (cv. 'Joaquin Gold') cotyledons. Plants were syringe-infiltrated with *A. citrulli* wild-type and GFP mutants at $10^3$ CFU/ml. Plants inoculated with sterilized water served as negative controls. Each treatment was inoculated on four watermelon and melon plants. Plants were incubated at 28°C. Pictures were taken 4 days after inoculation. The experiment was conducted twice with similar results.

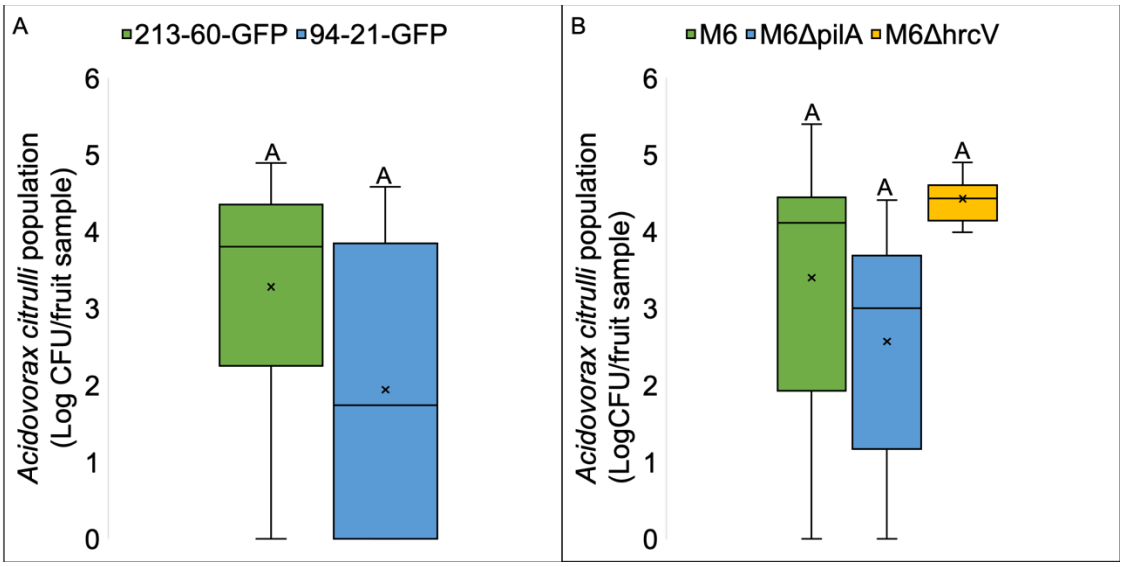


Fig 3.2 Box plot of *Acidovorax citrulli* populations at 3 h post swab-inoculation on melon (cv. Joaquin Gold) fruit tissues. A) Group I strain 213-60-GFP and group II strain 94-21-GFP were swab-inoculated at $10^8$ CFU/ml onto detached melon fruits (n=6). At 3 h post inoculation, fruits were surface disinfected with 0.5% NaOCl for 1 min. Then, the rind tissues were cut out. Tissue samples (diameter 0.7 cm, length 0.2 cm) were taken and crushed in sterilized $H_2O$ and plated on LB agar amended with kanamycin. Colonies were counted 48 h after incubation and converted to $Log_{10}$ CFU/fruit sample. B) Group I strains M6, M6 Δ*hrcV*, and M6 Δ*pilA* were swab-inoculated at $10^8$ CFU/ml onto detached melon fruits (n=6). Samples were processed as described above but subjected to DNA extraction followed by real-time PCR to determine *A. citrulli* population. 'x' represents the means and different letters indicate significant differences (P = 0.05) among treatments according to Tukey-Kramer's honestly significant difference test. The experiment was conducted twice with similar results.

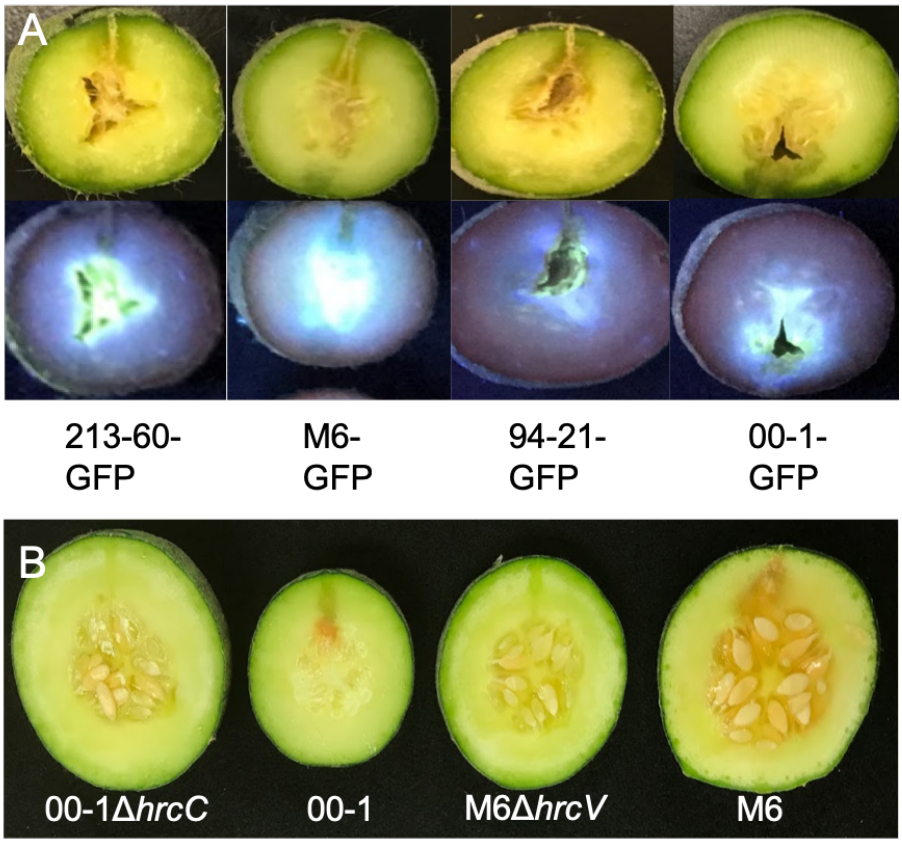


Fig 3.3 Bacterial fruit blotch symptoms observed on melon fruit mesocarp at 8 days post syringe-inoculation ($10^8$ CFU/ml). A) *Acidovorax citrulli* group I strains 213-60-GFP and M6-GFP and group II strains 94-21-GFP and 00-1-GFP. B) group I strains M6, M6Δ*hrcV*, group II strain 00-1, and 00-1Δ*hrcC*. Each treatment was inoculated on three melon fruits. The experiment was conducted twice with similar results

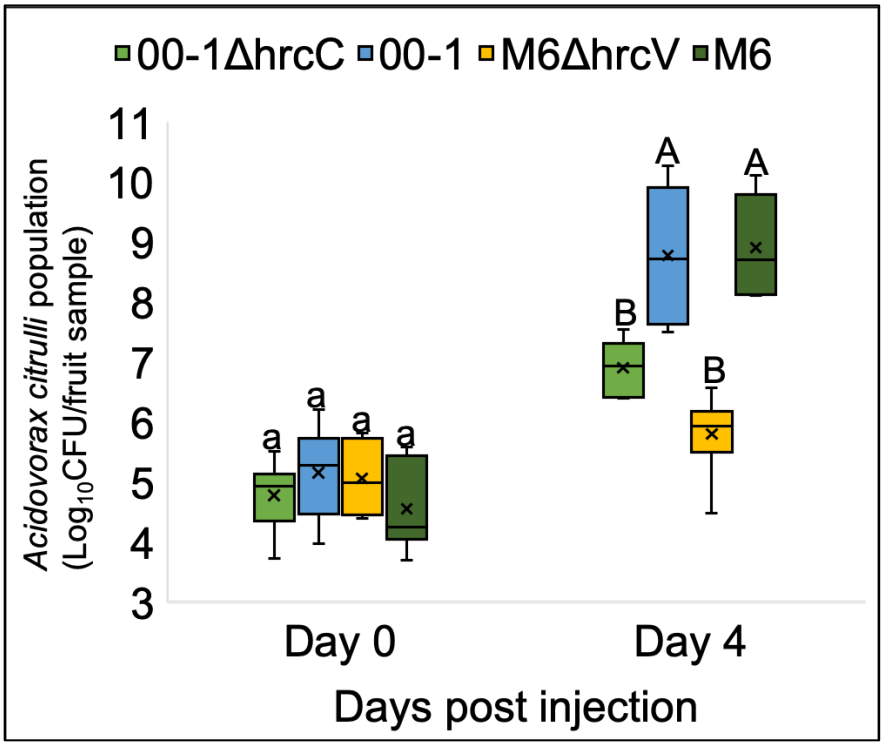


Fig 3.4 Role of the type 3 secretion system in *Acidovorax citrulli* melon (cv. 'Joaquin Gold') fruit colonization after syringe inoculation. Group I strains M6, M6 Δ*hrcV*, and group II strains 00-1, and 00-1Δ*hrcC* were injected (~ $10^4$ CFU) into melon fruit mesocarp. At 0 and 4 dpi, three samples (diameter 0.7 cm, length 1.2 cm) for each treatment were collected for DNA extraction followed by real-time PCR to determine *A. citrulli* population. 'x' represents the means. Different letters indicate significant differences (P = 0.05) among treatments according to Tukey-Kramer's honestly significant difference test. The experiment was conducted twice with similar results.

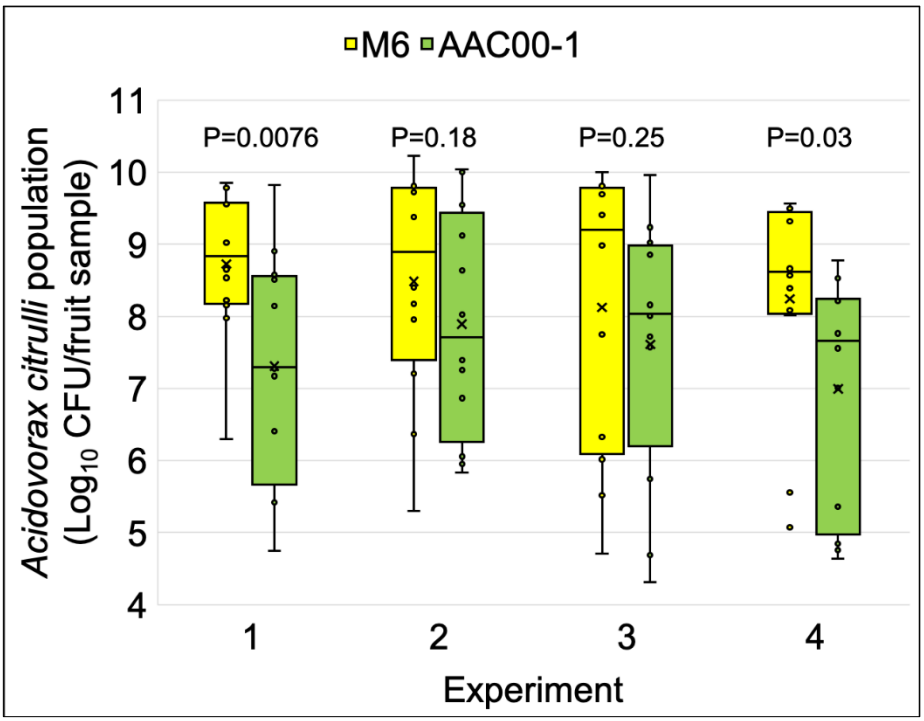


Fig 3.5 Box plot of *Acidovorax citrulli* populations at 20 days post swab-inoculation (dpi) on melon (cv. 'Joaquin gold') fruit tissues. Group I strain M6 and group II strains AAC00-1 were swab-inoculated at $10^8$ CFU/ml onto attached melon fruits in the greenhouse. At 20 dpi, four samples (diameter 0.7 cm, length 1.0 cm) from three fruits for each treatment were collected for DNA extraction followed by real-time PCR to determine *A. citrulli* population. The experiment was conducted four times. 'x' represents the means. P values according to Student t-test were labeled for each experiment.

# CHAPTER 4

# ROLES OF XOPJ AND HOPAF HOMOLOGS IN *ACIDOVORAX CITRULLI* VIRULENCE [1]

[1] Zhao, M., and Walcott R. R. To be submitted to *Phytopathology.*

## ABSTRACT

*Acidovorax citrulli* causes bacterial fruit blotch (BFB) of cucurbits. Despite the economic importance of BFB, the knowledge about the basic aspects of *A. citrulli*-plant interactions is relatively limited. Like many Gram-negative plant pathogenic bacteria, *A. citrulli* pathogenicity requires a functional type III secretion system to deliver effectors into the host cells. The objective of this study was to characterize the roles of XopJ and HopAF homologs in *A. citrulli* virulence. We did not find detectable effects of XopJ homologs on *A. citrulli* virulence on watermelon and melon cotyledons. The deletion mutant of XopJ homologs was less virulent on attached melon fruits compared to the wild-type, while their population levels on melon fruits were variable across experiments. However, the HopAF homolog may be important for *A. citrulli* colonization and symptom development on watermelon and melon cotyledons. Further characterization of the mechanisms by which these effectors interact with different hosts is needed.

## INTRODUCTION

Bacterial fruit blotch (BFB) is a disease that affects plant species in the family Cucurbitaceae, including watermelon and melon. BFB is caused by the Gram-negative bacterium, *Acidovorax citrulli* and in the early 1990s, it caused significant economic losses in US watermelon production (Latin and Rane 1990; Somodi et al. 1991). Subsequently, BFB outbreaks have occurred in many regions around the world, primarily on watermelon and melon crops, but also on other cucurbitaceous crop species (Burdman and Walcott 2012). Great efforts

have been expended to mitigate the impact of BFB, unfortunately, the efficacy of the current management approaches has been limited and BFB still poses a serious threat to commercial watermelon and melon production worldwide (Burdman and Walcott 2012).

*Acidovorax citrulli* strains can be divided into two distinct groups (I and II) based on carbon substrate utilization (Walcott et al. 2004), DNA fingerprint analysis (Burdman et al. 2005; Walcott et al. 2000; Yan et al. 2013), multilocus sequence analysis (Feng et al. 2009a; Yan et al. 2013), and fatty acid methyl ester analysis (Walcott et al. 2000). Comparative genome analysis revealed that the genome of the representative group I strain M6 is 0.5 Mb smaller than that of the group II strain AAC00-1 (Eckshtain-Levi et al. 2016). This difference was primarily explained by the presence of eight fragments, dispersed throughout the AAC00-1 genome (Eckshtain-Levi et al. 2016). The two *A. citrulli* groups also differ in their virulence on different cucurbit species (Walcott et al. 2004). For example, group I strains are moderately to highly aggressive on watermelon, melon, pumpkin, and squash while group II strains are highly aggressive on watermelon, but weakly aggressive on other cucurbit species (Walcott et al. 2004). Despite this, the mechanisms involved in *A. citrulli* host preference and virulence have not been elucidated.

Like many Gram-negative plant pathogenic bacteria, *A. citrulli* requires a hrp (hypersensitive response and pathogenicity) type III secretion system (T3SS) that translocates bacterial effector proteins directly into plant host cells (Bahar and Burdman 2010; Johnson et al. 2011). In general, type 3 secreted effectors

(T3Es) act as virulence factors by modifying host cellular targets, but can be recognized as avirulence factors if detected by cognate plant resistance receptors (Jones and Dangl 2006). Both group I and II *A. citrulli* strains require a functional T3SS for pathogenicity on cucurbit hosts (Bahar and Burdman 2010; Johnson et al. 2011).

Despite the economic importance of BFB, relatively little is known about the genetic and molecular determinants of *A. citrulli* virulence. Knowledge of these determinants may reveal important clues about evolutionary drivers of this bacterium and eventually lead to effective disease mitigation strategies. To date, there are no reliable sources for BFB host plant resistance, and the efficacy of chemical control is limited. Using unique T3Es from *A. citrulli* strains to identify resistance genes in host plants could be an effective approach for controlling BFB.

Group I and II *A. citrulli* strains differ in the arsenals of putative T3Es that they possess. Most notably, group II strains possess three putative T3Es (*Aave_2166*, *Aave_2708*, and *Aave_2938*; locus tags are from the annotation of AAC00-1, GenBank CP000512.1) that are truncated or absent in group I strains. These effectors belong to the YopJ/AvrRxv family. *Acidovorax citrulli* putative T3E genes *Aave_2708* and *Aave_2938* have the same nucleotide sequence with 99% identity to the *xopJ* effector gene from *Xanthomonas campestris* pv. *vesicatoria*. *Aave_2166* encodes a protein that is homologous to the *X. euvesicatoria* AvrBsT effector with 61% identity. Of eight group I *A. citrulli* strains tested, all had open reading frame (ORF) shifts (a 123-bp deletion) in

*Aave_2166,* and lacked *Aave_2708* and *Aave_2938* (Eckshtain-Levi et al. 2014). The first objective of this study was to determine the roles of XopJ homologs in group II *A. citrulli* virulence and host range.

Recently, Jiménez Guerrero et al. (2019) used sequence similarity analysis, machine learning, and transcriptomics data analysis to identify 58 T3Es in *A. citrulli* group I strain M6. In my initial screen of 49 T3Es from the AAC00-1 genome, *Aave_1373 (*HopAF homolog) and *Aave_4427* (HopBD homolog) were the only T3Es that occurred in *A. citrulli,* but not in other *Acidovorax* species. We also found all 20 *A. citrulli* genomes screened in silico contain a homolog of HopAF with a 100% sequence identity. Because HopAF homolog was initially considered as a unique effector in *A. citrulli* and also conserved among *A. citrulli* strains, we chose to characterize it. However, a more recent screen showed that genomes of *A. avenae* and *A. anthurii* also contain HopAF homologs. The second objective of this study was to determine the roles of HopAF homolog in *A. citrulli* virulence.

## MATERIALS AND METHODS

### Bacterial strains and inoculum preparation.

Bacterial strains and plasmids used in the study are listed in Table 4.1. *Acidovorax citrulli* strains were routinely cultured on nutrient agar (Becton Dickinson, Sparks, MD) at 28°C for 2 days and *Escherichia coli* strains on Luria-Bertani broth (LB, VWR chemicals, Solon, Ohio) or agar at 37°C for 1 day. When required, media were supplemented with kanamycin (Km) at 50 µg/ml,

tetracycline at 10 µg/ml, gentamycin at 100 µg/ml, and diaminopimelic acid (DAP) for *E. coli* Rho5 at 200 µg/ml. To prepare *A. citrulli* inocula, strains were cultured in nutrient broth at 28°C in a rotary shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm for approximately 16 h. Subsequently, the cultures were centrifuged at 16,100 x g for 1 min and the supernatants were decanted. The resulting pellets were resuspended in sterilized distilled water (sd$H_2O$). The bacterial concentrations were then adjusted to an optical density of 0.3 at 600 (~ $10^8$ colony forming units (CFU/ml) spectrophotometrically (Spectronic 20; Bausch and Lomb, Rochester, NY), and adjusted to the desired concentration by 10-fold serial dilutions.

