Comparative Analysis of Type III Secreted Effector Genes Reflects Divergence of *Acidovorax citrulli* Strains into Three Distinct Lineages

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ABSTRACT

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Acidovorax citrulli causes bacterial fruit blotch of cucurbits, a serious economic threat to watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*) production worldwide. Based on genetic and biochemical traits, *A. citrulli* strains have been divided into two distinct groups: group I strains have been mainly isolated from various non-watermelon hosts, while group II strains have been generally isolated from and are highly virulent on watermelon. The pathogen depends on a functional type III secretion system for pathogenicity. Annotation of the genome of the group II strain AAC00-1 revealed 11 genes encoding putative type III secreted (T3S)

effectors. Due to the crucial role of type III secretion for *A. citrulli* pathogenicity, we hypothesized that group I and II strains differ in their T3S effector repertoire. Comparative analysis of the 11 effector genes from a collection of 22 *A. citrulli* strains confirmed this hypothesis. Moreover, this analysis led to the identification of a third *A. citrulli* group, which was supported by DNA:DNA hybridization, DNA fingerprinting, multi-locus sequence analysis of conserved genes, and virulence assays. The effector genes assessed in this study are homologous to effectors from other plant-pathogenic bacteria, mainly belonging to *Xanthomonas* spp. and *Ralstonia solanacearum*. Analyses of the effective number of codons and gas chromatography content of effector genes relative to a representative set of housekeeping genes support the idea that these effector genes were acquired by lateral gene transfer. Further investigation is required to identify new T3S effectors of *A. citrulli* and to determine their contribution to virulence and host preferential association.

Bacterial fruit blotch (BFB) of cucurbits is caused by the gram-negative, seedborne bacterium *Acidovorax citrulli* (formerly *A. avenae* subsp. *citrulli*) (30,42). This disease gained importance after the occurrence of devastating outbreaks in watermelon fields in the United States during the late 1980s (4,20,30,32). Since then, the pathogen has spread worldwide and to other hosts in the cucurbit family such as melon, squash, pumpkin, and cucumber (1). To date, there is no reliable source of disease resistance to this highly destructive pathogen, and chemical control has limited efficacy for disease management (4). Therefore, *A. citrulli* represents a serious threat to the cucurbit industry, primarily to watermelon and melon production (4,21). Despite the economic importance of BFB, little is known about basic aspects of *A. citrulli* pathogenicity and factors that influence host–pathogen interactions.

A. citrulli strains can be divided into at least two well-differentiated groups: group I strains are generally moderately to highly virulent on non-watermelon plants (mainly melon), whereas group II strains have been mainly isolated from and are highly virulent to watermelon plants. Group II strains are weakly to moderately virulent on other cucurbits (28). This distinction is

clearly supported by a variety of DNA fingerprinting techniques, including pulsed-field gel electrophoresis (PFGE) and repetitive polymerase chain reaction (rep-PCR), carbon utilization profiles, and fatty acid methyl ester (FAME) analysis by gas chromatography (GC) (3,38,39).

Many gram-negative plant-pathogenic bacteria utilize a functional type III secretion system (T3SS) for pathogenicity and hypersensitive response (HR) induction in susceptible and resistant plants, respectively. The genes encoding the components of the type III secretion apparatus are named hypersensitive response and pathogenicity (*hrp*) genes and are located in large (generally 20- to 25-kb) clusters (5). Similarly, *A. citrulli* strains possess *hrp* gene clusters, and mutagenesis analyses revealed that *A. citrulli* requires a functional T3SS to infect cucurbit plants and to elicit an HR in incompatible tomato and tobacco plants (1,17). Pathogenic bacteria utilize the T3SS to secrete effector proteins into the plant cell. Although no reports about functional characterization of *A. citrulli* effectors have been published to date, studies with many type III secreted (T3S) effectors from several plant-pathogenic bacteria have revealed that most contribute to suppress plant defense responses and to manipulate the host cell metabolism to benefit the pathogen (27,45).

