Further Characterization of Genetically Distinct Groups of *Acidovorax citrulli* Strains

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ABSTRACT

Bacterial fruit blotch of cucurbits (BFB) is caused by the gram-negative bacterium *Acidovorax citrulli*, whose populations can be distinguished into two genetically distinct groups, I and II. Based on visual assessment of BFB severity on cucurbit seedlings and fruit after inoculation under greenhouse conditions, group I *A. citrulli* strains have been reported to be moderately to highly virulent on several cucurbit hosts, whereas group II strains have exhibited high virulence on watermelon but low virulence on other cucurbits. Additionally, group I strains are recovered from a range of cucurbit hosts, while group II strains are predominantly found on watermelon. The goal of this research was to develop tools to characterize and rapidly distinguish group I and II *A. citrulli* strains. We first sought to determine whether quantification of *A. citrulli* colonization of cucurbit seedling tissue reflects the differences between group I and II strains established by visual assessment of BFB symptom severity. Spray inoculation of melon seedlings with cell suspensions containing approximately 1×10^4 CFU/ml

resulted in significantly higher ($P = 0.01$) population growth of M6 (group I; mean area under population growth curve [AUPGC] = 43.73) than that of AAC00-1 (group II; mean AUPGC = 39.33) by 10 days after inoculation. We also investigated the natural spread of bacterial cells and the resulting BFB incidence on watermelon and melon seedlings exposed to three group I and three group II *A. citrulli* strains under mist chamber conditions. After 5 days of exposure, the mean BFB incidence on melon seedlings exposed to representative group II *A. citrulli* strains was significantly lower (25 and 3.98% in experiments 1 and 2, respectively) than on melon seedlings exposed to representative group I strains (94.44 and 76.11% in experiments 1 and 2, respectively), and on watermelon seedlings exposed to representative group I and II strains (70 to 93.33%). Finally, we developed a polymerase chain reaction assay based on the putative type III secretion effector gene, *Aave_2166*, to rapidly distinguish group I and II *A. citrulli* strains. This assay will be important for future epidemiological studies on BFB.

Bacterial fruit blotch of cucurbits (BFB) is caused by the gram-negative bacterium *Acidovorax citrulli* (formerly *A. avenae* subsp. *citrulli*) (Schaad et al. 2008; Willems et al. 1992). Although the disease was first reported in the mid-1960s (Schaad et al. 1978), it gained importance in the late 1980s when it caused up to 100% yield losses in commercial watermelon fruit production fields in several states in the United States (Latin and Rane 1990; Maynard and Hopkins 1999; Somodi et al. 1991; Wall and Santos 1988). Currently, BFB occurs sporadically on a range of cucurbit crops but, due to its destructive potential, it poses a serious threat to the production of cucurbit seed, seedling transplants, and fruit worldwide (Burdman and Walcott 2012).

A. citrulli populations can be distinguished into two groups (I and II) based on biochemical (carbon substrate utilization) and genetic analyses, including pulse-field gel electrophoresis (PFGE), repetitive-element polymerase chain reaction (rep-PCR), and multilocus sequence typing (Burdman et al. 2005; Feng et al. 2009; Walcott et al. 2004). More recently, these groups have been shown to differ in their repertoire of type III secretion (T3S) effectors (Eckshtain-Levi et al. 2014). Additionally, a third group was identified, consisting of only two strains (ZUM4000 and ZUM4001) that were weakly virulent on watermelon, melon, and squash seedlings (Eckshtain-Levi et al. 2014). Group I and II *A. citrulli* strains differ in virulence on watermelon and nonwatermelon hosts (Burdman et al. 2005; Feng et al. 2009; O'Brien and Martin 1999; Walcott et al. 2004). Group I strains exhibit a moderate to high level of virulence on a range of cucurbits, whereas group II strains have been reported as highly virulent on watermelon but weakly virulent on other cucurbitaceous species (Burdman et al. 2005; O'Brien and Martin

1999; Walcott et al. 2004). To date, the difference in virulence between group I and II strains has been investigated based on disease severity on seedlings of various cucurbit species using visual rating scales (Burdman et al. 2005; Hopkins and Thompson 2002a; O'Brien and Martin 1999; Walcott et al. 2004). Disease severity rating scales have also been routinely used for assessing host plant resistance to BFB (Bahar et al. 2009; Hopkins and Thompson 2002a; Wechter et al. 2011). Unfortunately, these assays lack reproducibility across experiments and between laboratories. Having multiple independent evaluators that are trained to score BFB severity helps reduce the variability between experiments. Nevertheless, this approach does not allow comparisons of disease severity data across independent studies. Thus far, there have been no attempts to develop more precise assessment tools for *A. citrulli* aggressiveness. Several studies have used tissue colonization assays to assess the roles of specific genes in *A. citrulli* virulence (Johnson 2010; Johnson and Walcott 2013; Johnson et al. 2011); however, these assays have not been used to compare the virulence of group I and group II strains.