**Deletion of XopJ homologs in the *A. citrulli* group II strain AAC00-1.**

To investigate the role of XopJ homologs in *A. citrulli* virulence and host preference, deletion mutants were generated. A deletion mutant of *Aave_2166* was made in AAC00-1 by G.V. Minsavage (University of Florida) as follows. A 2,968 bp fragment was amplified using primer set 2166JF/2166JR (Table 4.2) and cloned into pGEMT-easy. From the clone, a central fragment of 1,278 bp containing the entire *Aave_2166* ORF was excised using *Hind*III. The clone was re-circularized by ligation and the deleted fragment was cut out using *Apa*I/*Spe*I and cloned into the pOK1 vector digested with *Apa*I/*Xba*I. The pOK1 clone was then used for conjugation into AAC00-1 for the final mutation steps to generate 00-1Δ2166.

A XopJ double mutant (deletions of *Aave_2166* and *Aave_2708*) was made by XiaoXiao Zhang (Chinese Academy of Agricultural Sciences) based on

00-1Δ2166 using suicide plasmid pk18*mobsacB*. To accomplish this, the *Aave_2708* flanking sequences (597 bp and 585 bp) were amplified from AAC00-1 DNA using primer sets 2708-1F/2708-1R and 2708-2F/2708-2R (Table 4.2) as described by Zhang et al. (2018).

A XopJ triple mutant (deletions of *Aave_2166, Aave_2708,* and *Aave_2938*) was generated using the p3692 vector based on *A. citrulli* XopJ double mutant (deletions of *Aave_2166* and *Aave_2708* in AAC00-1). To accomplish this, the *Aave_2938* flanking sequences (1,000 bp each) and FRT marker were amplified from AAC00-1 DNA using primer sets 2938Up-FWD/2938Up-REV, 2938Down-FWD/2938Down-REV, and FRT-FWD/FRT-REV (Table 4.2) and Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, MA). The amplicons were ligated and cloned into the vector p4916 between *Bam*HI and *Sal*I restriction sites using Gibson Assembly® Master Mix (NEB) to generate p4916-2938. The Gibson reaction mixture was transformed into *E. coli* strain DH5α and the resulting transformants were screened for the presence of 2938Up and 2938Down fragments using colony PCR. The plasmids were extracted from overnight cultures inoculated with putative transformants using the EZNA® Plasmid Mini Kit (Omega Biotek, Norcross, GA) and confirmed by sequencing with primers M13F and M13R (Eurofins Genomics). Then, the LR reaction was performed by mixing the p4916-2938 and p3692 with the LR Clonase™ II enzyme mix (Invitrogen, Carlsbad, CA) overnight, and then transformed into *E. coli* strain DH5α and plated on LB amended with tetracycline and kanamycin. The resulted plasmid p3692-2938 was transformed into *E. coli* strain Rho5 and

plated on LB amended with DAP, tetracycline, and kanamycin. Bi-parental mating was carried out by plating a 1:1 mixture of *E. coli* strain Rho5::p3692-2938 and the *A. citrulli* XopJ double mutant on the LB agar with DAP. After 24 h, the mixed culture was streaked onto LB amended with kanamycin, and the resulted colonies were patched onto LB with kanamycin and LB with tetracycline. The colonies that grew only on LB amended with kanamycin were further confirmed by colony-PCR using primer set 2938Up-FWD/2938Down-REV. AAC00-1 wild-type DNA amplified with this primer set produced a 3,122 bp band and DNA from the mutant, designated as 00-1ΔxopJTriple, produced two amplicons of sizes 2,040 bp and 3,440 bp.

To generate a complemented strain of 00-1ΔxopJTriple, the full-length sequences of *Aave_2166* and *Aave_2708* including their putative promoter regions were PCR amplified with primer set 2166CompF/2166CompR and 2708CompF/2708CompR, respectively, using Phusion® High-Fidelity DNA Polymerase. The fragments were inserted into pBBR1MCS-5 between the *EcoR*I and *Sal*I restriction sites using Gibson Assembly® Master Mix to generate pBBR1MCS5_2166Comp and pBBR1MCS5_2708Comp. The plasmid was transformed into *E. coli* strain DH5α, confirmed by sequencing with the M13F and M13R primers, and transformed into 00-1ΔxopJTriple via *E. coli* strain Rho5 as described above. The transformants were selected on LB agar amended with kanamycin and gentamycin and confirmed by colony PCR.

**The role of XopJ in *A. citrulli* colonization of watermelon and melon cotyledons.**

To determine the role of XopJ in virulence, the ability of *A. citrulli xopJ* triple deletion mutant to colonize on watermelon and melon seedlings was tested. Two-week-old watermelon (cv. Crimson Sweet) (Johnny's Selected Seeds, Winslow, ME) and melon (cv. Joaquin Gold) (Rogers Brand, Syngenta, Greensboro, NC) cotyledons were syringe-infiltrated with $10^4$ CFU/ml of bacterial suspensions of AAC00-1 and 00-1ΔxopJTriple. Plants were maintained in a plastic box (45 cm x 59.8 cm x 28.4 cm, IRIS USA, Inc, Pleasant Prairie, WI) in an incubator (Percival, Perry, IA) at 28°C and 100% relative humidity (RH) for three days. Cotyledons (n = 3/time point/strain) were sampled at 0, 2, 3 days after infiltration. Total DNA was extracted from two leaf discs (diameter 0.7 cm) of inoculated cotyledons using Synergy™ 2.0 Plant DNA Extraction Kits (OPS Diagnostics LLC, Lebanon, NJ). DNA samples were eluted in 50 µl sd$H_2O$ and 5 µl of each sample was used for quantitative real-time PCR using *A. citrulli* specific BOX primers and probe as previously described (Ha et al. 2009). Cycle threshold (Ct) values less than 35 were considered positive for *A. citrulli* and were converted to $Log_{10}$ CFU/leaf discs based on a standard curve. The experiment was conducted twice. The student t-test was conducted on the *A. citrulli* concentration data using JMP statistical analysis software (version Pro 14; SAS Institute Inc., Cary, NC).

**The role of XopJ in *A. citrulli* colonization of attached melon fruits after swab inoculation assay.**

To determine the role of XopJ homologs on melon fruits by the natural infection process, immature melon (cv. 'Joaquin Gold') fruits at 5-8 days after pollination were inoculated with *A. citrulli* strain 00-1 and 00-1ΔxopJTriple in experiments 1 to 4 by applying bacterial suspension ($10^8$ CFU/ml) to the entire fruit surface using sterilized cotton tip applicators (Puritan, Guilford, ME). In experiments 5 and 6, 00-1ΔxopJTriple complemented with *Aave_2166* (XopJTM+2166), and 00-1ΔxopJTriple complemented with *Aave_2708* (XopJTM+2708) were included. For experiments 1 and 2, each fruit was incubated in a plastic bag for 60 h to maintain high humidity, followed by removal of the plastic bag and incubation for 20 days under greenhouse conditions including daily temperatures ranging from 22 to 28°C, RH of 80%, and with natural diurnal light cycles. For experiments 3 to 6, fruits were not bagged after inoculation. Fruits were harvested at 20 dpi, and surface disinfected with 0.5% NaOCl using sterilized cotton balls. Fruits were cut longitudinally into halves and the halves were cut vertically into quarters, and photographed. One tissue sample from each quarter was excised using a sterilized cork borer (diameter 0.7 cm) and removed from the cork borer using a sterilized wooden stick. The fruit tissue cores were cut to 1.0 cm long from the rind end using a sterilized scalpel. Four fruit tissue core samples per fruit from three fruits for each strain were collected and subjected to DNA extraction as described above. DNA was used for real-time PCR using *A. citrulli* specific primers 3979F/3979R and 3979 probe

(Zivanovic and Walcott 2017) to determine *A. citrulli* population. Percentages of fruits exhibited symptoms of watersoaked lesions, yellow halos, and internal lesions were also recorded. The experiment was conducted six times. The student t-test was conducted on *A. citrulli* population data from two treatments from experiments 1 to 4. Analysis of variance (ANOVA) was conducted for data from four treatments from experiments 5 and 6 using JMP.

**Deletion of HopAF homolog in *A. citrulli* group II strain A5 and group I strain M6.**

To determine the role of the HopAF homolog in *A. citrulli* virulence, deletion mutants from group II strain A5 and group I strain M6 were generated using the pK18*mobsacB* suicide vector. Group II strain A5 was used as it was relatively more genetically tractable than strain AAC00-1. HopAF deletion in strain A5 was made by Linlin Yang (Chinese Academy of Agricultural Sciences) as described by Zhang et al. (2018) using primer sets 1373-1F/1373-1R and 1373-2F/1373-2R (Table 4.2). The primer sequences were designed according to the *Aave_1373* gene from the AAC00-1 genome (GenBank accession CP000512.1). A5ΔHopAF::Complement was made using procedures as described above using pBBR1MCS-5 and primer sets 1373CompF/1373CompR and confirmed by sequencing.

HopAF (locus tag *APS58_3109* from M6 chromosome; GenBank accession CP029373.1) was deleted in strain M6 as described above with a few modifications. The HopAF flanking sequences (700 bp each) were amplified from M6 DNA with primer pairs 1373A-FWD/1373A-REV and 1373B-FWD/1373B-

REV (Table 4.2) using Phusion® High-Fidelity DNA Polymerase. The amplicons were ligated and cloned into pK18*mobsacB* between the *EcoR*I and *Sal*I restriction sites using Gibson Assembly® Master Mix to generate pK18ΔHopAF-M6. The plasmid was transformed into *E. coli* strain DH5α and plated on LB Km X-gal IPTG medium for blue-white screening. Plasmids were extracted from overnight cultures inoculated with white transformants using EZNA® Plasmid Mini Kit and the transformant was confirmed by sequencing with primers M13F and M13R. The pK18ΔHopAF-M6 was transformed into *E. coli* strain Rho5 and plated on LB amended with DAP and kanamycin. Bi-parental mating was carried out by plating a 1:1 mixture of *E. coli* strain Rho5::pK18ΔHopAF-M6 and M6 on the LB with DAP. After 24 h, the mixed culture was streaked onto LB with kanamycin to select for first crossover products. A single colony from the resulting first-crossover products was cultured overnight in LB broth to allow for second-crossover events. One hundred microliters of the overnight culture and a 10-fold dilution of the culture were plated on M9 agar medium (Sambrook et al. 1989) amended with 0.4% sodium citrate and 10% sucrose. After 3 days of incubation at 28°C, colonies from the M9 plates were patched onto LB with and without kanamycin. The colonies that grew only on LB were further screened by colony-PCR using primer set 1373OutF2/1373OutR2. M6 wild-type DNA amplified with primer set 1373OutF2/1373OutR2 produced a 2,278 bp band and DNA from the mutant (designated as M6ΔHopAF) produced a 1,645 bp band.

**Effect of HopAF on *A. citrulli* colonization and virulence on watermelon and melon cotyledons.**

To determine the role of HopAF homolog in *A. citrulli* virulence, the ability of *A. citrulli* mutants to colonize on watermelon and melon seedlings was tested as described above with slight modifications. Strains A5 and A5ΔHopAF were syringe-infiltrated into melon and watermelon cotyledons at $10^3$ CFU/ml and *A. citrulli* populations were determined at 3 dpi. On the other hand, the virulence tests for strains M6 and M6ΔHopAF were conducted separately from A5 strains and a lower concentration was used. They were syringe-infiltrated into melon and watermelon cotyledons at $10^2$ CFU/ml and *A. citrulli* populations were determined at 4 dpi. Cotyledons were photographed and BFB severity (lesion area/ total cotyledon area x100) (%) was determined using ImageJ (NIH, Bethesda, MD). Three samples (leaf disc diameter = 0.7 cm) from each treatment were collected for DNA extraction followed by real-time PCR using *A. citrulli* specific primers 3979F/3979R and 3979 probe (Zivanovic and Walcott 2017) to determine *A. citrulli* population. The experiment was conducted at least twice. ANOVA was conducted on *A. citrulli* concentration and disease severity data using JMP. The effect of treatment was compared using the Tukey-Kramer's honestly significant difference test.

**RESULTS**

**Effect of XopJ on *A. citrulli* colonization of watermelon and melon cotyledons.**

XopJ triple mutant and wild-type AAC00-1 grew to similar population

levels on watermelon and melon seedlings (Fig 4.1). The average $Log_{10}$ CFU per leaf disc for AAC00-1 and Δ*xopJ*Triple on watermelon cotyledons were 4.61 and 4.76 at 0 dpi (P = 0.65), 8.82 and 8.77 at 2 dpi (P =0.45), and 9.05 and 9.14 at 3 dpi (P=0.09), respectively. The average $Log_{10}$ CFU per leaf disc for AAC00-1 and ΔxopJTriple on melon cotyledons were 4.42 and 4.88 at 0 dpi (P = 0.11), 8.79 and 8.93 at 2 dpi (P =0.69), and 9.16 and 9.35 at 3 dpi (P=0.38), respectively.

**Effect of XopJ on *A. citrulli* colonization of attached melon fruits after swab inoculation assay.**

In experiments 1 and 2, the population levels of AAC00-1 and 00-1ΔxopJTriple at 20 dpi were not significantly different. The average $Log_{10}$ CFU per fruit sample for AAC00-1 and 00-1ΔxopJTriple were 7.6 and 7.1, respectively. In experiments 3 and 4, the XopJ triple mutant grew significantly (P = 0.001 and P < 0.0001, respectively) less than AAC00-1 (Fig 4.2A). The average $Log_{10}$ CFU per fruit sample for AAC00-1 and 00-1ΔxopJTriple were 7.3 and 4.6, respectively. However, when the two complemented strains were included (experiments 5 and 6), there was no significant difference in population levels (Fig 4.2B) among the four strains. The average $Log_{10}$ CFU per fruit sample for AAC00-1, 00-1ΔxopJTriple, XopJTM+2166, and XopJTM+2708 were 4.4, 4.0, 4.6, and 4.5, respectively.

The XopJ triple mutant was less virulent than the wild-type in terms of symptom development. For experiments 1 to 4, all (12/12) AAC00-1-inoculated fruits showed surface water-soaked lesions and 11 showed internal lesions. One fruit inoculated with AAC00-1 developed yellow halos on the rind surfaces. For

00-1ΔxopJTriple inoculated melon fruits, 45% (5/11) showed surface watersoaked lesions, 18% (2/11) showed internal lesions, and 0 showed yellow halos.

**Effect of HopAF on *A. citrulli* colonization and virulence on watermelon and melon cotyledons.**

*Acidovorax citrulli* group II strain A5ΔHopAF reached significantly lower population levels in melon cotyledons than A5, but grew similarly to the wild-type in watermelon cotyledons (Fig 4.3). The average $Log_{10}$ CFU per leaf disc for A5 and A5ΔHopAF were 9.35 and 6.80 on melon cotyledons, and 9.32 and 9.48 on watermelon cotyledons respectively. In terms of disease severity, A5ΔHopAF was less virulent on both melon (lesion area percentage = 11%) and watermelon (56%) cotyledons than A5 on melon (31%) and watermelon (91%) cotyledons. However, the complemented strain of A5ΔHopAF grew similarly as the A5ΔHopAF mutant (P = 0.15), instead of the wild-type level (Fig 4.4).

Since we were unable to complement A5ΔHopAF, we made a second HopAF homolog mutant in the *A. citrulli* group I strain M6. M6ΔHopAF grew significantly less than M6 in watermelon cotyledons, but grew similarly to the M6 in melon cotyledons. The average $Log_{10}$ CFU per leaf disc for M6 and M6Δ1373 were 5.99 and 4.00 on watermelon cotyledons, and 7.36 and 7.96 on melon cotyledons. Both M6 and M6ΔHopAF displayed low levels of virulence on watermelon cotyledons at 4 dpi (lesion area percentage = 9% and 1%, respectively), while on melon cotyledons, M6ΔHopAF was significantly less virulent (27%) than M6 (89%) (P = 0.0017).

## DISCUSSION

T3Es are translocated into host cells by the T3SS which is regulated by the hrp genes. *Acidovorax citrulli* strains contain more than 50 T3Es and are among the “richest” bacterial plant pathogens in terms of T3E arsenals (Jiménez-Guerrero et al. 2019). In this study, we examined the roles of XopJ and HopAF homologs in *A. citrulli.*

*Acidovorax citrulli* group II strains possess three unique effectors (*Aave_2166, Aave_2708,* and *Aave_2938*) that are absent or truncated in group I strains. We did not find detectable effects of XopJ homologs on melon and watermelon cotyledon colonization by *A. citrulli* after syringe infiltration. These three effectors belong to the YopJ/AvrRxv family that have cysteine proteinase or acetyltransferase enzyme activity (Lewis et al. 2011). We have subsequently found that both group I and II *A. citrulli* strains contain additional effectors that belong to the YopJ family (e.g., *Aave_0889*) or effectors that function as a cysteine proteinase (e.g., *Aave_4359,* XopD homolog). In addition, recently, group I strain M6 was reported to contain a unique XopJ-like effector gene (*APS58_1966*) which is not present in AAC00-1 (Jiménez-Guerrero et al. 2019). Virulence of plant pathogenic bacteria can be multifactorial, depending on numerous and functionally redundant T3Es (Kvitko et al. 2009). This provides a possible explanation for why the deletion of three XopJ effectors did not affect *A. citrulli* virulence on watermelon and melon seedlings.

Our results were in agreement with other studies that showed that knocking out XopJ-like effectors does not alter *in planta* bacterial population

sizes (Abrahamian et al. 2018; Noël et al. 2003). Abrahamian et al. (2018) reported that AvrBsT from the XopJ family affects the fitness of *X. perforans* only under field conditions. In the field trials, *X. perforans* wild-type strains had a significantly higher recovery rate than *avrBsT* mutant strains, and were capable of spreading longer distances across field plots compared with *avrBsT* mutant strains (Abrahamian et al. 2018).

Despite these findings, the role of XopJ homologs in *A. citrulli* colonization of melon fruit is unclear. We observed that 00-1ΔxopJTriple were less virulent on melon fruits than AAC00-1 in terms of symptom development. However, the population levels of the XopJ triple mutant were significantly less than AAC00-1 in two out of six experiments. The inconsistency of the data was mainly due to the highly variable population levels of AAC00-1 in each experiment. Fluctuating environmental conditions in the greenhouse may have also contributed to the differences across experiments.

We also tested the role of the HopAF homolog in *A. citrulli* colonization of melon and watermelon cotyledons after syringe infiltration. The results indicated that the HopAF homolog contributed to BFB development on watermelon and melon seedlings. A5ΔHopAF was less virulent on both hosts, but showed reduced populations in melon cotyledons. Similarly, effectors in the AvrBs3/PthA family in *Xanthomonas* and HopPtoM in *Pseudomonas* promoted lesion formation in susceptible hosts, without a commensurate effect on bacterial population growth (Badel et al. 2003; Yang et al. 1994).

For unknown reasons, the complemented strains of A5ΔHopAF that we generated did not restore wild-type virulence levels. Thus, we generated a HopAF mutant in strain M6. M6ΔHopAF was less virulent, but had a similar population level as the wild-type strain on melon cotyledons. However, both the wild-type and mutant strains were less virulent on watermelon. While the BFB severity levels on watermelon were not significantly different, the population levels of M6ΔHopAF were significantly less than M6 on watermelon cotyledons. This indicates that HopAF contributes to BFB seedling disease development.