The sequencing and annotation of the genome of the *A. citrulli* group II strain AAC00-1 by the Joint Genome Institute (GenBank NC_008752) revealed the presence of at least 11 genes encoding putative T3S effectors, based on their homology to other plant-pathogenic bacterial genes. Due to the demonstrated role of effectors in host–pathogen interactions, we hypothesize that differences in the arsenal of T3S effector genes between group I and II

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strains of *A. citrulli* may play a role in determining preferential association of the pathogen with different cucurbits.

In the present study, we performed an extensive comparative sequence analysis of the aforementioned 11 T3S effector genes using a set of 22 *A. citrulli* strains. This set included group I and II strains isolated from different cucurbit hosts and from various geographical locations. Here, we show that the effector sequences exhibited low codon usage bias and scatter GC content compared with a large set of housekeeping genes, which implies lateral gene transfer (LGT) as the mechanism responsible for the acquisition of T3S effector genes by *A. citrulli*. Phylogenetic analyses of the assessed effector gene sequences revealed that the strains clustered into two distinct groups that concurred with the established groups I and II. Interestingly, two strains, ZUM4000 and ZUM4001 (hereafter, ZUM strains), were shown to belong to a distinct clade. In addition, DNA from these strains yielded distinguishable rep-PCR profiles and were consistently less virulent than group I and II strains on watermelon, melon, and squash seedlings. DNA:DNA hybridization assays confirmed that the ZUM strains belong to the *A. citrulli* species. Altogether, the data presented here support the existence of a third group of pathogenic *A. citrulli* strains.

MATERIALS AND METHODS

Bacterial strains. *A. citrulli* strains used for sequencing of T3S effector genes and other assays are listed in Table 1. The *A.*

citrulli type strain DSM17060 (American Type Culture Collection 29625) (42) was used as reference for DNA:DNA hybridization. *A. citrulli* strains were generally grown in nutrient broth (NB) (Difco, Franklin Lakes, NJ) or nutrient agar (NA; NB containing agar at 15 g/liter) at 28°C, unless stated otherwise. *Escherichia coli* DH5α (12) was used for cloning and sequencing. This strain was grown in lysogeny broth (LB) broth (Difco) or LB agar (LB containing agar at 15 g/liter) at 37°C.

Cloning and sequencing of effector genes. The predicted open reading frame (ORF) for each putative T3S effector was amplified by PCR with primers that were designed based on the AAC00-1 genome sequence (GenBank NC_008752). PCR primers used in this study are listed in Supplemental Table 1. ORFs of effector genes were PCR amplified, cloned, and sequenced using primers corresponding to the predicted start and stop of translation regions. To allow reliable sequencing of the entire ORF, some of the cloned fragments were also sequenced with internal primers. When PCR products could not be obtained for some strains, new PCR primers corresponding to regions from outside or inside of the coding sequences were designed. PCR was performed in an Eppendorf (Hamburg, Germany) Thermal Cycler using REDTaq ready mix (Sigma-Aldrich, St. Louis, MO) in 50-μl reaction volumes, according to the manufacturers' instructions. The PCR thermal profile consisted of an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 40 s at X°C, and elongation at 72°C for Y seconds (X and Y = annealing tem-

TABLE 1. List of *Acidovorax citrulli* strains used in this study and distribution of the 11 putative T3S effector genes and the housekeeping gene *gltA* in these strains