PFGE and rep-PCR have been routinely used to distinguish group I and II *A. citrulli* strains. Although PFGE is robust and highly discriminatory, it is also labor intensive and requires expensive equipment (Olive and Bean 1999). rep-PCR is less laborious than PFGE but it was reported to have lower discriminatory power for some bacteria (Olive and Bean 1999). A simple, rapid, and robust method to distinguish group I and II *A. citrulli* strains would greatly improve the efficiency of assessing the threat posed to specific cucurbit crop species. Hence, the objectives of this research were to develop reliable tools to characterize and rapidly distinguish group I and II *A. citrulli* strains. More specifically, our objectives were to (i) compare population growth dynamics of representative group I and group II *A. citrulli* strains on melon seedlings as the putative differential host, (ii) compare the relative incidence of BFB on

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watermelon and melon seedlings exposed to inoculum of group I and II *A. citrulli* strains under natural spread and infection conditions; and (iii) develop a PCR-based assay to rapidly distinguish group I and II *A. citrulli* strains.

MATERIALS AND METHODS

Host species and cultivars. Two-week-old seedlings of 'Crimson Sweet' watermelon and 'Joaquin Gold' melon were established in 250-ml plastic pots or 36- or 48-cell polystyrene flats (Speedling Inc., Sun City, FL) filled with pasteurized potting mix (90% composted pine bark and 10% vermiculite) under greenhouse conditions. Two-week-old seedlings were chosen because, at this stage, plants are highly susceptible to infection by *A. citrulli*. Additionally, 2-week-old seedlings are routinely used for virulence assays and resistance screens (Hopkins and Thompson 2002a).

Bacterial strains, growth conditions, and inoculum preparation. *A. citrulli* strains used in this study (Table 1) were grown routinely on nutrient agar (Difco, Becton, Dickinson and Co., Sparks, MD) for 48 h or in nutrient broth (NB) (EMD Chemicals Inc., Gibbstown, NJ) overnight at 28 to 30°C. *A. citrulli* inoculum was prepared by adding cells from an overnight NB broth culture to sterilized distilled water. The cell suspension was adjusted to approximately 1×10^8 CFU/ml spectrophotometrically (optical density at 600 nm of approximately 0.3) (Spectronic 20; Bausch and Lomb, Rochester, NY) and 10-fold serial dilutions were conducted to generate the desired cell concentrations.

Temporal population dynamics of representative group I and II *A. citrulli* strains in melon cotyledons after spray inoculation. Temporal population dynamics of *A. citrulli* strains M6 (group I) and AAC00-1 (group II) were monitored on 2-week-old melon seedlings after spray inoculation with bacterial cell suspensions. Suspensions of each strain (approximately 1×10^4 CFU/ml) were sprayed onto seedlings until run-off and the plants were then placed in plastic bags and incubated in a growth chamber (Percival, Perry, IA) for 24 h at 30°C and approximately 100% relative humidity (RH), with fluorescent light for 12 h/day. Subsequently, the plastic bags were removed and the seedlings were incubated in a growth chamber at 30°C and 70 to 80% RH with 12 h of fluorescent light daily for nine additional days. A single cotyledon was collected from three plants at 4, 6, 8, and 10 days postinoculation (dpi). To eliminate surface inoculum, each cotyledon was placed in a separate tube with 10 ml of sterilized distilled water and incubated with shaking at 30°C for 2 h at 200 rpm. Subsequently, the cotyledons were air dried on paper towels for several minutes, and three leaf disks (5 mm in diameter) were collected from each cotyledon. Each leaf disk was crushed in 500 µl of sterilized distilled water and, after centrifugation for 3 min at $16,100 \times g$, the pellet was used for microbial DNA extraction using the Ultra Clean Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions, with the exception that the cells were lysed thermally at 65°C for 10 min with vortex mixing every 2 to 3 min. *A. citrulli* populations in leaf disks were quantified by TaqMan real-time PCR (qPCR) using proprietary *A. citrulli*-specific primers and probe (UGA3979) at concentrations of 0.3 and 0.5 µM/reaction, respectively. PCR amplification was conducted using the iQ Supermix reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions and the following thermal profile: 95°C for 180 s and 40 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s. Cycle threshold values below 33 were recorded for each sample and converted into CFU/disk based on a standard dilution curve (Fig. 1). Bacterial population data were plotted over time to generate population growth curves using Sigma Plot 10.0 (Systat Software Inc., San Jose, CA). Area under population growth curves (AUPGC) data were calculated and used to compare the population growth dynamics of each strain. The experiment was conducted twice. A generalized linear mixed model in analysis of variance (ANOVA) was used to test for interaction between experiment and strain. Due to the absence of significant interaction, fixed-effects

ANOVA was then performed on pooled data using the general linear model (GLM) procedure. All analyses were conducted in SAS (version SAS 9.2; SAS Institute Inc., Cary, NC).