HopAF is present in the genomes of many diverse plant pathogenic bacteria, including *Pseudomonas syringae* and *Xanthomonas* species. In *P. syringae* pv. tomato (*Pto*) DC3000, HopAF was reported to function as a deamidase involved in pattern-triggered immunity suppression by blocking ethylene production in *Arabidopsis thaliana* (Washington et al. 2016). Additionally, HopAF suppresses the effector-triggered immunity (ETI) triggered in tobacco by HopAD1 (Castañeda-Ojeda et al. 2017). The *Pto* DC3000 HopAF effector has also been reported to completely suppress the HopA1-dependent hypersensitive response (Guo et al. 2009). However, how HopAF homolog functions in cucurbit hosts remains unknown.

In conclusion, we did not find detectable effects of XopJ homologs on *A. citrulli* virulence on watermelon and melon cotyledons. However, the HopAF homolog may be important for *A. citrulli* colonization and symptom development on watermelon and melon cotyledons.

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Table 4.1 Strains and plasmids used in this study.

| Strains and plasmids | Relevant characteristics | Reference or source |
|---|---|---|
| *Acidovorax citrulli* | | |
| AAC00-1 | Wild-type, group II | R. Walcott collection |
| 00-1ΔxopJTriple | Triple knockout mutants, deletion of *Aave_2166, Aave_2708,* and *Aave_2938* in AAC00-1, $Km^R$ | This study |
| XopJTM+2166 | XopJ triple mutant complemented with *Aave_2166,* $Km^R$, $Gm^R$ | This study |
| XopJTM+2708 | XopJ triple mutant complemented with *Aave_2708,* $Km^R$, $Gm^R$ | This study |
| M6 | Wild-type, group I | (Burdman et al. 2005) |
| M6ΔHopAF | Deletion mutant of HopAF homolog in M6 | This study |
| A5 | Wild-type, group II | T. Zhao collection |
| A5ΔHopAF | Deletion mutant of HopAF homolog in A5 | T. Zhao collection |
| *Escherichia coli* | | |
| DH5α | $F^-$φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r^-_K$ $m^+_K$ ) *phoA glnV44* | NEB, Ipswich, MA |
| Rho5 | attTn7::*pir116*$^+$ *thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA* integrated RP4-2 $Tc^r$::Mu (λ*pir*$^+$)Δ*asd*::*FRT* Δ*aphA*::*FRT* | (Kvitko et al. 2012) |
| Plasmid | | |
| pGEM-T | $Amp^R$ | Promega Inc |
| pOK1 | $Sp^R$, $SacB^+$, suicide vector | (Huguet et al. 1998) |
| p4916 | PCR8GW-Kan-3, $Km^R$, $Sp^R$ | (Traore 2014) |
| p3692 | pLVC18L/SacB/CcdB, $Tet^R$, destination vector, suicide vector | (Traore 2014) |
| pK18*mobsacB* | Suicide vector, $Km^R$, $sacB^+$ | (Schäfer et al. 1994) |
| pBBR1MCS-5 | Broad-host range cloning vector, $Gm^R$ | (Kovach et al. 1995) |

$Km^R$, $Gm^R$, $Sp^R$, $Amp^R$, and $Tet^R$ indicate resistant to kanamycin, gentamycin, spectinomycin, ampicillin, and tetracycline, respectively.

Table 4.2 Oligonucleotide primers used in this study.

| Primer | Sequence (5' – 3') | Function |
|---|---|---|
| 2166JF | GTGCAGCCGCATGATGGTGTCGAT | To amplify a fragment of 2940 bp containing the entire *Aave_2166* open reading frame (ORF) |
| 2166JR | CGAGCTTCTGCAAACCGAAGCCCA | |
| 2165F1 | CACGAACATGATGGACGAGG | To confirm the deletion of 00-1Δ2166 |
| 2167R1 | ATTGGCTGAAATCGCGGATC | |
| 2708-1F | CTATGACATGATTACGAATTCCGGTTGCTGACGATTGATT | To amplify 597 bp upstream of *Aave_2708* |
| 2708-1R | CACCATTTGCCTATGACTGGCGGCTTTGAAACGCATAGAC | |
| 2708-2F | GTCTATGCGTTTCAAAGCCGCCAGTCATAGGCAAATGGTG | To amplify 585 bp downstream of *Aave_2708* |
| 2708-2R | CAGGTCGACTCTAGAGGATCCTCCGCTCACTCCGTAACCC | |
| 2708LongF | TGGGTCTATGCGTTTCAAAGC | To confirm the deletion of *Aave_2708* in 00-1ΔXopJDouble |
| 2708LongR | GCGATCTTCTTGCCCATGAG | |
| 2938Up-FWD | CGCCCTTATTTAAATGGATCCTGGCCACGCTGGAGTGGGT | To amplify 1,000 bp upstream of *Aave_2938* |
| 2938Up-REV | AGCAGCTCCAGCCTACACAAAATGGTGAACTTGATGTCCG | |
| FRT-FWD | CGGACATCAAGTTCACCATTTTGTGTAGGCTGGAGCTGCT | To amplify the FRT region |
| FRT-REV | CACAGTCACTGTAGGAGATCTATCCTCCTTAGTTCCTATT | |
| 2938Down-FWD | AATAGGAACTAAGGAGGATAGATCTCCTACAGTGACTGTG | To amplify 1,000 bp downstream of *Aave_2938* |
| 2938Down-REV | AATTCGCCCTTACTAGTCGAGCCGGCCAATGAACAGGAAA | |
| 2938UpFRT-F | CAGCTACGACAATGCACTGG | To confirm the deletion of 00-1ΔxopJTriple |
| 2938UpFRT-R | GAACTTCGAAGCAGCTCCAG | |

| | | |
|---|---|---|
| 2166ComF | TCCCCCGGGCTGCAGGAATTGGTTGCGCTTCACTATCGAT | To amplify full length of *Aave_2166* ORF |
| 2166ComR | GGGCCCCCCCTCGAGGTCGATCATTCGATAGCTTTTCTGA | |
| 2708ComF | TCCCCCGGGCTGCAGGAATTACTTCCTTTGTTTTGACATG | To amplify full length of *Aave_2708* ORF |
| 2708ComR | GGGCCCCCCCTCGAGGTCGACTATGACTGGCGATCAGAGA | |
| 1373A-FWD | CTATGACATGATTACGAATTGGCGCCCTGCTGATCCAGCG | To amplify 700 bp upstream of HopAF homolog from strain M6 |
| 1373A-REV | GCCCAAAGTGGTTACCTCCTGGTCATTGGAAATTTCCAAA | |
| 1373B-FWD | TTTGGAAATTTCCAATGACCAGGAGGTAACCACTTTGGGC | To amplify 700 bp downstream of HopAF homolog from strain M6 |
| 1373B-REV | CTTGCATGCCTGCAGGTCGAACCCAATCAATCCAATTAAT | |
| 1373OutF2 | GCACCGAGAACTGCGAATAG | To screen for putative M6ΔHopAF |
| 1373OutR2 | CCCTTCAGGTTCTCTTCCGT | |
| 1373-1F | CTATGACATGATTACGAATTCCCGCGTTCACCTGCACTTC | To amplify 580 bp upstream of HopAF homolog from strain A5 |
| 1373-1R | CGCCCAAAGTGGTTACCTCCTGGACGTTGGTCATTGGAAA | |
| 1373-2F | TTTCCAATGACCAACGTCCAGGAGGTAACCACTTTGGGCG | To amplify 531 bp downstream of HopAF homolog from strain A5 |
| 1373-2R | CAGGTCGACTCTAGAGGATCCCCGCACCATTCCATTATTACGAG | |
| 1373OutF1 | GCAGGTCGTTGTAGGTGTTG | To confirm mutation of A5ΔHopAF |
| 1373OutR1 | ATCTATTGAATGCCCGCGTG | |
| 1373ComF | CGCGGTGGCGGCCGCTCTAGTTACCTCCTTCCTGGCGATA | To amplify full length of HopAF ORF from strain A5 |
| 1373ComR | GTCGACGGTATCGATAAGCTGGCGGCAGACTTCGGGACGG | |

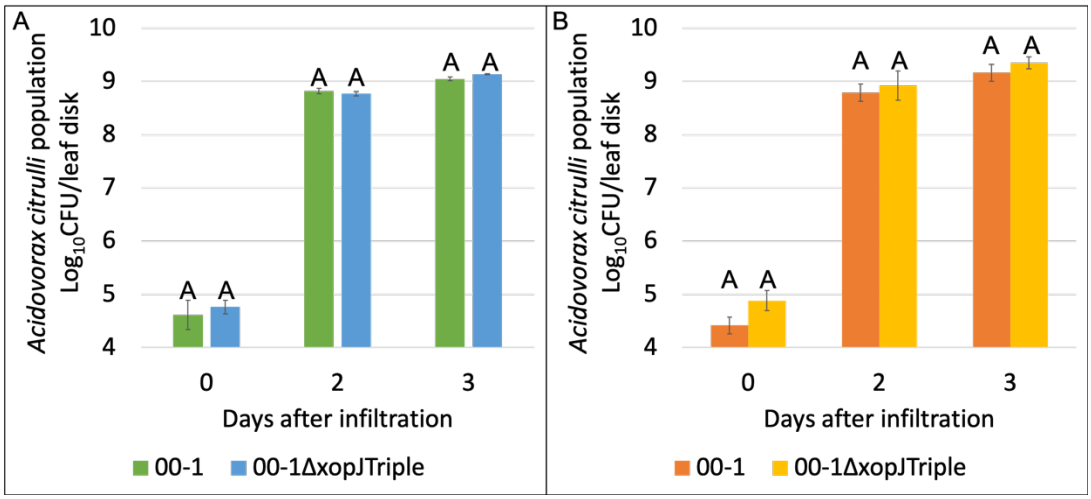


Fig 4.1. The effect of XopJ on *Acidovorax citrulli* colonization of (A) watermelon (cv. 'Crimson Sweet') and (B) melon (cv. 'Joaquin Gold') cotyledons. For each strain, four 2-week-old watermelon and melon seedlings were syringe infiltrated at $10^4$ CFU/ml and incubated at 100% relative humidity for 3 days. Four cotyledons were sampled at 0, 2, and 3 days after infiltration for DNA extraction followed by real-time PCR to determine *A. citrulli* population. The bars and lines represent the means and standard errors, respectively. Different letters indicate significant differences (P = 0.05) between treatments according to Tukey-Kramer's honestly significant difference test. The experiment was conducted twice with similar results.

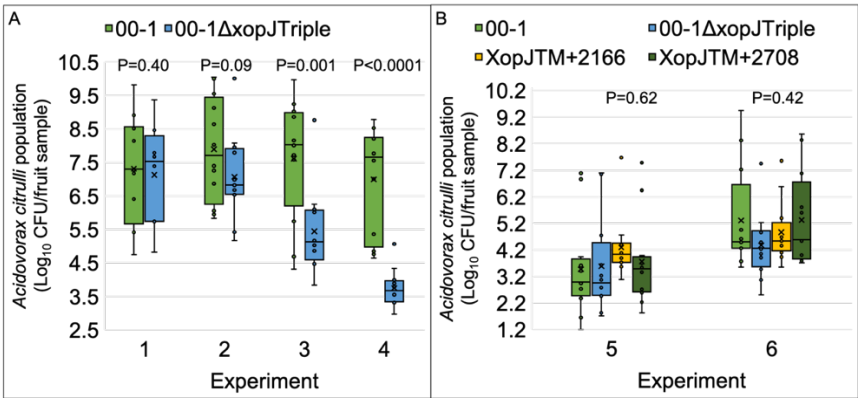


Fig 4.2. The effect of XopJ homologs on *Acidovorax citrulli* colonization of attached melon fruits after swab inoculation assay. Box plot of *A. citrulli* populations at 20 days post swab-inoculation (dpi) on melon (cv. 'Joaquin Gold') fruit tissues. Strains were swab-inoculated at $10^8$ CFU/ml onto the attached melon fruits in the greenhouse. For experiments 1 and 2, each fruit was incubated in a plastic bag for 60 h to maintain high humidity, followed by removal of the plastic bag and incubation in the greenhouse for 20 days with natural diurnal light cycles. For experiments 3 to 6, fruits were not bagged after inoculation. At 20 dpi, four samples (diameter 0.7 cm, length 1.0 cm) per fruit from three fruits for each treatment were collected for DNA extraction followed by real-time PCR to determine *A. citrulli* population. 'x' represents the means. A) shows four experiments conducted using strains AAC00-1 and 00-1ΔxopJTriple. B) shows two experiments conducted using strains AAC00-1, 00-1ΔxopJTriple, XopJ triple mutant complemented with *Aave_2166* (XopJTM+2166), and XopJ triple mutant complemented with *Aave_2708* (XopJTM+2708). P values according to the Student t-test (A) and analysis of variance (B) were labeled for each experiment.

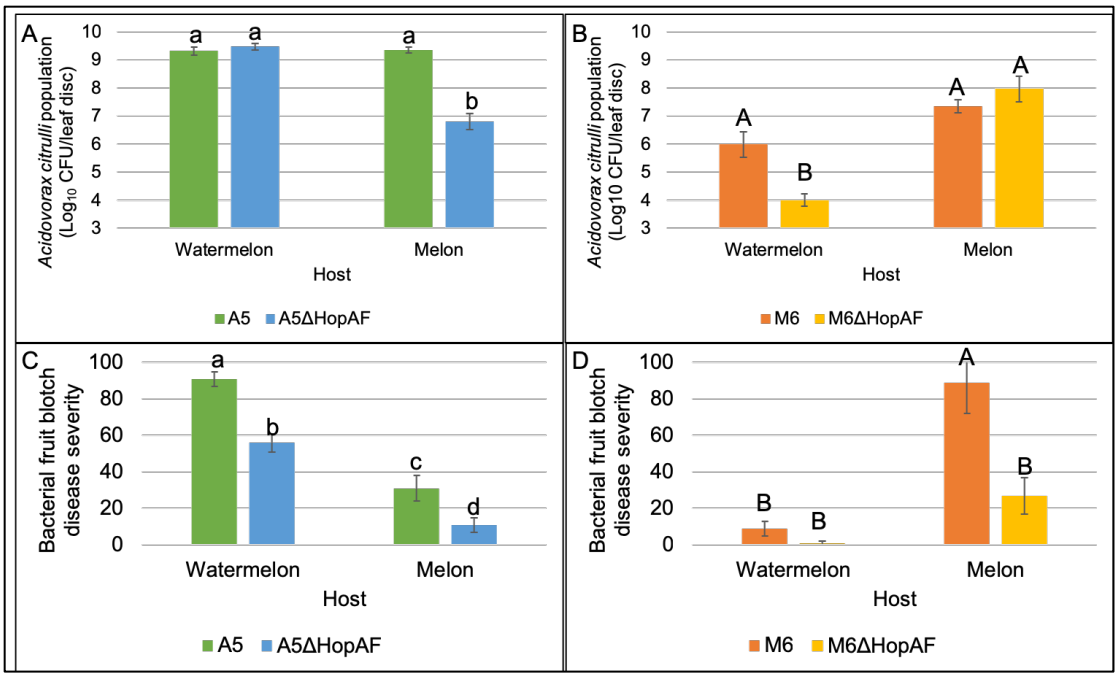


Fig 4.3. The effect of the HopAF homolog on *Acidovorax citrulli* colonization and virulence on watermelon and melon cotyledons. *Acidovorax citrulli* population levels (A and B) and BFB disease severity (C and D) on watermelon (cv. 'Crimson Sweet') and melon (cv. 'Joaquin Gold') seedlings at 3 days post inoculation (dpi) (A and C) for group II strain A5 and A5ΔHopAF and at 4 dpi (B and D) for group I strain M6 and M6ΔHopAF. Leaf discs from three cotyledons were sampled for DNA extraction from each treatment. The *A. citrulli* population was quantified by real-time PCR. Cotyledons (n=6) were photographed and disease severity (percentage of lesion area/ total cotyledon area) was determined using ImageJ. The bars and lines represent the means and standard errors, respectively. Different letters indicate significant differences (P = 0.05) between treatments according to Tukey-Kramer's honestly significant test. The experiment was conducted twice with similar results.

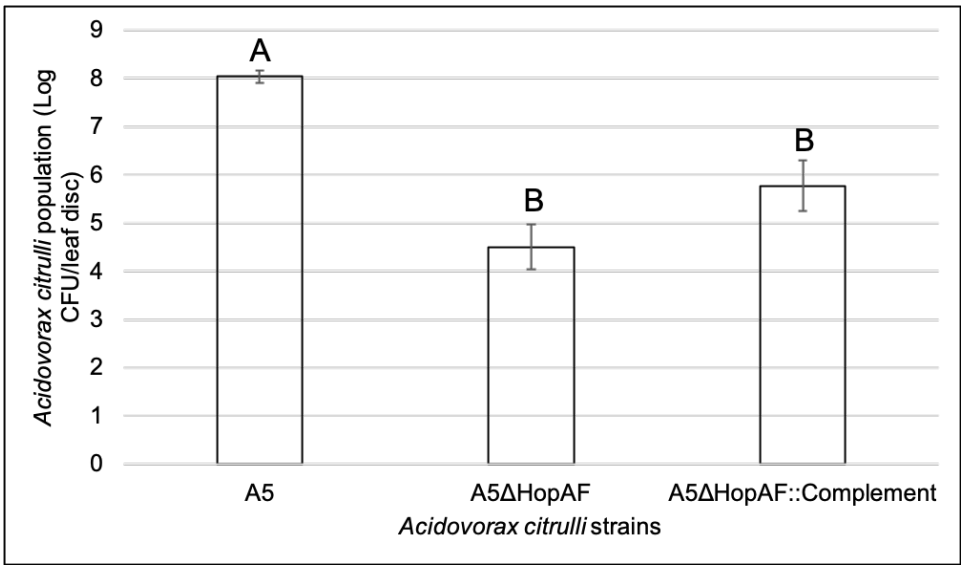


Fig 4.4. The effect of the HopAF homolog on *Acidovorax citrulli* group II strain A5 colonization of melon cotyledons. For group II strain A5, A5ΔHopAF, and A5ΔHopAF::Complement, three 2-week-old melon (cv. 'Joaquin Gold') seedlings were syringe infiltrated at $10^3$ CFU/ml and incubated at 100% relative humidity for 3 days. Leaf discs from three cotyledons for each treatment were sampled at 3 days after infiltration for DNA extraction followed by real-time PCR to determine *A. citrulli* population. The bars and lines represent the means and standard errors, respectively. Different letters indicate significant differences (P = 0.05) between treatments according to Tukey-Kramer's honestly significant test. The experiment was conducted twice with similar results.

# CHAPTER 5

# NOVEL VIRULENCE DETERMINANTS OF *ACIDOVORAX CITRULLI* IDENTIFIED USING AN IMMATURE WATERMELON FRUIT ASSAY [1]

[1] Zhao, M., and Walcott R. R. To be submitted to *Phytopathology*.

## ABSTRACT

*Acidovorax citrulli* causes bacterial fruit blotch (BFB) of cucurbits and can be assigned to two genetically distinct groups: I and II. BFB represents a serious threat to cucurbit crop production worldwide, especially watermelon and melon. Watermelon fruits are often associated with group II *A. citrulli* strains. However, no fruit-specific virulence determinants have been reported for *A. citrulli*. The objective of this study was to identify unique virulence determinants of *A. citrulli* group II strains. We found that only group II strains induced water-soaked lesions on immature watermelon fruit tissues. By screening an *A. citrulli* AAC00-1 transposon mutant library with a detached watermelon fruit assay, we found six non-pathogenic *A. citrulli* mutants. These mutants were also non-pathogenic on watermelon foliage and unable to induce a hypersensitive response (HR) on tobacco leaves. Three of the mutants were disrupted in type 3 secretion system associated genes. Two additional mutants were further studied but the genes responsible for the HR-negative phenotype could not be elucidated.

## INTRODUCTION

Bacterial fruit blotch (BFB) infects plant species in the family Cucurbitaceae, including watermelon and melon. BFB is caused by the Gram-negative bacterium, *Acidovorax citrulli* and in the early 1990s, it caused significant economic losses in US watermelon production (Latin and Rane 1990; Somodi et al. 1991). Subsequently, BFB outbreaks have occurred in many regions around the world, primarily on watermelon and melon crops, but also on other cucurbitaceous crop species (Burdman and Walcott 2012). Great effort has

been expended to mitigate the impact of BFB, unfortunately, the efficacy of the current management approaches has been limited, and BFB still threatens commercial watermelon and melon production worldwide (Burdman and Walcott 2012).

*Acidovorax citrulli* strains can be divided into two distinct groups (I and II) based on carbon substrate utilization (Walcott et al. 2004), DNA fingerprint analysis (Burdman et al. 2005; Walcott et al. 2000; Yan et al. 2013), multilocus sequence analysis (Feng et al. 2009a; Yan et al. 2013), and fatty acid methyl ester analysis (Walcott et al. 2000). Comparative genome analysis revealed that the genome of the representative group I strain M6 is 0.5 Mb smaller than that of the group II strain AAC00-1 (Eckshtain-Levi et al. 2016). This difference was primarily explained by the presence of eight fragments, dispersed throughout the AAC00-1 genome (Eckshtain-Levi et al. 2016). The two *A. citrulli* groups also differ in their virulence on different cucurbit species (Walcott et al. 2004). For example, group I strains are moderately to highly aggressive on watermelon, melon, pumpkin, and squash while group II strains are highly aggressive on watermelon, but weakly aggressive on other cucurbit species (Walcott et al. 2004). Despite this, the mechanisms involved in *A. citrulli* host preference and virulence have not been elucidated.

Little is known about the factors that influence the range of plant species affected by group I and II *A. citrulli* strains. One explanation for the differences is the unique type III secreted effectors (T3Es) present in strains of each group. Like many Gram-negative plant pathogenic bacteria, *A. citrulli* requires a hrp

(hypersensitive response and pathogenicity) type III secretion system (T3SS) that translocates bacterial effector proteins directly into plant host cells (Bahar and Burdman 2010; Johnson et al. 2011). In general, T3Es act as virulence factors by modifying host cellular targets, but can be recognized as avirulence factors if detected by cognate plant resistance receptors (Jones and Dangl 2006). Both group I and II *A. citrulli* strains require a functional T3SS for pathogenicity on cucurbit hosts (Bahar and Burdman 2010; Johnson et al. 2011).

We recently developed a detached melon fruit assay that can clearly distinguish group I and II *A. citrulli* strains based on symptom development (Yan et al. 2017). Using this assay, we observed a consistent difference in pathogenicity between representative group I and II *A. citrulli* strains. More specifically, only group I strains (n=4) induced water-soaked lesions on melon cv. 'Joaquin Gold' fruits.

The natural association of group I and II *A. citrulli* strains with melon and watermelon fruits strongly suggests host preference and tissue specificity. Additionally, in field experiments, we observed that watermelon fruits were often associated with group II *A. citrulli* strains. However, no fruit-specific virulence determinants have been reported for *A. citrulli* on watermelon. Hence, the objective of this study was to develop an immature watermelon fruit assay to distinguish group I and II *A. citrulli* strains and use it to identify unique virulence determinants of *A. citrulli* group II strains.

## MATERIALS AND METHODS

### Bacterial strains and inoculum preparation.

Bacterial strains and plasmids used in the study are listed in Table 5.1. *Acidovorax citrulli* strains were routinely cultured on nutrient agar (NA) (Becton Dickinson, Sparks, MD) at 28°C for 2 days and *Escherichia coli* strains on Luria-Bertani broth (LB, VWR chemicals, Solon, Ohio) or agar at 37°C for 1 day. When required, media were supplemented with kanamycin at 50 µg/ml, tetracycline at 10 µg/ml, and diaminopimelic acid (DAP) for *E. coli* Rho5 at 200 µg/ml.

To prepare *A. citrulli* inocula, strains were cultured in nutrient broth at 28°C in a rotary shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm for approximately 16 h. Subsequently, the cultures were centrifuged at 16,100 x g for 1 min and the supernatants were decanted. The resulting pellets were resuspended in sterilized distilled water (sd$H_2O$). The bacterial concentrations were then adjusted to an optical density of 0.3 at 600 (~ $10^8$ colony forming units (CFU/ml) spectrophotometrically (Spectronic 20; Bausch and Lomb, Rochester, NY), and adjusted to the desired concentration by 10-fold serial dilutions.

### Pathogenicity of group I and II *A. citrulli* strains on immature detached watermelon fruit.

To determine the suitability of a detached immature watermelon fruit assay for distinguishing group I and II *A. citrulli* strains, three group I (AAC98-17, AAC92-300, and AAC203-16) and three group II (AAC211-2, AAC96-6, and AAC92-303) *A. citrulli* strains were tested. Watermelon (cv. ‘Crimson Sweet’) (Johnny’s Selected Seeds, Winslow, ME) plants were grown in pots filled with

plant growth media (Sun gro® Horticulture, Agawam, MA) under greenhouse conditions. In greenhouses, the conditions were set to maintain a day temperature of 28°C and a night temperature of 22°C. The daytime temperatures reached a maximum of 38°C during the summer months. The relative humidity (RH) was approximately 80% and plants were watered daily. At anthesis, open female flowers were pollinated by rubbing pollen from the anthers of male flowers onto the stigmas of female flowers and the resulting fruits were allowed to develop for 4 to 7 days. Immature detached watermelon fruits were harvested, washed with tap water, surface-disinfected with 70% ethanol, cut into halves longitudinally, and placed on 1-cm-high sterilized plastic blocks. Then, fruit halves (n=15) were pin-prick inoculated with 10 µl bacterial suspensions ($10^8$ CFU/ml) of group I and II *A. citrulli* strains. Immature detached watermelon fruits inoculated with sd$H_2O$ served as negative controls. After inoculation, watermelon fruits were incubated in a transparent clamshell box (Solo Container Corporation, Mason, MI) under conditions of 28°C and 100% RH with continuous fluorescent light in an incubator (Percival, Perry, IA) for eight days. Diameters of the resulting lesions on watermelon fruit rinds were measured at 8 days post inoculation (dpi). This experiment was conducted twice. Analysis of variance (ANOVA) was conducted followed by Tukey-Kramer's honestly significant difference (HSD) test ($P = 0.05$) to determine the effect of bacterial strain and group on mean lesion diameter size.

**The role of type 3 secreted effectors in immature watermelon fruit infection by *A. citrulli*.**

To determine the effect of type 3 secreted effectors on *A. citrulli* pathogenicity on watermelon fruits, AAC00-1 and AAC00-1Δ*hrcC* (non-pathogenic T3SS mutant) (Johnson et al. 2011) were pin-prick inoculated on detached immature watermelon (cv. 'Crimson Sweet') fruits as described above, and fruit halves were visually examined for water-soaked lesions 8 dpi. Each strain was inoculated onto four watermelon fruits and the experiment was conducted three times. The percentages of fruit halves that develop water-soaked lesions were recorded.

**Generation of a transposon mutant library of *A. citrulli* AAC00-1.**

A mini-transposon random insertion mutant library of *A. citrulli* AAC00-1 was generated using the EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (Epicentre, Madison, WI). AAC00-1 was grown overnight in 4 ml NB at 28°C for approximately 16 h, and 400 µl of which was sub-cultured in 4 ml NB, and grown to the exponential phase ($OD_{600nm}$ = 0.5-0.7). Cells were pelleted by centrifuging at 16,100 x g for 1 min and washed with 300mM filtered sucrose four times. One microliter of the EZ-Tn5™ <R6Kγori/KAN-2>Tnp transposome was added to the washed AAC00-1 cells, and electroporation was performed using a Gene Pulser (Biorad, Hercules, CA) with a setting of 1800 V. Electroporated cells were immediately recovered by adding 1 ml of NB, transferred to a sterilized 14 ml test tube and shaken at 28°C for 2 h. Transformants were plated on NA with kanamycin (NAK), and after 48 h at 28°C, individual colonies were patch-plated

onto NAK, and stored in 96-well plates (MedSupply Partners, Atlanta, GA) containing 15% glycerol.

**Screening random insertion transposon library of *A. citrulli* AAC00-1.**

To identify genes that allowed AAC00-1 to infect watermelon fruits, the AAC00-1 transposon library was screened for loss of pathogenicity using the immature watermelon fruit assay. Colonies of individual transposon mutants on NAK plates were suspended in sd$H_2O$. Immature watermelon fruit halves were pin-prick inoculated with 10 µl of the bacterial suspensions and incubated at 28°C and 100% RH. The transposon mutants that did not develop symptoms on watermelon fruits were tested by the pin-prick inoculation at least three times.

The impaired genes in the non-pathogenic mutants were identified by a plasmid rescue procedure followed by sequencing Tn5 flanking regions using transposon-specific primers. Specifically, the genomic DNA of the selected mutants were extracted, digested using *Kpn*I and *Sph*I, phosphorylated, then self-ligated using T4 DNA ligase (New England Biolabs Inc., Ipswich, MA). The ligation mix was chemically transformed into *pir*$^+$ *E. coli* MaHI. The transformants were selected on LB agar with kanamycin. Plasmids from the transformants were sequenced (Eurofins Genomics, Louisville, KY) using EZ-Tn5 <R6Kγori/KAN-2> transposon-specific primers. Sequences were BLASTed against the AAC00-1 genome sequence (GenBank accession CP000512.1).

To confirm single insertion for selected transposon mutants (87D3, 90A1, 90E2, and 88A11), the whole genomes were sequenced using the MicrobeNG service. Transposon sequence (2,001 bp) was BLASTed against the genome

sequences, and the number of hits and the inserted sites were identified.

**Deletion of *Aave_0691* and *Aave_0692-0697* in *A. citrulli* AAC00-1*.***

The transposon mutant 87D3 was disrupted in gene *Aave_0691.* To determine the role of *Aave_0691* in *A. citrulli* virulence*,* a marker exchange mutant *Aave_0691* was generated using the p3692 vector and named 00-1Δ0691-A11. However, the mutant caused BFB lesions on watermelon seedlings and a hypersensitive response (HR) on tobacco, which was not consistent with the transposon mutant 87D3. To further investigate the role of *Aave_0691* in *A. citrulli* pathogenicity, an unmarked deletion of *Aave_0691* was made using the pk18*mobsacB* vector and named 00-1Δ0691-A2. To accomplish this, the *Aave_0691* flanking sequences (1,000 bp each) were amplified from AAC00-1 DNA with primer pairs 0691A-F/0691A-R and 0691B-F/0691B-R (Table 5.3) using Phusion® High-Fidelity DNA Polymerase (NEB). The amplicons were ligated and cloned into the suicide vector pK18*mobsacB* between the *EcoR*I and *Sal*I restriction sites using Gibson Assembly® Master Mix (NEB) to generate pK18Δ0691. The plasmid was transformed into *E. coli* strain DH5α and plated on LB kanamycin X-gal IPTG for blue-white screening. Plasmids were extracted from overnight cultures inoculated with white transformants using the EZNA® Plasmid Mini Kit (Omega Biotek, Norcross, GA) and confirmed by sequencing with primers M13F and M13R. The pK18Δ0691 was transformed into *E. coli* strain Rho5 and plated on LB amended with DAP and kanamycin. Bi-parental mating was carried out by plating a 1:1 mixture of *E. coli* strain Rho5::pK18Δ0691 and AAC00-1 on LB with DAP. After 24 h, the mixed culture was streaked onto

LB with kanamycin to select for first crossover products. A single colony from the resulting first-crossover products was cultured overnight in LB to allow for second-crossover events. One hundred microliters of the overnight culture and a 10-fold dilution of the culture were plated on M9 agar (Sambrook et al. 1989) amended with 0.4% sodium citrate and 10% sucrose. After 3 days of incubation at 28°C, colonies from the M9 plates were patched onto LB with and without kanamycin. The colonies that grew only on LB were further screened by colony-PCR using primer set 0691OutF/0691OutR. AAC00-1 DNA amplified with primer set 0691OutF/0691OutR produced a 4,383 bp band and DNA from the mutant (designated as 00-1Δ0691-A2) produced a 2,394 bp band.

Because 00-1Δ0691-A2 still caused BFB on watermelon seedlings and an HR on tobacco, the five genes downstream from *Aave*_0691, *Aave_0692* – *Aave_0696* were deleted as described above using primer sets 0692A-FWD/0692A-REV and 0697B-FWD/0697B-REV (Table 5.3). The mutant, designated as 00-1Δ*Aave_0692-6*-B9, was confirmed by PCR using primer set 0692OutF/0696OutR (Table 5.3). The DNA from the mutant 00-1Δ*Aave_0692-6*-B9 amplified with the primer set 0692OutF/0697OutR yielded a 2,259 bp band, while AAC00-1 wild-type DNA produced a 6,676 bp band.

**Deletion of *Aave_4431* in *A. citrulli* AAC00-1*.***

The transposon mutant 90E2 was disrupted 4 bp before the start codon of *Aave_4431.* To investigate the role of *Aave_4431* in *A. citrulli* pathogenicity, *Aave_4431* was deleted to generate AAC00-1Δ4431-E2. The deletion process was as described above using primer sets 4431A-FWD/4431A-REV and 4431B-

FWD/4431B-REV (Table 5.3). To select for the deletion mutants, the second crossover colonies grown on M9 plates were patch-plated onto LB with and without kanamycin. Colonies that did not grow on kanamycin were selected for mutation confirmation using primer set 4431OutF/4431OutR. AAC00-1 DNA amplified with primer set 4431OutF/4431OutR produced a 3,772 bp band and DNA from the mutant (designated as 00-1Δ4431) produced a 2,497 bp band.

**Swimming motility assays.**

Because 90E2 was disrupted in the flagellar gene cluster, we tested the swimming motility of *A. citrulli* AAC00-1, 00-1Δ4431, and 90E2. Single colonies were stab-inoculated into soft agar medium (10 g tryptone, 5 g NaCl, 3 g agar per liter). Plates were incubated at 28°C for 1 day. The diameter of the zone of migration was measured in two directions on four different plates. The experiment was conducted twice. Analysis of variance (ANOVA) was conducted on the diameter of migration zone data using JMP statistical analysis software (version Pro 14; SAS Institute Inc., Cary, NC). The effect of strain on the diameter of migration zone data was compared using the Tukey-Kramer's honestly significant difference (HSD) test.

**Hypersensitive response assays.**

To determine if the *A. citrulli* mutant strains could induce a hypersensitive response (HR) on tobacco (*Nicotiana tabacum*), bacterial suspensions ($10^8$ CFU/ml) were syringe-infiltrated into tobacco leaves (Schaad et al. 2001). AAC00-1 served as the positive control and sd$H_2O$ served as the negative

control. The results were recorded 24 h after inoculation. The experiment was conducted at least twice.

**Pathogenicity test of *A. citrulli* mutants on watermelon seedlings.**

The pathogenicity of the *A. citrulli* mutant strains was tested as follows. Two-week-old watermelon (cv. 'Crimson Sweet') cotyledons were syringe-infiltrated with $10^3$ CFU/ml of bacterial suspensions. Four seedlings were inoculated with each strain. Plants inoculated with sd$H_2O$ served as negative controls. Plants were maintained in a plastic box (45 cm x 59.8 cm x 28.4 cm, IRIS USA, Inc, Pleasant Prairie, WI) in an incubator (Percival) at 28°C and 100% RH for four days. Pictures of watermelon cotyledons were taken at 4 dpi. The experiment was conducted twice.

**Re-construction of 0691Tn5 and 4431Tn5 transposon mutants in AAC00-1.**

Because the unmarked knockout mutants of *Aave_0691* and *Aave_4431* did not show the same virulence phenotypes as the Tn5 transposon mutants 87D3 and 90E2, insertion mutants were created to duplicate the insertion event of the transposon mutants using the p3692 vector. To accomplish this, sequences including the 2,001 bp transposon and ~400-500 bp flanking sequences from 87D3 and 90E2 were amplified with Phusion® High-Fidelity DNA Polymerase using primer sets 4916-0691Tn5-FWD/4916-0691Tn5-REV and 4916-4431Tn5-FWD/4916-4431Tn5-REV, respectively. The amplicons were cloned into the vector p4916 between *Bam*HI and *Sal*I restriction sites using Gibson Assembly® Master Mix and the Gibson reaction mixture was transformed into *E. coli* strain DH5α. The resulting transformants were screened for the

presence of the inserted fragments using colony PCR. The plasmids were extracted from overnight cultures inoculated with putative transformants using the EZNA® Plasmid Mini Kit and the transformants were confirmed by sequencing with primers M13F and M13R. Then, the LR reaction was performed by mixing the p4916 constructs and p3692 with the LR Clonase™ II enzyme mix (Invitrogen, Carlsbad, CA) overnight, and then transformed into *E. coli* strain DH5α and plated on LB with tetracycline and kanamycin. The resulted plasmids p3692-0691Tn5 and p3692-4431Tn5 were transformed into *E. coli* strain Rho5 and plated on LB with DAP, tetracycline, and kanamycin. Bi-parental mating was carried out by plating a 1:1 mixture of *E. coli* strain Rho5::p3692-0691Tn5 or Rho5::p3692-4431Tn5 and the *A. citrulli* AAC00-1 on LB with DAP. The mating mixtures were grown in LB broth with kanamycin overnight and then spread-plated on LB agar with kanamycin. Single colonies were patched-plated on LB with kanamycin and LB with tetracycline. A mutant that grew only on LB with kanamycin was selected and designated as 00-1Δ4431Tn5. It was confirmed using primer set 4431OutF/KAN-2 FP-1, yielding 1,222 bp, while AAC00-1 wild-type DNA did not produce an amplicon. We have not yet generated a 00-1Δ0691Tn5 mutant.

## RESULTS

### Pathogenicity of group I and II *A. citrulli* strains on immature detached watermelon fruits.

Immature detached watermelon fruits (cv 'Crimson Sweet') inoculated with group II *A. citrulli* strains developed water-soaked lesions by 4 dpi. In contrast,

the group I strains induced little or no water-soaking on the rinds of immature watermelon fruits. Mean diameters of water-soaked lesions induced by group II strains AAC211-2, AAC96-6, and AAC92-303 were 2.73, 2.73, and 3.13 mm, respectively, whereas the mean diameters of lesions induced by group I *A. citrulli* strains AAC98-17, AAC92-300, and AAC203-16 were 0, 0, and 0.20 mm, respectively. The mean lesion diameters induced by each group II *A. citrulli* strains were significantly higher than the lesions induced by group I ($p < 0.0001$) (Fig 5.1).