Comparison of natural infection of watermelon and melon seedlings by representative group I and II *A. citrulli* strains. Six 2-week-old melon and watermelon seedlings were spray inoculated until run-off with cell suspensions (approximately 1×10^6 CFU/ml) of representative group I (M6, AU9, and AAC98-17) and group II (AAC00-1, AAC94-21, and AAC206-102) *A. citrulli* strains. After inoculation, seedlings were placed in plastic bags and incubated at 20 to 25°C and 100% RH for 24 h, with 6 to 8 h of fluorescent and incandescent light daily. Subsequently, the bags were removed and the plants were transplanted into the center and corners of separate 36- or 48-cell planting trays containing alternately positioned, uninoculated 2-week-old watermelon and melon seedlings. Each planting tray contained 15 to 20 watermelon and 15 to 20 melon plants. Plants were incubated for 5 days in a mist chamber at 20 to 25°C and >90% RH, with 6 to 8 h of fluorescent and incandescent light daily. The light cycle was altered relative to the spray-inoculation experiment to avoid inadvertent increases in temperature that occurred with prolonged light duration in the mist chamber. Seedlings were sprayed twice daily with a stream of sterilized water to promote splash dispersal of bacterial cells. The proportion of watermelon and melon seedlings showing at least one BFB lesion on cotyledons or leaves was recorded each day and the experiment was conducted twice. BFB incidence on melon and watermelon seedlings after 5 days of exposure to inoculum was compared for each experiment by conducting ANOVA using the GLM procedure. Additionally, a *t* test of least significant difference was used for pairwise comparisons of relative BFB incidence. Statistical analyses were conducted in SAS (version SAS 9.2).

Development of a PCR assay to distinguish group I and II *A. citrulli* strains. Eckshtain-Levi et al. (2014) reported that the putative T3S effector, *Aave_2166* (*AvrBsT Xanthomonas euvesicatoria* ortholog), was present in 12 group II *A. citrulli* strains, while 8 group I strains possessed a 123-bp deletion in this locus (from 72 bp upstream to 51 bp downstream of the predicted start codon). Based on this information, we designed two forward primers: G2AcFwd, a group II-specific primer, and G12AcFwd, a primer that amplified a portion of *Aave_2166* in strains of both *A. citrulli* groups. One reverse primer, G12AcRev, was used with both forward primers and it annealed to template DNA 291 and 254 bp downstream from G2AcFwd and G12AcFwd, respectively (Table 2; Fig. 2). The specificity of the PCR assay was evaluated using genomic DNA from 25 group I and 30 group II *A. citrulli* strains, ZUM4000 and ZUM4001 (recently identified group III *A. citrulli* strains), a strain of *A. avenae* (AAA99-2), and *X. euvesicatoria* (XCV04-01) (Table 1). Cells from a single colony of a 48-h-old culture of each strain were suspended in 50 µl of sterilized distilled water, incubated at 100°C for 10 min, and used as template DNA for PCR. Conventional PCR was conducted using MasterAmp Tfl DNA polymerase (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions. All primers were used at a concentration of 0.5 µM/reaction, and the following thermal profile was employed: 95°C for 5 min; 29 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 30 s; 95°C for 30 s; 55°C for 40 s; and 72°C for 5 min. For high-throughput screening, qPCR was conducted using SYBR Green Master Mix (Bio-Rad Laboratories) according to the manufacturer's instructions. All primers were used at the concentration of 0.3 µM/reaction, and the following thermal profile was employed: 95°C for 120 s (optics off); 40 cycles of 95°C for 20 s (optics off), 55°C for 20 s (optics off), and 72°C for 30 s (optics on); and a melt curve analysis step (ramping from 60 to 95°C at 0.2°C/s).

RESULTS

Temporal population dynamics of representative group I and II *A. citrulli* strains in melon cotyledons after spray inoculation. Populations of the *A. citrulli* group I strain M6 were

greater than those of the group II strain AAC00-1 in melon cotyledons at 4 and 6 dpi. However, at 8 dpi, populations of both strains reached approximately 1×10^7 CFU/disk. Although mean AAC00-1 populations (approximately 1×10^7 CFU/disk) were lower than those of M6 (5×10^7 CFU/disk) at 10 dpi, there was high variability in population estimates for both strains at this time point. M6 populations ranged from approximately 1×10^7

to approximately 5×10^7 CFU/disk at each time point, while the population densities of AAC00-1 increased from approximately 1×10^6 CFU/disk at 4 dpi to approximately 1×10^7 CFU/disk at 8 and 10 dpi (Fig. 3A). AUPGC was significantly greater for M6 (mean AUPGC = 43.73) than for AAC00-1 (mean AUPGC = 39.33) in melon cotyledons ($P = 0.01$) (Fig. 3B).