**The role of type 3 secreted effectors in immature watermelon fruit infection by *A. citrulli*.**

At 4 days after pin-prick inoculation with approximately $10^6$ CFU, AAC00-1 induced water-soaked lesions in 100% (n=4) of the inoculated watermelon fruits. In contrast, 00-1Δ*hrcC* did not induce water-soaked lesions in all four watermelon fruits tested at 8 dpi (Fig 5.2).

**Screening random insertion transposon library of *A. citrulli* AAC00-1.**

10,080 AAC00-1 Tn5 transposon mutants were generated. After screening 1,013 transposon mutants, six strains (Table 5.2) were found to be non-pathogenic on watermelon fruits. Additionally, these mutants did not cause disease on watermelon seedlings nor elicit an HR on tobacco leaves.

BLAST results of the whole-genome sequences showed that there was only one insertion of the transposon for 87D3, 90A1, 90E2, and 88A11 Tn5 mutants. We selected Tn5 mutants 87D3 and 90E2 to further characterize *Aave_0691* and *Aave_4431*.

**Characterization of *Aave_0691.***

To characterize mutant 87D3, a marker exchange mutant of *Aave_0691* was generated and designated as 00-1Δ*Aave_0691*-A11. This mutant was infiltrated into watermelon cotyledons for pathogenicity tests and tobacco leaves for HR tests. Interestingly, 00-1Δ*Aave_0691*-A11 caused disease on watermelon seedlings and induced an HR on tobacco while the transposon mutant 87D3 did not (Fig 5.3). To eliminate the effect of the antibiotic marker, an unmarked deletion of *Aave_0691* was made. However, this mutant 00-1Δ*Aave_0691*-A2 still caused disease on watermelon seedlings (Fig 5.3A) and induced an HR on tobacco (Fig 5.3B). Then, to test if the phenotype of 87D3 was due to downstream genes, an unmarked deletion of genes *Aave_0692 – Aave_0696* was generated. This deletion mutant caused disease on watermelon seedlings (Fig 5.3A) and induced an HR on tobacco (Fig 5.3B).

**Characterization of *Aave_4431.***

The Tn5 transposon insertion was localized 4 bp before the start codon of *Aave_4431* (flagellar hook-associated protein 3, hap3/flgL) for the 90E2 transposon mutant. Thus, a swimming motility assay was conducted. 90E2 consistently produced a significantly larger zone of swimming motility compared to the wild-type AAC00-1, while deleting *Aave_4431* eliminated its swimming motility. The average diameter for Tn5 mutant 90E2, AAC00-1, and 00-1Δ4431 were 9.9, 18.6, and 1.5 mm, respectively (Fig 5.4).

When *A. citrulli* strains were syringe-infiltrated into 2-week-old watermelon cotyledons, 00-1Δ4431 showed decreased BFB severity relative to wild-type

AAC00-1, while 90E2 consistently did not induce BFB symptoms (Fig 5.5A). While AAC00-1 and 00-1Δ4431 were positive for HR induction in tobacco, transposon mutant 90E2 was HR negative (Fig 5.5B). 00-1Δ4431Tn5 was generated to re-create the insertion event of the transposon 90E2, and it failed to induce an HR on tobacco and cause BFB symptoms on watermelon cotyledons, which was consistent with the original Tn5 mutant 90E2.

**DISCUSSION**

Both *A. citrulli* group I and II strains can induce BFB lesions on the foliage of watermelon and melon. However, here we showed that only group II strains induced water-soaked symptoms on immature watermelon fruits. We used an immature detached watermelon fruit assay to screen an *A. citrulli* AAC00-1 random insertion transposon library for loss of pathogenicity. Six mutants failed to cause lesions on watermelon fruits. They were also non-pathogenic on watermelon foliage and unable to induce an HR on tobacco leaves.

Three transposon mutants were disrupted in T3SS associated genes. The T3SS is the main determinant of *A. citrulli* virulence on watermelon fruits, which is consistent with previous studies on cucurbit foliage (Bahar and Burdman 2010; Johnson et al. 2011; Liu et al. 2012). The three Tn5 mutants (*Aave_0464* (HrpE/YscL family), *Aave_0463* (*hrcN*), and *Aave_0446* (*hpaB*)), along with the 00-1Δ*hrcC* confirmed that T3SS is required for *A. citrulli* pathogenicity on watermelon fruits.

The *A. citrulli* AAC00-1 Tn5 mutant 88E6 was disrupted in the *Aave_0464* gene, which belongs to the HrpE/YscL family. The nomenclature of this T3SS

protein family varies across all genera (Pallen et al. 2006), including HrpE in *P. syringae*, HrpL in *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), and HrpF in *Ralstonia solanacearum*. A hrpE mutant of *P. syringae* pv. *syringae* 61 failed to elicit an HR in both *N. tabacum* and *N. benthamiana* (Ramos et al. 2007). HrpE interacts with T3E HopV1 (Tian 2010). The cognate chaperone of HopV1, ShcV was shown to interact with HrcN ATPase, and HrpE was inferred to negatively regulate HrcN (Tian 2010).

The *A. citrulli* AAC00-1 Tn5 mutant 90H6 was disrupted in the *Aave_0463* gene (*hrcN*). *hrcN* encodes for T3S apparatus ATPase, which provides energy for effector translocation. In addition to energizing the translocation process, HrcN is also involved in the timing of effector release by dissociating effectors bound to the global chaperone HpaB (Lorenz and Büttner 2009). Consistent with this study, Liu et al. (2012) screened a Tn5 mutant library of *A. citrulli* group I strain FC440 for altered pathogenicity on cucumber cotyledons and found a non-pathogenic mutant that was impaired in *hrcN*. The FC440 Tn5 mutant was also unable to induce an HR in tobacco leaves.

The *A. citrulli* AAC00-1 Tn5 mutant 88A11 was disrupted in the *Aave_0446 (hpaB).* The *A. citrulli* HpaB sequence shared 60% identity with *R. solanacearum* and 53% with *Xcv*. HpaB is a general T3S chaperone that promotes the secretion of several T3Es and prevents the translocation of non-effectors of *Xcv* (Büttner et al. 2004) and *R. solanacearum* (Lonjon et al. 2017) into the plant cell. HpaB is strictly required for disease development and HR induction on tobacco plants (Büttner et al. 2004; Lonjon et al. 2017). *Acidovorax*

*citrulli* Tn5 88A11 mutant showed similar phenotypes as *hpaB* mutants of *Xcv* and *R. solanacearum.* Thus, the loss of pathogenicity phenotype may be explained by the defect of the secretion of numerous T3Es.

The other three Tn5 mutants, 90A1, 87D3, and 90E2 were disrupted at a transposase gene, *Aave_0691,* and 4 bp before *Aave_4431,* respectively. The Tn5 transposon mutants did not elicit an HR on tobacco. The unmarked knockout mutants of *Aave_0691* and *Aave_4431* did not show the same virulence phenotypes as the corresponding Tn5 transposon mutants. On the other hand, the insertion mutant 00-1Δ4431Tn5, generated to duplicate the insertion event of 90E2, did not elicit an HR on tobacco. However, the mechanisms of these Tn5 mutants being HR-negative were unclear. *Aave_0691* is relatively close (approximately 239 - 269 kb) to the T3SS gene cluster (*Aave_0444* - *Aave_0474*). So it is possible that the transposon insertion affected the transcription or translation of the T3SS genes. For the 90E2 mutant, the transposon was inserted between *Aave_4430* and *Aave_4431,* which was located in the flagellar gene cluster. A hypothetical protein gene *fliTX* was located in the flagellar gene cluster of *X oryzae* pv. *oryzae* and its deletion mutant, Δ*fliTX,* lost the ability to elicit an HR on tobacco (Yu et al. 2017). Thus, it is possible that the HR-negative phenotype of 90E2 was due to altered gene expression of genes in the flagellar gene cluster.

*Aave_4431* is required for *A. citrulli* swimming motility. This was consistent with the report from Kim et al. (2008), a mutation of HAP3 in *Vibrio vulnificus* led to a complete loss of motility. The virulence phenotype of 00-1*ΔAave_4431* was

also consistent with the report from Liu et al. (2012) that their HAP3 transposon mutant in the *A. citrulli* group I strain FC440 showed reduced virulence on cucumber cotyledons. However, further experiments are needed to explain the lack of pathogenicity of 90E2.

In conclusion, T3SS of *A. citrulli* is essential for inducing an HR on tobacco and causing BFB symptoms on watermelon foliage and fruits. Tn5 transposon mutants 87D3 and 90E2 failed to induce an HR on tobacco, and this was further confirmed by targeted insertion mutation to reproduce the Tn5 insertion event. However, the clean knockout mutants of the inserted genes associated with 87D3 and 90E2, *Aave_0691* and *Aave_4431,* respectively, did not show the same phenotypes, suggesting that *Aave_0691* and *Aave_4431* are not important for HR induction. Other genes affected by the Tn5 transposon insertion await further characterization.

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Table 5.1 Strains and plasmids used in this study.

| Strains and plasmids | Relevant characteristics | Reference or source |
|---|---|---|
| *Acidovorax citrulli* (AAC) | | |
| AAC00-1 | Wild-type, group II | R Walcott collection |
| 00-1Δ*hrcC* | *hrcC* deletion mutant | (Johnson et al. 2011) |
| 00-1Δ*Aave_0691*-A11 | *Aave_0691* deletion mutant, $Km^R$ | This study |
| 00-1Δ*Aave_0691*-A2 | *Aave_0691* deletion mutant | This study |
| 00-1Δ*Aave_0692-6* | *Aave_0692-Aave_0696* deletion mutant | This study |
| 00-1Δ4431 | *Aave_4431* deletion mutant | This study |
| 00-1Δ4431Tn5 | Insertion mutant to reproduce 90E2 mutant, $Km^R$ | This study |
| AAC211-2 | Wild-type, group II | R. Walcott collection |
| AAC96-6 | Wild-type, group II | R. Walcott collection |
| AAC92-303 | Wild-type, group II | R. Walcott collection |
| AAC98-17 | Wild-type, group I | R. Walcott collection |
| AAC92-300 | Wild-type, group I | R. Walcott collection |
| AAC203-16 | Wild-type, group I | R. Walcott collection |
| *Escherichia coli* | | |
| DH5α | $F^-$φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r^-_K$ $m^+_K$ ) *phoA glnV44* | NEB, Ipswich, MA |
| Rho5 | attTn7::*pir116*$^+$ *thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA* integrated RP4-2 $Tc^r$::Mu (λ*pir*$^+$)Δ*asd*::*FRT* Δ*aphA*::*FRT* | (Kvitko et al. 2012) |
| MaHI | $F^-$φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r^-_K$ $m^+_K$ ) *phoA glnV44* attTn7::*pir116*+ | (Kvitko et al. 2012) |
| Plasmid | | |
| p4916 | PCR8GW-Kan-3, $Km^R$, $Sp^R$ | (Traore 2014) |
| p3692 | pLVC18L/SacB/CcdB, $Tet^R$, destination vector, suicide vector | (Traore 2014) |

| | | |
|---|---|---|
| pK18*mobsacB* | Suicide vector, $Km^R$, $sacB^+$ | (Schäfer et al. 1994) |

$Km^R$, $Sp^R$, and $Tet^R$ indicate resistant to kanamycin, spectinomycin, and tetracycline, respectively.

Table 5.2 AAC00-1 Tn5 transposon mutants that failed to cause disease on immature watermelon fruits by pin-prick inoculation.

| Mutant | Insertion locus |
|---|---|
| 88E6 | *Aave_0464* (Type 3 secretion (T3S) apparatus protein, HrpE/YscL family, *hrp* (hypersensitive response and pathogenicity)) |
| 90H6 | *Aave_04e* (*hrcN,* T3S apparatus ATPase*)* |
| 90A1 | Transposase (16 matches in 00-1 genome) |
| 87D3 | *Aave_0691* (hypothetical protein; also annotated as type 6 secreted protein) |
| 90E2 | The insertion point was 4 bp before the start codon of *Aave_4431*, flagellar hook-associated protein 3 |
| 88A11 | *hpaB* (hrp-associated) |

Table 5.3 Oligonucleotide primers used in this study.

| Primer | Sequence (5' – 3') | Function |
|---|---|---|
| 0691Up-FWD | CGCCCTTATTTAAATGGATCCCGCGTTCACGTTTGCGCGC | To amplify 1,000 bp upstream of *Aave_0691* |
| 0691Up-REV | AGCAGCTCCAGCCTACACAAGGCTGTGCTCGCTGCAAGCT | |
| 0691FRT-FWD | AGCTTGCAGCGAGCACAGCCTTGTGTAGGCTGGAGCTGCT | To amplify 1,471 bp FRT-Kan region |
| 0691FRT-REV | GTCAAACCGGGGCAACTTCATATCCTCCTTAGTTCCTATT | |
| 0691Down-FWD | AATAGGAACTAAGGAGGATATGAAGTTGCCCCGGTTTGAC | To amplify 1,000 bp downstream of *Aave_0691* |
| 0691Down-REV | AATTCGCCCTTACTAGTCGACGGGCGCACACCCATCTCGG | |
| 0689-confirmF | AGCTCATGGTGATGCAGACT | To confirm the deletion of 00-1ΔAave_0691-A11 |
| FRT-confirmR | TTCTGCGGACTGGCTTTCTA | |
| 0691A-F | CTATGACATGATTACGAATTtcacgtttgcgcgctcgcat | To amplify 1,000 bp upstream of *Aave_0691* |
| 0691A-R | ccggggcaacttcactatccggtcatggctgtgctcgctg | |
| 0691B-F | cagcgagcacagccatgaccggatagtgaagttgccccggt | To amplify 1,000 bp downstream of *Aave_0691* |
| 0691B-R | CTTGCATGCCTGCAGGTCGAcacacccatctcggcgcagc | |
| 0691OutF | GCCTTGCGCTACCTGGATA | To screen for putative Δ*Aave_0691* colonies |
| 0691OutR | TTCTCAAGCCGGGATTTGGT | |
| 0692A-FWD | GGTACCCGGGGATCCTCTAGATGGCCGAACCATCATCGGC | To amplify 1,000 bp upstream of *Aave_0692* |
| 0692A-REV | GTTGGCATGCATACTGAATGCATTTCGAACTCCTTGCTCT | |
| 0697B-FWD | AGAGCAAGGAGTTCGAAATGCATTCAGTATGCATGCCAAC | To amplify 1,000 bp downstream of *Aave_0696* |
| 0697B-REV | ACGACGGCCAGTGCCAAGCTCAGAAGACAACGGGCCCTTT | |
| 0692OutF | tgcagatcgaacgcttcaac | To screen for putative |

| | | |
|---|---|---|
| 0696OutR | gtgatagccgcccgtgtg | Δ*Aave_0692-6* colonies |
| 4431A-FWD | CTATGACATGATTACGAATTgttcaccgtgcccgccagca | To amplify 1,000 bp upstream of *Aave_4431* |
| 4431A-REV | ttgtctcgttcggcttagccgctcatggcgtgctcggtag | |
| 4431B-FWD | ctaccgagcacgccatgagcggctaagccgaacgagacaagcgg | To amplify 1,000 bp downstream of *Aave_4431* |
| 4431B-REV | CTTGCATGCCTGCAGGTCGAcggcgtcgagcaggcgctcg | |
| 4431OutF | CGCTTCCACAACAACGATCT | To screen for putative |
| 4431OutR | ATCACGCGTAATGTCTTGGC | Δ*Aave_4431* colonies |
| KAN-2 FP-1 | ACCTACAACAAAGCTCTCATCAACC | To confirm the mutation of 00-1Δ4431Tn5 together with 4431OutF |

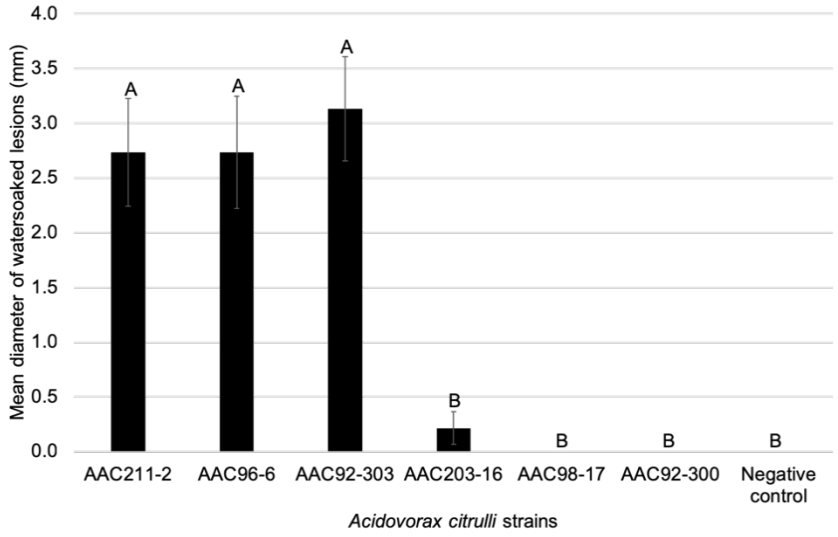


Fig 5.1 Mean lesion diameters induced by six different *Acidovorax citrulli* strains representing groups I and II on detached watermelon (cv. 'Crimson Sweet') fruits after pin-prick inoculation. Group I strains include AAC203-16, AAC98-17, and AAC92-300. Group II strains include AAC211-2. AAC96-6, and AAC92-303. Female watermelon flowers were pollinated and, after 4 to 7 days, fruits were harvested, cut in halves longitudinally, and pin-prick inoculated with 10 µl of cell suspensions (approximately $10^8$ CFU/ml) of each strain. Fruit halves were incubated for 8 days at 28°C and 100% relative humidity and lesion diameters were measured. Each treatment was replicated in 15 different fruit halves. Bars represent the mean lesion diameter, and lines represent the standard error of the mean. Different letters indicate significant differences (P = 0.05) among treatments according to Tukey-Kramer's honestly significant difference test. This experiment was conducted twice with similar results.

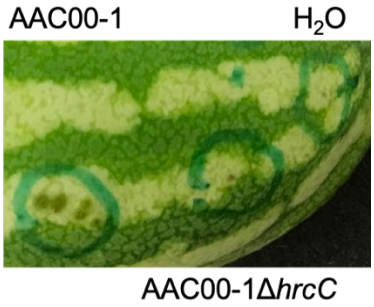


Fig 5.2 Role of the *Acidovorax citrulli* type 3 secretion system on detached immature watermelon (cv. 'Crimson Sweet') fruits after pin-prick inoculation. The strains include group II strains AAC00-1 and AAC00-1Δ*hrcC*. Under greenhouse conditions, female flowers were pollinated and, after five days, watermelon fruits were harvested, cut in halves longitudinally, and pinprick inoculated with 10 µl of cell suspensions (approximately $1 \times 10^8$ CFU/ml). Fruit halves were incubated at 28°C and 100% relative humidity and the picture was taken at 8 days post inoculation. Each treatment was replicated in four different fruit halves. This experiment was conducted three times with similar results.

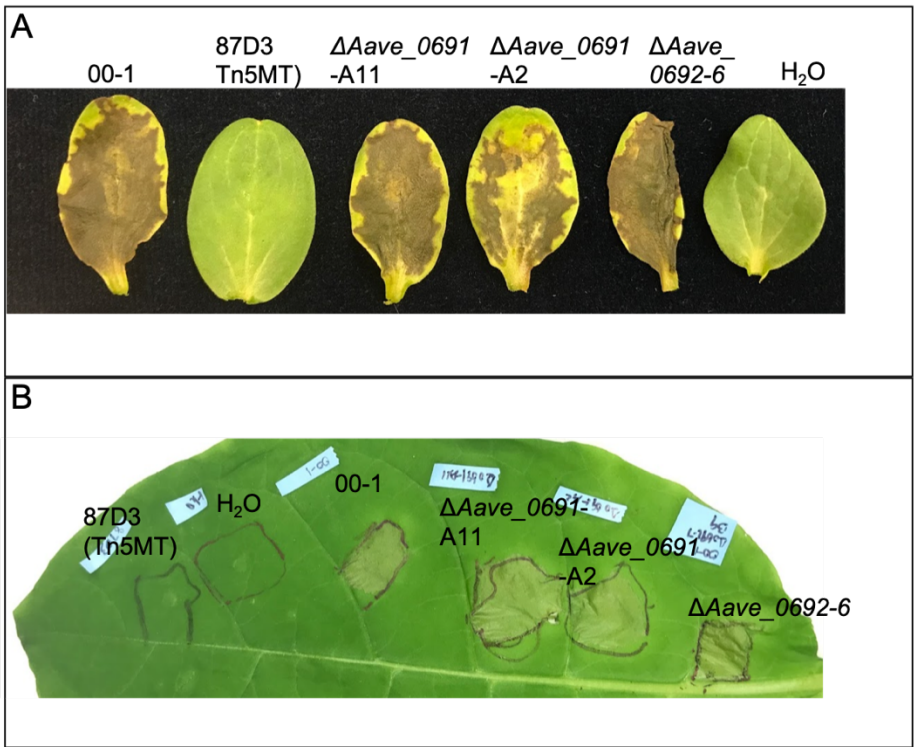


Fig 5.3 Virulence and hypersensitive response (HR) induction of *Acidovorax citrulli* mutants 87D3 and deletion mutants of *Aave_0691* and *Aave_0692-6*. A) *Acidovorax citrulli* mutants of *Aave_0691* and *Aave_0692-6* showed similar virulence as AAC00-1, while the 87D3 mutant showed a loss of virulence on watermelon cotyledon with the syringe-infiltration assay. B) The HR of tobacco leaves induced by *A. citrulli* strains AAC00-1, 87D3, 00-1Δ*Aave_0691*-A11, 00-1Δ*Aave_0691*-A2, and 00-1Δ*Aave_0692-6*. Each strain was syringe infiltrated into tobacco leaf panels at $10^8$ CFU/ml, and the picture was taken 24 h after infiltration. The experiments were conducted twice.

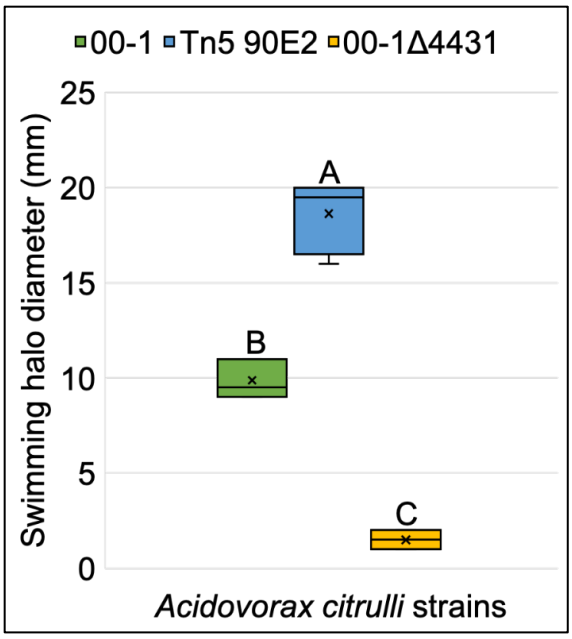


Fig 5.4 Box plot of swimming motility of *Acidovorax citrulli* strains AAC00-1, its Tn5 transposon mutant 90E2, and the deletion mutant 00-1Δ4431. Single colonies were stab-inoculated into soft agar medium (10 g tryptone, 5 g NaCl, 3 g agar per liter). Plates were incubated at 28°C for 1 day. The diameter of the zone of migration was measured in two directions on four different plates. 'x' represents the means. Different letters indicate significant differences (P = 0.05) among treatments according to Tukey-Kramer's honestly significant difference test. The experiment was conducted twice with similar results.

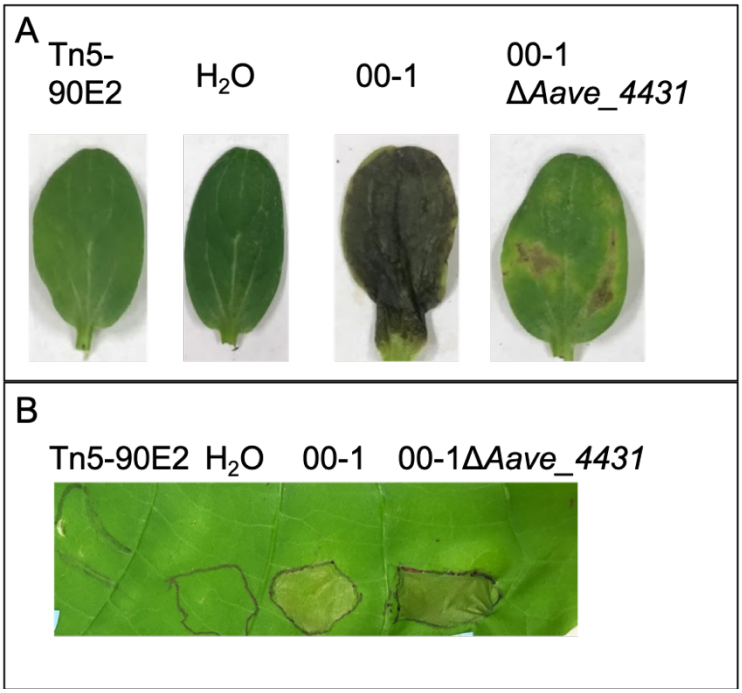


Fig 5.5 Virulence and hypersensitive response (HR) induction of *Acidovorax citrulli* strains AAC00-1, 90E2, and 00-1Δ4431 mutant. A) *Aave_4431* mutant showed reduced virulence, while the 90E2 mutant showed a loss of virulence on watermelon cotyledon with the syringe-infiltration assay. B) The HR of tobacco induced by *A. citrulli* strains AAC00-1, 90E2, and 00-1Δ4431. Each strain was syringe infiltrated into tobacco leaf panels at $10^8$ CFU/ml, and the picture was taken 24 h after infiltration. The experiment was conducted twice with similar results.

# CHAPTER 6

# *ACIDOVORAX CITRULLI* IS SENSITIVE TO ELEVATED TEMPERATURES DURING EARLY STAGES OF WATERMELON SEED GERMINATION [1]

[1] Zhao, M., and Walcott R. R. Submitted to *Seed Science and Techonology*, 5/13/2019.

**Abstract**

Bacterial fruit blotch (BFB), caused by *Acidovorax citrulli,* is a seed-transmitted disease of cucurbit crop species. During seed-to-seedling transmission of BFB, *A. citrulli* initially grows as a saprophyte on germinating seeds and subsequently switches to a pathogenic mode. We investigated the effect of temperature on the colonisation of germinating watermelon seeds/seedlings by *A. citrulli*. Watermelon seeds were vacuum-infiltrated with $10^6$ CFU/ml *A. citrulli*, germinated at 28°C and 100% relative humidity, and transferred to 40°C at different time intervals. Mean BFB incidence was significantly lower for seeds that were sown at 28°C and transferred to 40°C at 3 days after sowing (das), compared to seeds incubated constantly at 28°C. Inoculated seeds showed reduced mean BFB transmission percentages when transferred from 28°C to 40°C at 3 das, regardless of initial *A. citrulli* concentration. Moreover, the effect of increased temperature on BFB seedling transmission was reversible regardless of the initial *A. citrulli* seed inoculum concentration. Furthermore, the *A. citrulli* population on germinating watermelon seedlings that were transferred from 28°C to 40°C at 3 das was significantly lower than that of seedlings maintained at 28°C. We conclude that *A. citrulli* cells associated with germinating watermelon seeds are more sensitive to elevated temperature during the first 3 das relative to the later days. This information might inform the development of novel seed treatment strategies for BFB management.

**Keywords:** BFB, seed-to-seedling transmission, seed pathology, temperature

## Introduction

Bacterial fruit blotch (BFB) is a devastating plant disease caused by the Gram-negative, seedborne bacterium, *Acidovorax citrulli (syn. Acidovorax avenae* subsp*. citrulli)* (Schaad et al. 2008; Schaad et al. 1978; Willems et al. 1992) that affects a wide range of hosts in the Cucurbitaceae family, including watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai).

*Acidovorax citrulli*, the causal agent of BFB was first isolated by Webb and Goth in 1965 at the Regional Plant Introduction Station in Griffin, Georgia, USA (Webb and Goth 1965). The phytopathogenic bacterium, isolated from seedlings of watermelon plant introductions (PI 174103 and PI174104) collected from Turkey, was unidentified, but determined to be seedborne (Webb and Goth 1965). Subsequently, Schaad et al. (1978) identified the causal agent as *Pseudomonas pseudoalcaligenes* subsp*. citrulli* and designated the type strain, American Type Culture Collection (ATCC) 29625. The first natural, commercial BFB outbreak occurred in the Mariana Islands in 1987 on watermelon fruits (Wall and Santos 1988) and in 1989, the disease was observed in commercial watermelon fields in Florida (Somodi et al. 1991) and Indiana (Latin and Rane 1990). Subsequently, BFB outbreaks have occurred worldwide, primarily on watermelon and melon crops, resulting in significant economic losses (Burdman and Walcott 2012). BFB has a wide geographical distribution, likely due to the global production and trade of cucurbit seeds. BFB occurs sporadically but has

the potential to negatively impact the global cucurbit industry, including seed, transplant, and fruit production. In fact, *A. citrulli* is a quarantined pest in Israel, China, and Europe Union countries (Feng et al. 2013). *A. citrulli* was recently added to the European Plant Protection Organization (EPPO) region quarantine A1 List (EPPO 2018).

Because infested seeds are important sources of primary inoculum for BFB epidemics, seed treatment is a critical component of disease management. In general, commercial seed treatments include thermotherapy, and various antimicrobial chemicals including peroxyacetic acid, HCl, and CaOCl (Fan et al. 2017; Hopkins et al. 1996; Hopkins et al. 2003; Johnson et al. 2011). Overall, most seed treatments reduce seedborne inoculum on seed surfaces; but fail to eliminate bacteria from seeds that are internally contaminated by *A. citrulli* without negatively affecting seed germination or seedling establishment (Feng et al. 2009b; Rane and Latin 1992). Thus, at present, seed treatments alone have not been able to control BFB.

Seed-to-seedling transmission, defined in this study as disease development and measured by incidence during the seedling phase, is an important component of the BFB disease cycle. Dutta et al. (2012b) reported that seed inoculum loads as low as 10 *A. citrulli* CFU/seed could lead to BFB seed-to-seedling transmission under greenhouse conditions. Hence, there is a low tolerance for seedborne *A. citrulli* inoculum. Additionally, Johnson et al. (2011) suggested that *A. citrulli* switches from saprophytic to pathogenic growth on watermelon seeds approximately 4 days after sowing (das). They showed that a

non-pathogenic type 3 secretion system (T3SS) mutant of *A. citrulli* retained the ability to colonize germinating watermelon seeds at wild-type levels, even though it was incapable of inciting disease. Finally, Dutta et al. (2014a) reported that by flower inoculation, bacterial pathogens, including *A. citrulli,* can infest the seeds of non-host plants and can be transmitted as epiphytes to resulting seedlings. Despite these reports, the process of seedling colonisation by *A. citrulli* and seed-to-seedling transmission of BFB is poorly understood.

To understand the factors that influence seed-to-seedling transmission of BFB, we investigated the effect of elevated temperature. Preliminary observations revealed that BFB seed-to-seedling transmission was reduced for artificially inoculated watermelon seeds germinated at 37°C compared to 28°C, we hypothesized that elevated temperature negatively affects the ability of *A. citrulli* to colonize certain stages of germinating watermelon seeds. Hence, the objective of the current study was to investigate how temperature affects *A. citrulli* colonisation of watermelon seeds/seedlings during the period when the bacterium is switching from saprophytic to pathogenic survival mode. Specifically, we aimed to test 1) the effect of temperature on seed-to-seedling transmission of BFB of watermelon, 2) if initial *A. citrulli* seed inoculum concentration affected BFB seedling transmission at different temperatures; 3) the effect of the duration of elevated temperature on BFB seedling transmission; and 4) the effect of temperature on *A. citrulli* population growth on germinating watermelon seeds examined by qPCR.

**Materials and methods**

*Bacterial cultures and inoculum preparation.* The *A. citrulli* group II strain AAC00-1 (Ron Walcott, University of Georgia, Athens GA) and group I strain M6 (Saul Burdman, Hebrew University of Jerusalem, Rehovot Israel) was routinely cultured on nutrient agar (Becton Dickinson, Sparks, MD). For seed inoculation, *A. citrulli* was cultured in nutrient broth at 28°C on a rotary shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm for 24 h. Subsequently, cultures were centrifuged at 16,100 g for 1 min and the supernatant was discharged. The resulting pellets were washed with and resuspended in sterilized distilled water ($sdH_2O$). The bacterial concentrations were then adjusted to an optical density of 0.3 at 600 (~ $10^8$ colony forming units (CFU/ml) (Spectronic 20; Bausch and Lomb, Rochester, NY).

*Effect of temperature on seed-to-seedling transmission of BFB of watermelon.* Watermelon seeds (cv. Crimson Sweet) were vacuum-infiltrated with cell suspensions (~$10^6$ CFU/ml) of *A. citrulli* strains AAC00-1 or M6 for 40 min (with interruptions in the vacuum at 10 min intervals). Treated seeds were air-dried overnight at room temperature and treated with the fungicide, Captan (active ingredient: 2-(trichloromethylsulfanyl)-3a,4,7,7a-tetrahydroisoindole-1,3-dione) (Hi-yield, Bonham, TX) (2.5 mg per seed) to limit the growth of saprophytic fungi. Seeds (*n*=30 for each treatment) were then sown individually, each in a separate capped 14-ml glass test tube on cotton balls saturated with sdH2O. Test tubes were incubated at 28°C and 100% relative humidity (RH) with continuous fluorescent light in a growth chamber (Percival, Perry, IA). The plants

incubated at 28°C for the entire experiment severed as the control treatment. At 3, 5, or 7 das, seedlings (*n*=30 for each time point) were transferred to 40°C and BFB incidence on cotyledons of the seedlings was visually evaluated daily until 14 das. Usually, at 3 das, radicles emerged from the watermelon seeds, and at 4-5 das, cotyledons developed. BFB incidence was calculated as follows: (Number of symptomatic seedlings / Number of germinated seeds) $\times$100. This experiment was carried out four times. Analysis of variance (ANOVA) was conducted on BFB incidence (%) data using JMP statistical analysis software (version Pro 13; SAS Institute Inc., Cary, NC). The effect of temperature switch time (3, 5, and 7 das) on BFB incidence was compared using Tukey-Kramer's honestly significant difference (HSD).

*Effect of initial A. citrulli inoculum concentration on seed-to-seedling transmission of BFB under two different temperature regimes.* Watermelon seeds (cv. Crimson Sweet) were individually vacuum-infiltrated with 10 µl *A. citrulli* strain AAC00-1 cell suspensions containing $10^8$, $10^6$, and $10^4$ CFU/ml ($10^6$, $10^4$, and $10^2$ CFU/seed) as previously described (Dutta et al. 2012b). Briefly, two holes were created in each seed with a sterilized dissecting needle (Carolina Biological Supply Company, Burlington, NC). The first hole was close to the micropylar end of the seed and the second was on the chalazal end of the seed. With the aid of a 1 ml pipette tip, a vacuum was applied to one hole, and the inoculum suspension was aspirated into the seed through the other hole. Inoculated seeds (*n*=30 seeds) were treated with Captan and planted individually in separate, capped test tubes as described above. Seeds were incubated at

28°C and 100% RH with continuous fluorescent light in a growth chamber. At 3 das, seedlings were transferred to a growth chamber set at a constant 40°C (all other conditions were identical). BFB incidence on seedlings was visually evaluated daily until 14 das, as described above. The experiment was carried out four times and BFB incidence data were used to compare the effect of initial inoculum concentration on BFB seed-to-seedling transmission using Tukey-Kramer's HSD.

*Effect of duration of elevated temperature on BFB seedling transmission.* To determine the effect of the duration of elevated temperature on BFB seedling transmission, watermelon seeds (*n*=30) were individually vacuum-infiltrated with *A. citrulli* strain AAC00-1 to achieve concentrations of $10^4$ or $10^2$ CFU/seed. Seeds were then treated with Captan and planted individually in capped test tubes with cotton balls saturated with $sdH_2O$. Seeds were incubated at 28°C and 100% RH with continuous fluorescent light and at 3 das, seedlings were transferred to 40°C (all other conditions were identical). At 5 das, seedlings were transferred to a 28°C incubator. Seeds inoculated with corresponding inoculum concentrations and maintained at 28°C for 14 days served as controls. BFB incidence was visually observed and recorded at 14 das. The experiment was carried out three times. BFB incidence data at 14 das were used to compare the effect of the temperature regimes using Tukey-Kramer's HSD.

*Effect of temperature on A. citrulli population growth on germinating watermelon seeds examined by qPCR.* Watermelon seeds (cv. Crimson Sweet) were vacuum-infiltrated with AAC00-1 (~$10^2$ CFU/seed), treated with Captan

fungicide, as described above and planted individually in capped test tubes at 28°C and 100% RH. Seedlings were transferred to 40°C at 3 das and at 3, 5, 7, 9, 11, and 13 das, samples (*n* = 4 seedlings/treatment) were crushed individually in microcentrifuge tubes containing 1.1 ml of $sdH_2O$. Seven hundred microliters of the macerated tissue were transferred to a clean microcentrifuge tube and centrifuged at 16,100 x g for 1 min. The supernatant was discarded and the pellet was subjected to DNA extraction (E.Z.N.A Bacterial DNA Kit, OMEGA Bio-Tek, Norcross, GA). Total DNA from each sample was concentrated into 50 µl of elution buffer and 5 µl of each sample was used for quantitative real-time PCR using the BOX primer/probe set as previously described (Ha et al. 2009). The following thermal profile was used for amplification: denaturation at 95°C for 180 s, followed by 35 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 40 s (Ha et al. 2009). Cycle threshold (Ct) values less than 35 were considered positive for *A. citrulli* and were converted into $Log_{10}$ CFU/seed or seedling based on a standard curve (Ha et al. 2009). The experiment was carried out three times and mean bacterial populations were plotted over time to generate area under population growth curve (AUPGC) data. Student t-test was conducted on AUPGC data to compare the effects of different temperature regimes.