TABLE 1. Bacterial strains used to assess the specificity of the polymerase chain reaction (PCR) assay for distinguishing group I and II *Acidovorax citrulli* strains

Strain	Host	Source	Haplotype ^b	Group	PCR result ^a	
					12Ac	2Ac
AAC00-1	Citron	Walcott et al. 2000	A1 (A)	II	+	+
W1	Watermelon	Burdman et al. 2005	A1 (A)	II	+	+
AAC94-21	Watermelon	Walcott et al. 2000	A3 (C)	II	+	+
AAC92-3	Watermelon	Walcott et al. 2000	A4 (D)	II	+	+
AAC94-55	Watermelon	Walcott et al. 2000	A5 (E)	II	+	+
AAC94-87	Watermelon	Walcott et al. 2000	A6 (G)	II	+	+
Tw16	Watermelon	Eckshtain-Levi et al. 2014	A6 (G)	II	+	+
Tw148	Watermelon	Eckshtain-Levi et al. 2014	A6 (G)	II	+	+
AAC94-39	Watermelon	Walcott et al. 2000	A7 (J)	II	+	+
SaticoyB	Cantaloupe	Walcott et al. 2004	A8 (Q)	II	+	+
AAC94-48	Watermelon	Walcott et al. 2000	A9 (U)	II	+	+
AAC201-12	Watermelon	Walcott et al. 2004	A10 (T)	II	+	+
AAC205-22	Watermelon	Ha et al. 2009	A11 (W)	II	+	+
AAC94-12	Watermelon	Walcott et al. 2000	A11 (W)	II	+	+
AAC204-8	Watermelon	This study	A12 (Z)	II	+	+
AAC206-102	Unknown	Ha et al. 2009	A13	II	+	+
AAC208-20	Watermelon	This study	A14	II	+	+
AAC208-19	Watermelon	This study	A15	II	+	+
AAC205-14	Watermelon	Ha et al. 2009	A16	II	+	+
AAC211-36	Watermelon	This study	A17	II	+	+
AAC210-13	Unknown	This study	A18	II	+	+
AAC212-30	Unknown	This study	A19	II	+	+
W6	Watermelon	Burdman et al. 2005	A20	II	+	+
AAC208-47	Watermelon	This study	A21	II	+	+
AAC206-79	Unknown	Ha et al. 2009	A22	II	+	+
7a1	Watermelon	Eckshtain-Levi et al. 2014	A23	II	+	+
AAC211-29	Unknown	This study	A24	II	+	+
AAC208-27	Watermelon	This study	A25	II	+	+
AAC213-49	Unknown	This study	A26	II	+	+
AAC210-11	Unknown	This study	A27	II	+	+
AAC203-51	Unknown	This study	B1	I	+	–
AAC92-301	Watermelon	Walcott et al. 2000	B1 (F)	I	+	–
AAC92-305	Watermelon	Walcott et al. 2000	B2 (I)	I	+	–
AAC92-300	Watermelon	Walcott et al. 2000	B3 (K)	I	+	–
AU2	Rockmelon	Walcott et al. 2000	B4 (L)	I	+	–
AU9	Rockmelon	Walcott et al. 2000	B5 (M)	I	+	–
AAC206-94	Melon	This study	B5 (M)	I	+	–
AAC98-17	Pumpkin	Walcott et al. 2000	B6 (N)	I	+	–
AAC99-5	Cantaloupe	Walcott et al. 2004	B7 (O)	I	+	–
AAC200-23	Watermelon	Walcott et al. 2004	B8 (P)	I	+	–
AAC203-57	Watermelon	Eckshtain-Levi et al. 2014	B9 (R)	I	+	–
AAC200-30	Cantaloupe	Walcott et al. 2004	B10 (S)	I	+	–
AAC207-42	Watermelon	This study	B11 (V)	I	+	–
AAC202-66	Melon	Walcott et al. 2004	B12 (X)	I	+	–
AAC203-11	Watermelon	This study	B13 (Y)	I	+	–
Tw76	Melon	Eckshtain-Levi et al. 2014	B13 (Y)	I	+	–
AAC207-41	Unknown	This study	B15	I	+	–
AAC206-2	Watermelon	Ha et al. 2009	B16	I	+	–
AAC206-1	Watermelon	Ha et al. 2009	B17	I	+	–
AAC206-95	Unknown	Ha et al. 2009	B19	I	+	–
M1	Melon	Burdman et al. 2005	B21	I	+	–
5	Watermelon	Eckshtain-Levi et al. 2014	B21	I	+	–
M6	Melon	Burdman et al. 2005	B21	I	+	–
AAC208-5	Gourd	This study	B22	I	+	–
AAC208-26	Watermelon	This study	B23	I	+	–
ZUM4000	Watermelon	Eckshtain-Levi et al. 2014	NA	III	+	+
ZUM4001	Watermelon	Eckshtain-Levi et al. 2014	NA	III	+	+
XCV04-01 ^c	Pepper	This study	ND	NA	–	–
AAA99-2 ^d	Maize	Fessehaie and Walcott 2005	ND	NA	–	–