**Results**

*Effect of temperature on seed-to-seedling transmission of BFB of watermelon.* The effect of *A. citrulli* strain on BFB incidence was not significant (P = 0.16), hence, data from the two strains were pooled for statistical analysis.

Increasing the incubation temperature from 28°C to 40°C at 3 das significantly reduced BFB seed-to-seedling transmission on watermelon seeds artificially inoculated with *A. citrulli* (Fig 6.1). At 14 das, we observed a significantly ($P = 0.03$) lower mean BFB incidence (54%) for seedlings transferred to 40°C at 3 das compared to seeds incubated at 28°C (89%) for the entire experiment. However, BFB incidences were not significantly different among seedlings that were germinated at 28°C and then transferred to 40°C at 3 (54%), 5 (69%), or 7 das (71%) (Fig 6.1).

*Effect of initial A. citrulli inoculum concentration on seed-to-seedling transmission of BFB under two different temperature regimes.* Increasing the incubation temperature from 28°C to 40°C at 3 das reduced BFB seed-to-seedling transmission regardless of initial *A. citrulli* seed inoculum concentration. Seeds inoculated with $10^6$, $10^4$, and $10^2$ *A. citrulli* CFU/seed had significantly ($P < 0.0001$) lower mean BFB incidences (51%, 32%, and 10%, respectively) when transferred from 28°C to 40°C at 3 das (Fig 6.2), compared to seeds with similar inoculum levels, but incubated at 28°C for 14 days (90%, 80%, and 58%, respectively).

*Effect of duration of elevated temperature on BFB seedling transmission.* The effect of increased temperature on BFB seedling transmission was reversible regardless of the initial *A. citrulli* seed inoculum concentration. For watermelon seeds inoculated with $10^4$ *A. citrulli* CFU/seed, increasing incubation temperature from 28°C to 40°C at 3 das significantly ($P=0.0012$) reduced mean BFB seedling incidence (47%), compared to seedlings incubated at 28°C for 14

days (92%); but returning seedlings to 28°C at 5 das increased BFB incidence (89%) (Fig 6.3).

For seeds inoculated with $10^2$ *A. citrulli* CFU/seed, increasing seedling incubation temperature from 28°C to 40°C at 3 das also significantly ($P < 0.0001$) reduced mean BFB seedling incidence (12%), compared to the seedlings incubated at 28°C for 14 days (80%). Returning watermelon seedlings to 28°C at 5 das increased BFB incidence (64%) (Fig 6.3).

*Effect of temperature on A. citrulli population growth on germinating watermelon seeds.* For seedlings maintained at 28°C for the entire experiment, the average *A. citrulli* population density increased from $1.62 \times 10^4$ CFU/seedling at 3 das to $8.91 \times 10^6$ CFU/seedling at 13 das (Fig 6.4). However, *A. citrulli* populations increased only slightly when seedlings were transferred from 28°C to 40°C, reaching a density of $4.57 \times 10^4$ CFU/seedling at 13 das (Fig 6.4). Based on the AUPGC data, the *A. citrulli* population on germinating watermelon seedlings that were transferred from 28°C to 40°C at 3 das (AUPGC = 46) was significantly lower than that of seedlings maintained at 28°C (AUPGC = 52) ($P = 0.03$).

**Discussion**

Temperature has long been considered a key determinant in disease epidemic development. The effect of temperature on disease outbreaks varies depending on the pathosystem e.g., plant diseases caused by Pseudomonads generally prefer cooler temperatures (Moreno and Rojo 2014). However, there have been no detailed studies describing the effect of changes in temperature on

BFB seedling transmission. Here we report that elevated temperature (40°C) negatively affects the ability of *A. citrulli* to colonize germinating watermelon seeds. More interestingly, we observed that the effect of transferring seedlings to a higher temperature was more pronounced when done at an early phase than at later seedling developmental stages.

*Acidovorax citrulli* has been reported to grow at 41°C (Schaad et al. 2001), which is higher than many plant pathogenic bacteria. Recently, Silva et al. reported a differential effect of temperature on *in vitro* growth of representative group I and II *A. citrulli* haplotypes (Silva, G. M. et al. 2016). Specifically, of 18 group II strains tested, all grew at 40 and 41°C, whereas only 3 of 15 group I strains grew at 40°C (Silva, G. M. et al. 2016). Despite this, we did not observe a significant difference between representative group I (M6) and II (AAC00-1) strains in BFB seed-to-seedling transmission at elevated temperatures in preliminary studies (data not shown). Surprisingly, our preliminary work showed that BFB seed-to-seedling transmission on watermelon was lower at 37°C compared to 28°C, regardless of *A. citrulli* group (data not shown).

In the current study, we showed that BFB seed-to-seedling transmission was significantly reduced when seedlings that developed from artificially inoculated seeds were transferred from 28°C to 40°C at 3 das. However, when seedlings were transferred to 40°C later than 3 das there was no significant reduction in BFB incidence. These data suggest that *A. citrulli* cells in the spermosphere of watermelon seeds at 3 das were more sensitive to temperature stress. This phenomenon was observed for all initial seed inoculum

concentrations tested. We also observed a similar level of BFB incidence after watermelon seedlings were transferred from 28°C to 40°C at 3 das and returned to 28°C at 5 das (Fig 6.3). This indicates that the effect of elevated temperature at 3 das is reversible. In addition, we showed that when germinating seedlings were incubated at 40°C, *A. citrulli* populations increased more slowly than at 28°C (Fig 6.4). The ability of bacteria to colonize germinating seeds is an important step for pathogen/disease transmission to seedlings. Thus, we speculate that the lower disease incidence observed when artificially inoculated watermelon seeds were incubated at 40°C was due to lower *A. citrulli* population growth when exposed to elevated temperatures.

In summary, elevating temperature to 40°C at 3 das significantly, but reversibly reduced BFB seed-to-seedling transmission with watermelon seeds artificially inoculated with *A. citrulli*. These observations suggest that *A. citrulli* cells are sensitive to increased temperature during the initial stages of seed germination. In other words, we speculate that *A. citrulli* cells may be more vulnerable, or in a different physiological state at 0-3 das than at later stages of watermelon seedling development. Additionally, 3 das represents a vulnerable window for eliminating *A. citrulli* on germinating watermelon seedlings. Interestingly, this window coincides with, and may be related to, the switch from saprophytic to pathogenic growth reported for *A. citrulli* during seed-to-seedling transmission (Johnson et al. 2011). Johnson et al. reported that the ability of a non-pathogenic T3SS *A. citrulli* mutant, AAC00-1Δ*hrcC*, retained its ability to colonize germinating watermelon seeds. By 4 das its populations were not

significantly different from those of AAC00-1 (wildtype) or AAC00-1Δ*hrcC*comp (a complemented mutant strain) (Johnson et al. 2011). This saprophytic growth phase during the initial stage of germination is not unique to *A. citrulli,* as it was reported in other plant pathogenic bacteria, including *Pseudomonas syringae* pv. *syringae* B728a on beans (Hirano et al. 1999). Hirano et al. (1999) suggested that *P. syringae* pv. *syringae* does not behave like a parasite during bean germination, as similar population dynamics were observed for wild-type *P. syringae* pv. *syringae* and its T3SS mutants on germinating seedlings in field trials. Furthermore, Darrasse et al. showed that *Xanthomonas campestris* pv. *campestris* was seed-to-seedling transmitted in the non-host bean and reached populations of $1.9 \times 10^5$ CFU/leaf disc at 4 das (Darrasse et al. 2010). Interestingly, no significant plant defense responses were induced by the seed-associated bacteria in early stages of bean seedling development (Darrasse et al. 2010). Thus, they confirmed that bacterial colonisation of early-stage germinating seedlings does not depend on parasitism but rather, represents a transient saprophytic phase of growth (Darrasse et al. 2010). Based on the results of the current study, we posit that *A. citrulli* cells associated with germinating watermelon seeds may be more sensitive to elevated temperatures and possibly, other environmental stressors during the first 3 das, which coincides with the saprophytic growth phase. We hypothesize that this vulnerability declines as *A. citrulli* cells enter the pathogenic growth phase and suggest that transcriptome analysis of *A. citrulli* cells growing on watermelon seedlings at 3 and 5 das could aid the understanding of the factors that control *A.*

*citrulli* response to germinating watermelon seedlings. This line of research might lead to novel and effective strategies for managing seed-to-seedling transmission of BFB and other seedborne bacterial diseases.

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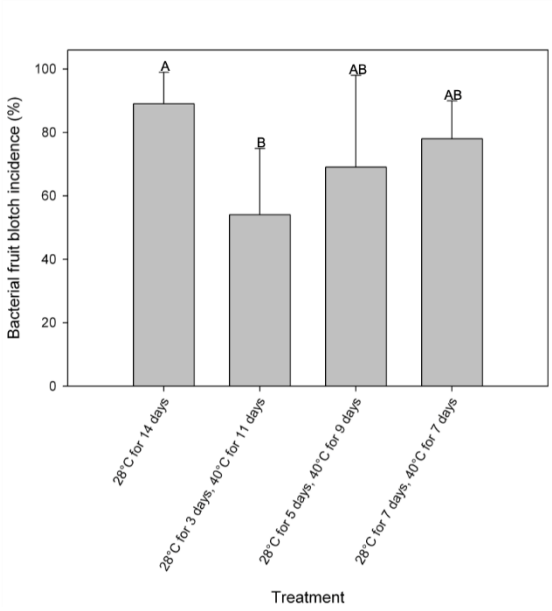


Fig 6.1 Bacterial fruit blotch incidence on watermelon seedlings that were vacuum infiltrated with *Acidovorax citrulli* and incubated at 28°C for 14 days, or incubated at 28°C and then transferred to 40°C at 3, 5, or 7 days after sowing (das). Watermelon seeds (*n* = 30) were vacuum-infiltrated with *A. citrulli* strain AAC00-1 and M6 cell suspensions (~$10^6$ CFU/ml) and then air-dried. Seeds were planted in individual capped, test tubes (one seed/tube) on cotton balls saturated with sterilized water. The experiment was carried out four times and the bars and lines represent the means and standard errors, respectively. Different letters indicate significant differences (P = 0.05) among treatments according to Tukey-Kramer's Honestly Significant Difference (HSD).

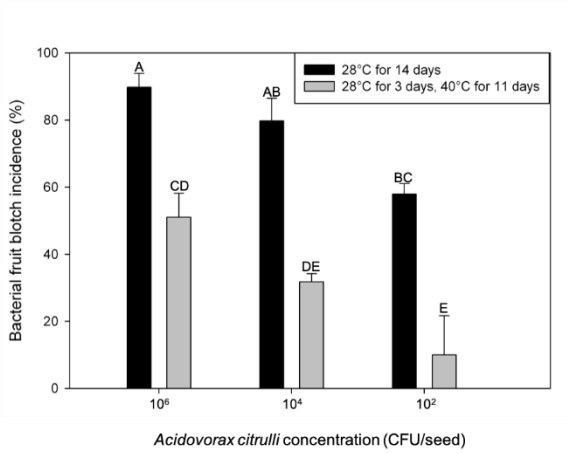


Fig 6.2 Effect of *Acidovorax citrulli* seed inoculum load on bacterial fruit blotch seedling transmission for watermelon seeds incubated at 28°C for 14 days after sowing (das), compared to seeds incubated at 28°C and transferred 40°C at 3 das. Seeds (*n*=30) were vacuum-infiltrated with *A. citrulli* strain AAC00-1 cell suspensions at $10^6$, $10^4$, and $10^2$ CFU/seed. The experiment was carried out four times and the bars and lines represent the means and standard errors, respectively. Different letters indicate significant differences (P = 0.05) among treatments according to Tukey-Kramer's HSD.

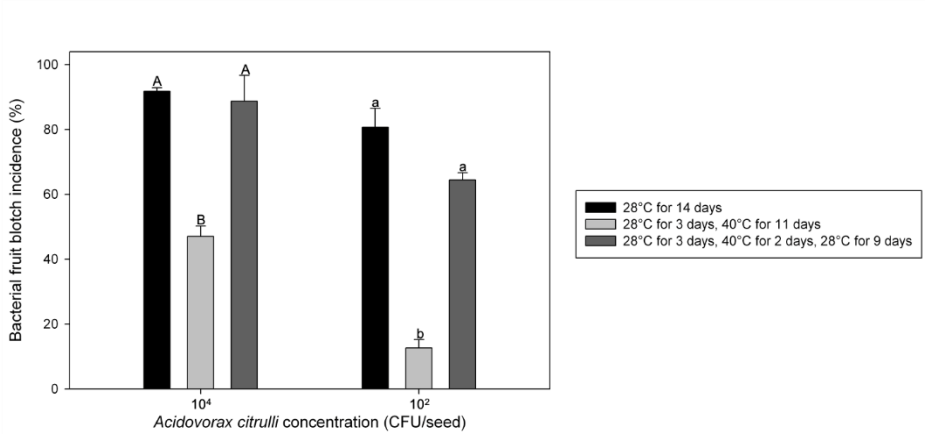


Fig 6.3 Bacterial fruit blotch seed-to-seedling transmission for artificially inoculated watermelon seed germinated at 28°C for 14 days after sowing (das); seeds germinated at 28°C for 3 das and then transferred to 40°C until 14 das; and seeds incubated at 28°C for 3 das, switched to 40°C for 2 days and then transferred to 28°C for 9 days. Seeds (*n* = 30) were vacuum-infiltrated with suspensions containing ~$10^4$ and $10^2$ CFU/seed of *Acidovorax citrulli* strain AAC00-1. The experiment was carried out three times and the bars and lines represent the means and standard errors, respectively. Different letters indicate significant differences (P = 0.05) among treatments according to Tukey-Kramer's HSD.

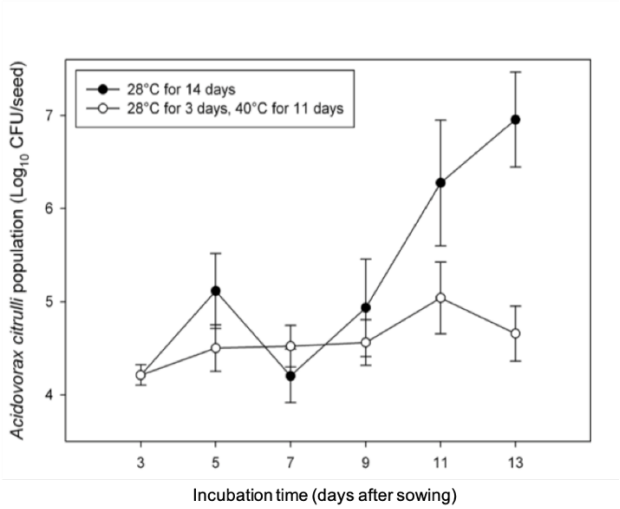


Fig 6.4 *Acidovorax citrulli* population growth on germinating watermelon seeds incubated at 28°C for 14 days after sowing (das), or 28°C for 3 das followed by 40°C for 11 das. Watermelon seeds were inoculated by vacuum-infiltration with *A. citrulli* strain AAC00-1 (~$10^2$ CFU/seed) and 3, 5, 7, 9, 11, and 13 das seedlings were crushed and subjected to microbial DNA extraction. DNA was subjected to quantitative real-time PCR using an *A. citrulli*-specific assay to estimate bacterial populations. Each data point represents the mean of three experiments, each with 4 replicates per treatment. Vertical lines represent standard errors of the means.

# CHAPTER 7

## SUMMARY

Bacterial fruit blotch (BFB) is an economically important seed-borne and seed-transmitted disease of cucurbitaceous plant species including watermelon and melon crops. BFB occurs sporadically but impacts the global cucurbit industry. It has the potential to affect the entire cucurbit production chain, including seed, transplant, and fruit production. *Acidovorax citrulli*, the causal agent of BFB, can be divided into two major groups, I and II (Zhao and Walcott 2018). Group II strains are associated with outbreaks in watermelon, while group I strains are associated with outbreaks in melon and other cucurbit crops. Additionally, group I strains are moderately to highly aggressive on a range of non- watermelon cucurbits, while strains in group II are highly aggressive on watermelon, but only mildly aggressive on other cucurbits. The natural association of group I and II *A. citrulli* strains with different cucurbit species strongly suggests host preference, evidenced by multiple population surveys (Feng et al. 2009a; Walcott et al. 2004; Yan et al. 2013) and anecdotal observations (Walcott et al. 2000). However, there are no direct data to support this host preference hypothesis under field conditions.

Despite the economic importance of BFB, little is known about fundamental aspects of *A. citrulli*-host interactions compared with other pathosystems. Importantly, significant yield losses by the disease are due to fruit

infection, but to date, most BFB research has focused on the vegetative plant stages. Moreover, it appears that the differences in host preferential association between group I and II strains are more pronounced in the fruit relative to the vegetative stages.

The overall goal of this study was to gain a better understanding of host preference and virulence of the two major groups of *A. citrulli*. Findings from these studies will provide basic knowledge about interactions between two *A. citrulli* groups and their cucurbit hosts, and might lead to the development of new strategies to manage BFB. Specific objectives were 1) to assess differences in cucurbit host preference between group I and II *A. citrulli* strains under field conditions; 2) to characterize melon fruit infection by *A. citrulli*; 3) to characterize the role of the putative type 3 secreted effector XopJ and HopAF homologs in *A. citrulli* virulence; 4) to identify unique virulence determinants of *A. citrulli* group II strains using an immature watermelon fruit assay; 5) to investigate the effect of elevated temperature on *A. citrulli* colonization of germinating watermelon seeds.

From 2017-2019, we used group I and II strains to initiate BFB outbreaks in field plots planted with four cucurbit species. At different times, we collected symptomatic tissues and assayed them for group I and II strains using a group-specific PCR assay. Binary distribution data analysis revealed that the odds of melon, pumpkin, and squash foliage infection by group I strains were 21.7, 11.5, and 22.1 times greater, respectively, than the odds of watermelon foliage infection by the group I strain ($p < 0.0001$). More strikingly, the odds of melon fruit infection by the group I strain were 97.5 times greater than watermelon fruit

infection by the same strain ($p < 0.0001$). Unexpectedly, some of the group II isolates recovered from the 2017 and 2019 studies were different from the group II strains used as inocula. These data confirmed that *A. citrulli* strains exhibited a preference for different cucurbit species, which was more pronounced in fruit tissues.

The field experiments showed significant differences in BFB incidence between representative group I and II *A. citrulli* strains on melon and watermelon fruits. However, no studies have explored why group I and II *A. citrulli* strains prefer melon and watermelon fruits, respectively. Thus, we aimed to investigate host preference among *A. citrulli* groups, focusing on melon fruits. Group I and II *A. citrulli* strains were inoculated on detached melon fruits by swab- and syringe-inoculation, and attached melon fruits by swab-inoculation. Group I and II *A. citrulli* strains penetrated melon fruit mesocarp tissues at similar levels 3 h after swab-inoculation, and colonized melon fruit tissues at similar population levels after syringe inoculation. By swab-inoculation of attached melon fruits, group I strains produced more severe symptoms and earlier. However, group II strains showed BFB symptoms at 10 and 20 dpi, but at a lower frequency. We conclude that bacterial penetration, colonization, and symptom development do not account for the host preference of *A. citrulli* on melon fruits. Further studies are needed to uncover the mechanisms of host preference of *A. citrulli.*

Despite the economic importance of BFB, knowledge about basic aspects of *A. citrulli*-plant interactions is relatively limited. Like many Gram-negative plant pathogenic bacteria, *A. citrulli* requires a functional type III secretion system for

pathogenicity to deliver effectors into the host cells. We created deletion mutants of XopJ and HopAF homologs to characterize their roles in *A. citrulli* virulence. We did not find detectable effects of XopJ homologs on *A. citrulli* virulence on watermelon and melon cotyledons. The deletion mutant of XopJ homologs was less virulent on attached melon fruits compared to the wild-type, while their population levels on melon fruits were variable across experiments. However, the HopAF homolog may be important for *A. citrulli* colonization and symptom development on watermelon and melon cotyledons. Further characterization of the mechanisms by which these effectors interact with different hosts is needed.