^a 12Ac = G12AcFwd/G12AcRev and 2Ac = G2AcFwd/G12AcRev.

^b *SpeI* pulse-field gel electrophoresis haplotype. Letters in parentheses represent previously reported haplotypes (Walcott et al. 2000, 2004) while letter/number combinations represent current haplotype designations. NA = not applicable and ND = not done.

^c *Xanthomonas euvesicatoria*.

^d *A. avenae*.

Fig. 1. Standard curve for converting cycle threshold (Ct) values obtained by quantitative real-time polymerase chain reaction (qPCR) to the concentration of *Acidovorax citrulli* cells (log₁₀CFU/cotyledon disk). Cotyledon disks (5 mm in diameter) from uninoculated 2-week-old watermelon seedlings were collected and macerated in 450 µl of sterile water and spiked with 50 µl of bacterial suspensions to obtain final concentrations ranging from 10⁵ to 10⁸ CFU/ml. Total microbial DNA was extracted and qPCR was conducted using an *A. citrulli*-specific TaqMan assay to obtain the Ct values. The experiment was performed four times. Linear regression was conducted and the equation for the regression line was used to calculate log₁₀CFU/cotyledon disk.

TABLE 2. Sequences and functions of oligonucleotide primers used as components of a polymerase chain reaction assay for distinguishing group I and II *Acidovorax citrulli* strains

Primer	DNA sequence (5'→3')	Function
G2AcFwd	CGATAGGGTTGGGTTCAAG	Group II-specific forward primer
G12AcFwd	CCGAAGAGATAAACTGCATC	Group I and II forward primer
G12AcRev	ACGTACTGCCGATTTTTC	Group I and II reverse primer

Comparison of natural infection of watermelon and melon seedlings by representative group I and II *A. citrulli* strains. To empirically investigate the differences in host preference between group I and II *A. citrulli* strains, we compared BFB incidence on watermelon and melon seedlings after exposure to three group I (M6, AAC98-17, and AU9) and three group II (AAC00-1, AAC206-102, and AAC94-21) strains under conditions that facilitated natural bacterial spread and infection for 5 days. Mean BFB incidence on melon seedlings exposed to group II *A. citrulli* strains (25 and 3.98% in experiments 1 and 2, respectively) was significantly lower ($P < 0.0001$ and $P = 0.004$ in experiments 1 and 2, respectively) than for melon seedlings exposed to group I strains (94.44 and 76.11% in experiments 1 and 2, respectively). Mean BFB incidence on melon seedlings exposed to group II *A. citrulli* strains was also significantly lower than for watermelon seedlings exposed to group I and II strains (70 to 93.33%) (Fig. 4). Additionally, there was a significant difference in the effects of group I and II strains on BFB incidence ($P < 0.0001$ and $P = 0.01$ in experiments 1 and 2, respectively), as well as a significant host effect ($P = 0.0002$ and 0.02 in experiments 1 and 2, respectively). There was also a significant group–host interaction related to BFB incidence ($P = 0.0001$ and 0.008 in experiments 1 and 2, respectively).

Discrimination of group I and II *A. citrulli* strains by PCR assay. Genomic DNA of *A. citrulli* strains M6 and AAC00-1 was amplified using a conventional PCR assay with primers designed in the current study. Using the group II-specific primer set (G2AcFwd/G12AcRev), the PCR assay yielded the expected 291-bp amplicon with genomic DNA from AAC00-1 but no amplicon was produced with genomic DNA from M6 (Fig. 5). The PCR assay conducted with the primer pair G12AcFwd/G12AcRev produced a 254-bp amplicon with genomic DNA from AAC00-1 and M6 (Fig. 5). A SYBR Green-based qPCR assay was used to evaluate additional group I and II *A. citrulli* strains, group III strains (ZUM4000 and ZUM4001), a strain of *A. avenae*, and a strain of *X. euvesicatoria* (Table 1). Using primers G2AcFwd/G12AcRev, all group II and III *A. citrulli* strains yielded the expected amplicon with a melting peak temperature of approximately 88°C. In contrast, there was no amplification of DNA from the 25 group I strains tested with this primer set. Primer set G12AcFwd/G12AcRev

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TTCTGCTGGGCCGATCGGTGCGAATGGACCAGCGTTGAAGTTTGCTCAGAGAAAGGTGGAAGCTTTCTGA
ATATTTGCAATTGAGAATGGTTT[ATG]AAGAATTTTCATGCGATCGATAGGGTTGGGTTCAAGCAGGAGCAG
TCGGCACGGCCGAAGAGATAAACTGCATCCGGACGAGGATCCACGTCTGCAGGATCGTTCGAGTTCCGCC
CACGGAAACTCCGGAGCACTCTCCGGCCTGTCTGCAAGAACTGCTGGAAGTGGAGAATTCGGGTTTCTGC
CAGAGCGTCCCCGTGAGAAGGCAATAGCTTTGCGCGAAGCACTGAACCGTGCGGAAAACACTCCTTCGGA
CCTGCGGGCATATGCCGAGGCCGCATTGACAGCAGCAAAAATCGGCAGTACGT[CAGACATGACGGATCTG
GATGTGCAAAACAAGCAATACCTGGCCGATGCATACACCCCGCTTCCCCGATCTCAAGCTGATTTGTC
ATGATTCCGCTCAGGCGTTTTTCTCGGATTTTCATGACTTCCGAAAAGCCGGTGTGGCGATCCCTCGTGCA
GTTGAGCCCCCATAGCTTGCATCATGTGGCCATCGATGTTCTGTTTGCAGGATGGGAAGCGCACGGCGGTG
GTGTTGGAGCCGGCAATTGGCTATGGAATGAGGTCAGACGGGAAGATAACGATGCTGGCCGGATACGAAC
CGCTGGGCAGGAATATTCAGAAATATCTCGGAGAAAACGGCGATATGGCGGTCTGTCAGTTGGGGGCCCA
GAAGTCGCATTACGACTGCATTATTTTTTCTTTGAATCTCGCCCTGTGTGCCTATCAGCGGGATGATGTG
ATCGATGGTCTGCATGAAAGGCTTCGTGGGCATTACAGATGTTTTTGCATCCGATGAAAATAAATCTCAAT
TGCATCAGAATATTGAATTTATAGACGGCACGACATTTTTTGCCTCCGGTTTTTTTCAAACATTGCGACGC
CAGGTCAACTATTTCTGAGGTTTTTGGGGAATCAGCCTGACATATCGGATCGAAATGTGAGCACCGGGAAT
GATAATCCCCGGGAAACGCTTTCCGAGCGGGTGGAAAATTTCCGTGTGGATCGAGGAGAAATGGCATGCA
GCATGTGATCGAAGCGTCACGTTTGAGAAAAATCAGAAAAGCTATCGAA[TTG]TTGTCTGATGGGCATGG
CGGGAACGAGTGCGCCGCAGCGTTAAAGGACGAACAAAGCGGCAGTAAGGCAGTAGCTGAACTGGCTTG

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Fig. 2. DNA sequence of *Acidovorax citrulli* gene *Aave_2166* showing the relative positions of the group II-specific forward primer G2AcFwd (highlighted gray), the forward primer G12AcFwd that anneals to the portion of *Aave_2166* in group I and II *A. citrulli* strains (underlined), and the reverse primer G12AcRev (highlighted gray, underlined); start and stop codons of *Aave_2166* locus (solid black border).

enabled amplification of genomic DNA from all tested *A. citrulli* strains, and all amplicons had the expected peak melting temperature of approximately 88°C. Attempts to amplify genomic DNA from *A. avenae* and *X. euvesicatoria* yielded negative results using both primer sets (Table 1).

DISCUSSION

A. citrulli populations can be distinguished into at least two groups based on molecular and biochemical analyses and visual assessment of disease severity on various cucurbit hosts (Burdman et al. 2005; Feng et al. 2009; O'Brien and Martin 1999; Walcott et al. 2004). According to these studies, group I *A. citrulli* strains are moderately to highly virulent on a range of cucurbit species, whereas group II strains are highly virulent on watermelon but mildly aggressive on other cucurbits. To date, most of the assays used to screen for BFB resistance have relied on visual assessment