Watermelon fruits are often associated with group II *A. citrulli* strains. However, no fruit-specific virulence determinants have been reported for *A. citrulli* on watermelon. We developed an immature watermelon fruit assay to distinguish group I and II *A. citrulli* strains and used the assay to identify unique virulence determinants of *A. citrulli* group II strains. We found that only group II strains induced water-soaked lesions on immature watermelon fruit tissues. By screening group II *A. citrulli* AAC00-1 transposon mutant library, we found six mutants that were non-pathogenic on watermelon fruits. After further characterization, they were also non-pathogenic on watermelon foliage and unable to induce a hypersensitive response (HR) on tobacco leaves. Three mutants disrupted type 3 secretion system (T3SS) associated genes. Two additional mutants were further characterized, but the explanation for the loss of HR induction remains unknown.

BFB is a seed-borne and seed-transmitted disease. Because infested seeds are important sources of primary inoculum for BFB epidemics, seed treatment is a critical component of disease management. However, at present, seed treatments alone have not been able to control BFB. Seed-to-seedling transmission is an important component of the BFB disease cycle. To understand the factors that influence seedling transmission of BFB, we investigated the effect of temperature on the colonization of germinating watermelon seeds/seedlings by *A. citrulli*. Watermelon seeds were vacuum-infiltrated with $10^6$ CFU/ml *A. citrulli*, germinated at 28°C and 100% relative humidity, and transferred to 40°C at different time intervals. Mean BFB incidence was significantly lower for seeds that were sown at 28°C and transferred to 40°C at 3 days after sowing (das), compared to seeds incubated constantly at 28°C. Inoculated seeds showed reduced mean BFB transmission percentages when transferred from 28°C to 40°C at 3 das, regardless of initial *A. citrulli* concentration. Moreover, the effect of increased temperature on BFB seedling transmission was reversible regardless of the initial *A. citrulli* seed inoculum concentration. Furthermore, the *A. citrulli* population on germinating watermelon seedlings that were transferred from 28°C to 40°C at 3 das was significantly lower than that of seedlings maintained at 28°C. We conclude that *A. citrulli* cells associated with germinating watermelon seeds are more sensitive to elevated temperature during the first 3 das relative to the later days. This information might inform the development of novel seed treatment strategies for BFB management.

Overall, we confirmed the host preference of *A. citrulli* under field conditions, demonstrated that the penetration, colonization, and symptom development did not account for the host preference of *A. citrulli* on melon fruits, determined that HopAF homolog but not XopJ homologs contributed to *A. citrulli* virulence on watermelon and melon cotyledons, showed that only group II strains induced lesions on detached watermelon fruits and confirmed the T3SS was important for fruit pathogenicity, and revealed that *A. citrulli* was sensitive to elevated temperatures during early stages of watermelon seed germination.

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APPENDICES

*ACIDOVORAX CITRULLI MUTANT* VALIDATION

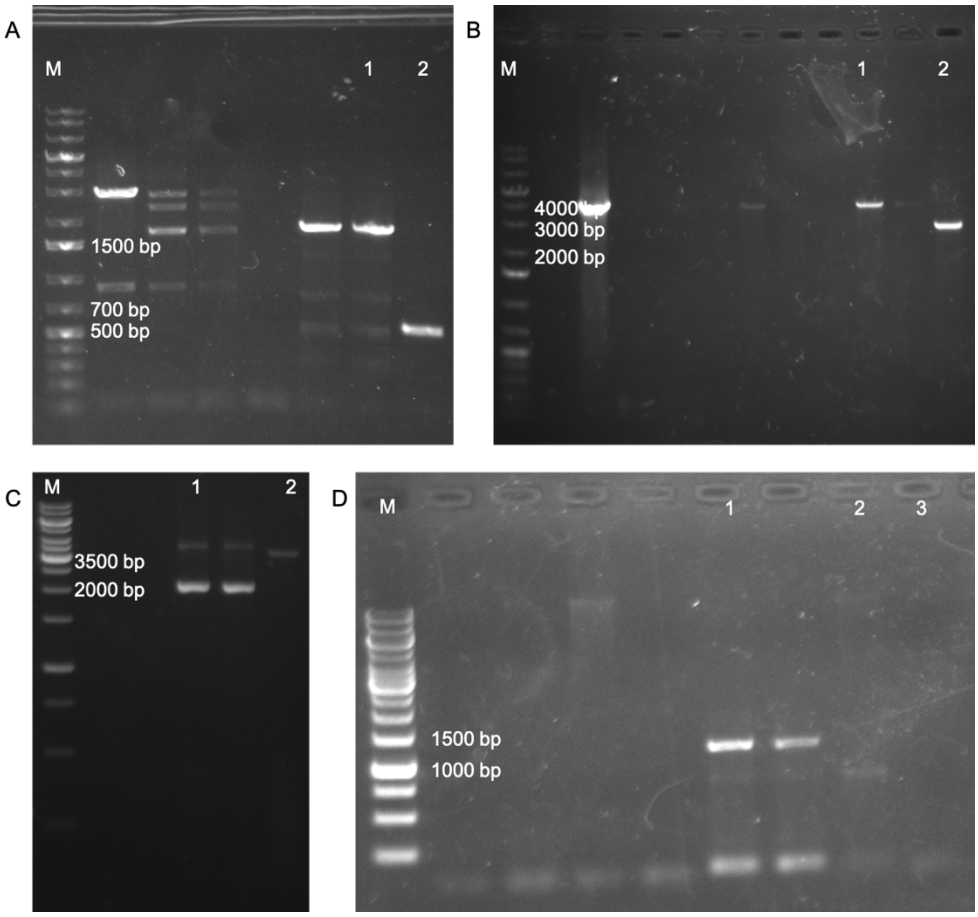


Fig A1. PCR confirmation of the *Acidovorax citrulli* mutant 00-1ΔxopJTriple (deletions of *Aave_2166*, *Aave_2178,* and *Aave_2938* sequentially in AAC00-1). A) 00-1Δ2166 was confirmed using primer set 2165F1/2167R1. AAC00-1 wild-type DNA amplified with primer set 2165F1/2167R1 produced a 1,828 bp band and DNA from the mutant 00-1Δ2166 produced a 550 bp band. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: AAC00-1 DNA; Lane 2: 00-1Δ2166 DNA. B) 00-1ΔXopJDouble (deletions of *Aave_2166* and *Aave_2708*) was

confirmed using primer set 2708LongF/2708LongR. AAC00-1 wild-type DNA amplified with primer set 2708LongF/2708LongR produced a 3,813 bp band and DNA from the mutant 00-1ΔXopJDouble produced a 2,691 bp band. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: AAC00-1 DNA; Lane 2: 00-1ΔXopJDouble DNA. 00-1ΔxopJTriple (deletions of *Aave_2166, Aave_2708,* and *Aave_2938*) was confirmed using primer sets 2938Up-FWD/2938Down-REV (C) and 2938UpFRT-F/2938UpFRT-R (D). C) AAC00-1 wild-type DNA amplified with primer set 2938Up-FWD/2938Down-REV produced a 3,122 bp band and DNA from the mutant 00-1ΔxopJTriple produced two amplicons of sizes 2,040 bp and 3,440 bp. Lane M: GeneRuler$^{TM}$ 1kb DNA Ladder; Lane 1: 00-1ΔxopJTriple DNA; Lane 2: AAC00-1 DNA. D) 00-1ΔxopJTriple yielded the expected 1,123 bp amplicon, while wild-type AAC00-1 and the negative control did not yield an amplicon. Lane M: GeneRuler$^{TM}$ 1kb DNA Ladder; Lane 1: 00-1ΔxopJTriple DNA; Lane 2: AAC00-1 DNA. Lane 3: negative controls ($H_2O$).

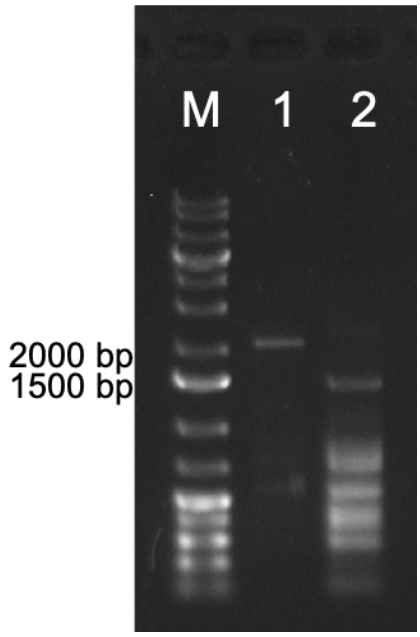


Fig A2. PCR confirmation of the *Acidovorax citrulli* mutant A5ΔHopAF using primer set 1373OutF/1373OutR. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: A5 DNA; Lane 2: A5ΔHopAF DNA. A5 wild-type DNA amplified with primer set 1373OutF/1373OutR produced a 2,109 bp band and DNA from the mutant A5ΔHopAF produced a 1,484 bp band.

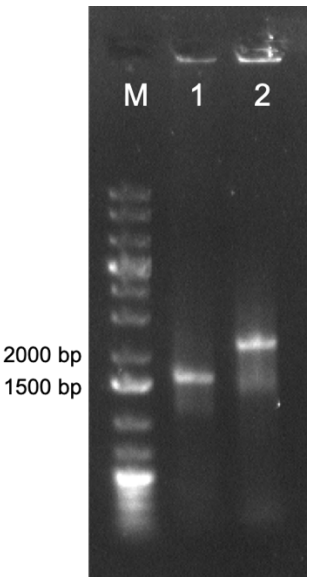


Fig A3. PCR confirmation of the *Acidovorax citrulli* mutant M6ΔHopAF using primer set 1373OutF2/1373OutR2. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: M6ΔHopAF DNA; Lane 2: M6 DNA. M6 wild-type DNA amplified with primer set 1373OutF2/1373OutR2 produced a 2,278 bp band and DNA from the mutant M6ΔHopAF produced a 1,645 bp band.

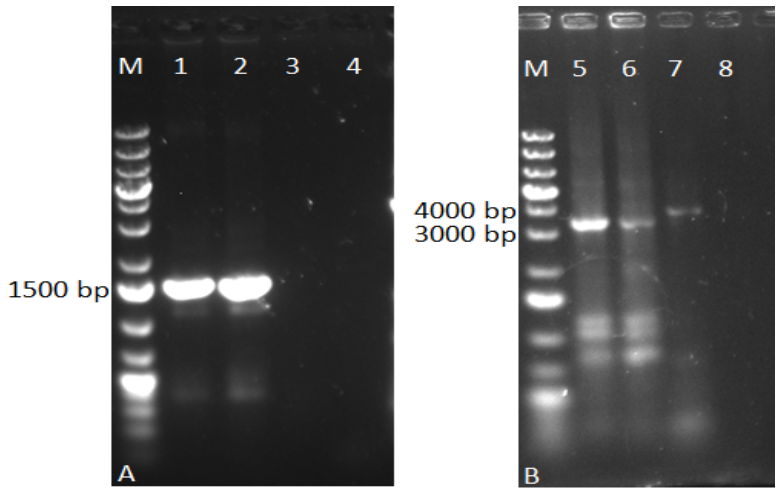


Fig A4. PCR confirmation of 00-1ΔAave_0691-A11 using primer set located upstream of the 0691-Up fragment and at the FRT-Kan region (0689-confirmF/FRT-confirmR) (A) and primer set 0691UpF/0691DownR (B). Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder. Lanes 1 and 5 (colony 0691A11) and 2 and 6 (colony 0691A12) show two individual mutants. Lanes 3 and 7 show the results of AAC00-1 DNA. Lanes 4 and 8 are negative controls ($H_2O$). A) Mutants yielded the expected 1,487 bp amplicon. Wild-type AAC00-1 and the negative control did not yield an amplicon. B) Wild-type AAC00-1 yielded the expected amplicon (3,989 bp). Mutants yielded the expected 3,400 bp amplicon.

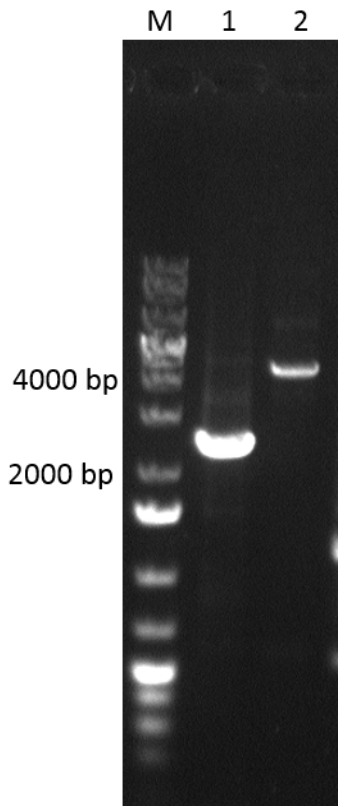


Fig A5. PCR confirmation of the *Acidovorax citrulli* mutant 00-1Δ*Aave_0691*-A2 using primer set 0691OutF/0691OutR. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: 00-1Δ*Aave_0691*-A2 DNA; Lane 2: AAC00-1 DNA. AAC00-1 DNA amplified with primer set 0691OutF/0691OutR produced a 4,383 bp band and DNA from the mutant 00-1Δ*Aave_0691*-A2 produced a 2,394 bp band.

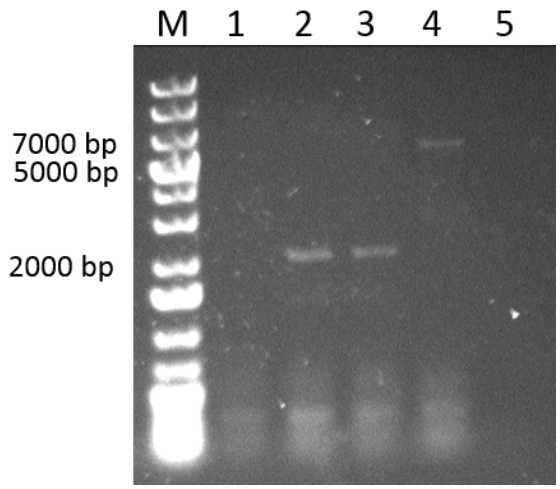


Fig A6. PCR confirmation of the *Acidovorax citrulli* mutant 00-1Δ*Aave_0692-6*-B9 using primer set 0692OutF/0696OutR. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: 00-1ΔAave_0692-6-B6 DNA; Lane 2: 00-1ΔAave_0692-6-B9 DNA; Lane 3: 00-1ΔAave_0692-6-C8 DNA; Lane 4: AAC00-1 DNA; Lane 4: negative control ($H_2O$); The DNA from the mutant 00-1ΔAave_0692-6-B9 amplified with the primer set 0692OutF/0696OutR yielded a 2,259 bp band, while AAC00-1 wild-type DNA produced a 6,676 bp band.

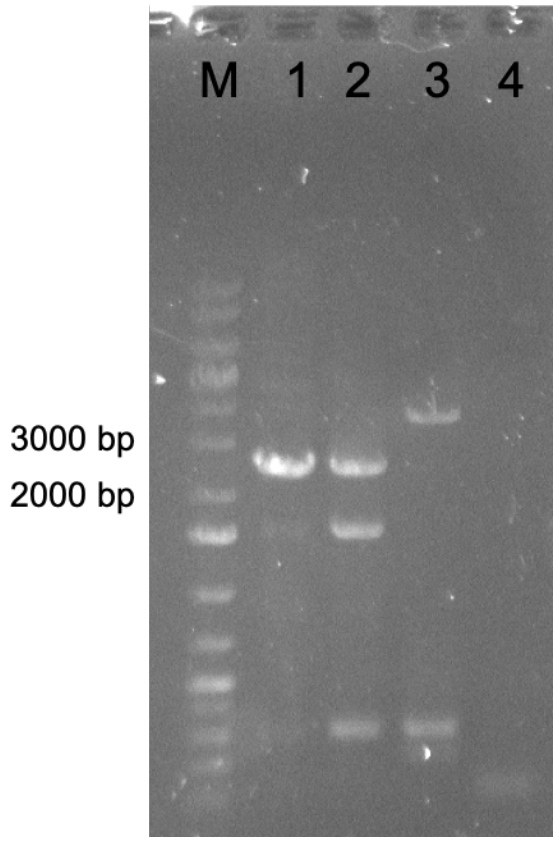


Fig A7. PCR confirmation of the *Acidovorax citrulli* mutant 00-1Δ4431 using primer set 4431OutF/4431OutR. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: 00-1Δ4431-E2 DNA; Lane 2: 00-1Δ4431-E10 DNA; Lane 3: AAC00-1 DNA; Lane 4: negative controls ($H_2O$). AAC00-1 DNA amplified with primer set 4431OutF/4431OutR produced a 3,772 bp band and DNA from the mutant 00-1Δ4431 E2 and E10 produced a 2,497 bp band.

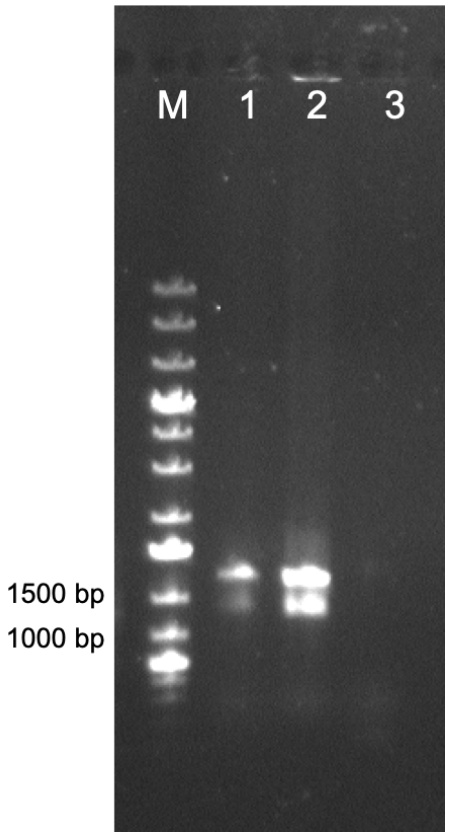


Fig A8. PCR confirmation of the *Acidovorax citrulli* mutant 00-1Δ4431Tn5 using primer set 4431OutF/KAN-2 FP-1. Lane M: GeneRuler$^{TM}$ 1kb Plus DNA Ladder; Lane 1: 00-1Δ4431Tn5; Lane 2: transposon mutant 90E2; Lane 3: AAC00-1 DNA. 00-1Δ4431Tn5 and 90E2 was confirmed using primer set 4431OutF/KAN-2 FP-1, yielding 1,222 bp, while AAC00-1 wild-type DNA did not produce an amplicon.