of BFB severity using rating scales (Araújo et al. 2005; Hopkins and Thompson 2002b; O'Brien and Martin 1999). Unfortunately, these assays lack reproducibility across experiments and laboratories. Efforts to alleviate this variability include the use of multiple independent evaluators who are trained to score disease severity prior to data collection. However, this does not allow disease severity data to be compared across independent studies. For these reasons, we sought to determine whether quantification of *A. citrulli* colonization of cucurbit seedling tissue reflects the differences in virulence between group I and II strains with higher precision and reproducibility. We observed that, after cotyledon infiltration with bacterial cell suspensions (approximately 1×10^4 CFU/ml), M6 and AAC00-1 reached similar population densities in watermelon and melon seedlings (data not shown). This suggests that, once in the plant apoplast, the differences in the arsenal of virulence factors possessed by the two strains do not affect tissue colonization. In contrast to our observations, Baltrus et al. (2012) reported that three closely related pathovars of *Pseudomonas syringae* (namely, *P. syringae* pv. *phaseolicola* 1448a and 1644R and *P. syringae* pv. *glycinea* R4), recovered from naturally infected French bean, mung bean, and soybean leaves, respectively, reached the highest population densities in their original hosts and significantly lower population densities in the nonhost species after leaf tissue infiltration with 10^4 CFU/ml. The authors further reported that the presence of two genes encoding the type III effectors *hopC1* and *hopM1* and the absence of another (*avrB2*) contributed to the host range differences between pathovars *glycinea* and *phaseolicola* (Baltrus et al. 2012).

Because infiltration of bacterial cells into the plant apoplast does not reflect the natural infection process, we further hypothesized that quantifying *A. citrulli* population dynamics in seedling tissues

Fig. 3. A, Temporal population dynamics of *Acidovorax citrulli* strains M6 (group I) and AAC00-1 (group II) in melon seedlings after spray inoculation. Two-week-old melon seedlings were spray inoculated until run-off with cell suspensions containing each strain at approximately 1×10^4 CFU/ml. Plants were incubated at 30°C and 70 to 80% relative humidity, with a 12-h light period, and three cotyledon disks were collected from three plants at 4, 6, 8, and 10 days after inoculation. Leaf disks were macerated and used for total microbial DNA extraction followed by quantitative real-time polymerase chain reaction using an *A. citrulli*-specific TaqMan assay. Data points represent means of the log₁₀ *A. citrulli* CFU/cotyledon disk and error bars represent the standard errors of the means. **B,** Bar chart of area under population growth curves (AUPGC) calculated for M6 and AAC00-1 on spray-inoculated melon seedlings. Bars represent mean AUPGC values and lines indicate standard errors of the means. Population growth of M6 was significantly higher ($P = 0.01$) than that of AAC00-1. The experiment was conducted twice and pooled data are presented.

Fig. 4. Mean bacterial fruit blotch (BFB) incidence in a mixed population of watermelon and melon seedlings exposed to group I (M6, AAC98-17, and AU9) and group II (AAC00-1, AAC206-102, and AAC94-21) *Acidovorax citrulli* strains under conditions that facilitated natural spread of bacterial cells and infection. Each bar represents mean BFB incidence and error bars represent the standard errors of the means. Two-week-old melon and watermelon seedlings spray inoculated with group I and group II *A. citrulli* strains, respectively, were placed in the center and corners of separate seedling trays with mixed populations of uninoculated 2-week-old watermelon and melon seedlings. Seedlings were incubated at >90% relative humidity and 20 to 25°C with light for 6 to 8 h/day and irrigated twice daily using a spray bottle. BFB incidence was recorded after 5 days of exposure, the experiment was conducted twice, and pooled data are presented; BFB incidence on melon seedlings exposed to group II strains was significantly lower ($P < 0.0001$ and $P = 0.004$ in experiments 1 and 2, respectively) than in any other treatment.

after spray inoculation would better reflect the differences in virulence between group I and II strains on melon, the putative differential host. As expected, we found that the AUPGC was significantly greater for strain M6 than for AAC00-1 (Fig. 3B), which implied that M6 had a greater ability to survive on and penetrate into the apoplast of the melon cotyledons than AAC00-1. However, despite the observed differences, there was substantial variability in population densities of both strains between plants and sampling times. In addition, the assay is labor intensive and costlier than visual assessment of BFB severity and, therefore, would not serve as an adequate replacement.

Group I strains have been recovered from a range of cucurbits, whereas group II strains are mainly recovered from watermelon (Burdman et al. 2005; Feng et al. 2009; Walcott et al. 2004). Based on this observation, we hypothesized that, in a natural infection assay, BFB incidence would be lower on melon seedlings exposed to group II strains than in all other *A. citrulli* group–cucurbit host combinations. As hypothesized, the relative BFB incidence on melon seedlings after 5 days of exposure to group II strains was significantly lower than on melon seedlings exposed to group I, or watermelon seedlings exposed to group I and II strains (Fig. 4). It would be interesting to investigate the incidence of BFB on watermelon and melon fruit exposed to group I and II *A. citrulli* strains, because fruit infection might more clearly delineate host preference among the groups. It would also be informative to repeat the natural infection assays under field conditions. Knowing the potential of an *A. citrulli* strain to naturally infect different cucurbit species would inform growers of the risks associated with having different cucurbits in close proximity in seed, seedling transplant, and fruit production systems. Finally, the natural infection assay confirmed previous observations of reduced BFB incidence with group II strains on melon. Therefore, this relatively simple assay could be useful for characterizing new *A. citrulli* strains.

Fig. 5. Differentiation of *Acidovorax citrulli* groups by polymerase chain reaction (PCR) using primer pair G12AcFwd/G12AcRev that amplifies a portion of the *Aave_2166* gene in group I and II strains, and primer pair G2AcFwd/G12AcRev that amplifies a portion of *Aave_2166* in group II strains only. Lane M contains a marker consisting of 15 chromatography-purified DNA fragments (ranging from 20,000 to 75 bp). PCR assay results obtained with the primer pair G12AcFwd/G12AcRev: lane 1, sterilized distilled water (negative control); lanes 2 and 3, genomic DNA of AAC00-1 (group II) and M6 (group I), respectively. PCR assay results obtained with the primer pair G2AcFwd/G12AcRev: lane 4, sterilized distilled water; lanes 5 and 6, genomic DNA of AAC00-1 (group II) and M6 (group I), respectively.

Thus far, whole-genome restriction enzyme digestion followed by PFGE and rep-PCR have been used for distinguishing group I and II *A. citrulli* strains (Burdman et al. 2005; Walcott et al. 2004). Because these methods are laborious and require expensive equipment (especially PFGE), we sought to design primers to rapidly distinguish group I and II strains using the PCR assay. We designed a primer pair, G12AcFwd/G12AcRev, that amplified a portion of the *Aave_2166* gene in all tested *A. citrulli* strains but yielded no amplicon with DNA from *A. avenae* and *X. euvesicatoria*. On the other hand, the group II-specific primer pair, G2AcFwd/G12AcRev, yielded amplicons with genomic DNA from the tested group II strains. Unexpectedly, amplicons were produced with both primer pairs using DNA from the ZUM strains (group III), even though these strains were reported to lack the *Aave_2166* gene (Eckshtain-Levi et al. 2014). The group III strains ZUM 4000 and ZUM4001 were isolated from watermelon seedlings in India and yielded variable results with *A. citrulli*-specific diagnostic PCR and serological assays. Unlike group I and II *A. citrulli* strains, *SpeI* restriction enzyme digestion of genomic DNA followed by PFGE failed to generate DNA fingerprints for the ZUM strains in multiple attempts. Additionally, rep-PCR DNA profiling clearly indicated a difference between group III and other representative *A. citrulli* strains (Eckshtain-Levi et al. 2014). Nevertheless, these strains were confirmed as *A. citrulli* by DNA-DNA hybridization assays. One explanation for the generation of an amplicon with the group II-specific primer set designed in this study is that a variant of the *Aave_2166* locus is present in the ZUM strains, even though it was not detected by Eckshtain-Levi et al. (2014). To detect the *Aave_2166* locus in the ZUM strains, Eckshtain-Levi et al. (2014) used three different forward primers in combination with a single reverse primer. Even though these combinations of primers yielded no amplicon with group III strains, it is possible that the ZUM strains possess polymorphisms in the *Aave_2166* flanking sequences (possibly complementary to the reverse primer) that prevented hybridization and subsequent amplification of the *Aave_2166* gene. This could explain why the primers designed in this study, that target internal regions of *Aave_2166*, generated an amplicon with DNA from the ZUM strains. Efforts are underway to sequence the genomes of one of the ZUM strains, which will yield insight into this discrepancy. Nevertheless, the ZUM strains appear to be of limited distribution and epidemiological importance because they exhibited low virulence on watermelon, melon, and squash seedlings (Eckshtain-Levi et al. 2014).

Recently, Zhong et al. (2016) published a primer set designed based on the *pilL* gene of AAC00-1 to discriminate group I and II *A. citrulli* strains. Although this PCR assay generated amplicons from 51 of 52 group II strains, it also generated an amplicon from three group I strains (Zhong et al. 2018). One of these three strains, AAC200-23, was included in the current study and was accurately identified as a group I member by our PCR assay. Additionally, the currently described assay is superior to that described by Zhong et al. (2016) because it allows for the positive detection of group I and group II strains, rather than relying on a negative result for group I strain determination. Multiplexing the two PCR assays described in the current study using TaqMan-based technology could yield a highly effective tool for diagnostic purposes and epidemiological studies.

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