Group	Strain	Country	Year ^a	Host	Haplotype ^b	Effector gene (<i>Aave</i> gene number) ^c						
						0277 1548 2173 2876 3452 3462	2166	2708	3062	3237	4728	<i>gltA</i>
I	M1 ^d	Israel	2000	Melon	B21	✓	✗	✗	✗	✓	✓	✓
I	M2 ^d	Israel	2002	Melon	B21	✓	✗	✗	✗	✓	✓	✓
I	M6 ^d	Israel	2002	Melon	B21	✓	✗	✗	✗	✓	✓	✓
I	5 ^e	Israel	2005	Melon	B21	✓	✗	✗	✗	✓	✓	✓
I	Tw76 ^f	Taiwan	1995–1999	Melon	B13 (Y)	✓	✗	✗	✗	✓	✓	✓
I	AAC98-17 ^g	United States	1999	Pumpkin	B6 (N)	✓	✗	✗	✗	✓	✓	✓
I	AAC203-57 ^h	Honduras	2003	Watermelon	B9 (R)	✓	✗	✗	✗	✓	✓	✓
I	AACAU-9 ⁱ	Australia	1999	Melon	B5 (M)	✓	✗	✗	✗	✓	✓	✓
III	ZUM4000 ^j	India	2008	Watermelon	nd	✓	✗	✗	✓	✗	✗	✓
III	ZUM4001 ^j	India	2008	Watermelon	nd	✓	✗	✗	✓	✗	✗	✓
II	AAC00-1 ^{g + i}	United States	1990	Watermelon	A1 (A)	✓	✓	✓	✓	✓	✓	✓
II	W1 ^d	Israel	2002	Watermelon	A1 (A)	✓	✓	✓	✓	✓	✓	✓
II	W2 ^d	Israel	2003	Watermelon	A13	✓	✓	✓	✓	✓	✓	✓
II	W4 ^d	Israel	2002	Watermelon	A13	✓	✓	✓	✓	✓	✓	✓
II	W6 ^d	Israel	2001	Watermelon	A20	✓	✓	✓	✓	✓	✓	✓
II	7a1 ^e	Israel	1995	Watermelon	A23	✓	✓	✓	✓	✓	✓	✓
II	Tw16 ^f	Taiwan	1995–1999	Watermelon	A6 (G)	✓	✓	✓	✓	✓	✓	✓
II	Tw148 ^f	Taiwan	1995–1999	Watermelon	A6 (G)	✓	✓	✓	✓	✓	✓	✓
II	AAC92-17 ^{g + i}	United States	1992	Watermelon	A4 (D)	✓	✓	✓	✓	✓	✓	✓
II	AAC94-12 ^g	United States	1994	Watermelon	A11 (W)	✓	✓	✓	✓	✓	✓	✓
II	AAC94-21 ^g	United States	1994	Watermelon	A3 (C)	✓	✓	✓	✓	✓	✓	✓
II	AAC206-102 ^k	China	2000	Melon	A13	✓	✓	✓	✓	✓	✓	✓

^a Haplotypes based on pulsed-field gel electrophoresis analyses. Letters in parenthesis represent previously reported haplotypes (38,39) while letter/number combinations represent 10 current haplotype designations; nd = nondetermined.

^b Names of genes are according to the annotation of the sequenced strain AAC00-1: ✓ = the gene is present in an intact form; ✗ = the gene could not be detected; and ✗ = the gene is present but has base deletions causing frame shifts relative to strain AAC00-1.

^c Year of isolation. For the strains isolated in Taiwan, there is not a record of the exact year of isolation.

^d Source of bacterial strain: Burdman et al. (3).

^e Source of bacterial strain: Isaac Assouline.

^f Source of bacterial strain: Hsiou-Chen Huang.

^g Source of bacterial strain: Walcott et al. (39).

^h Source of bacterial strain: Ron Walcott.

ⁱ Source of bacterial strain: Walcott et al. (38).

^j Source of bacterial strain: Bert Woudt.

^k Source of bacterial strain: Feng et al. (10).

temperatures and elongation times are detailed in Supplemental Table 2). A final extension step was performed at 72°C for 10 min. PCR products were cloned into plasmid pTZ57R/T (Fermentas, Burlington, Canada) using the InsTAclone kit (Fermentas). Plasmids were sequenced at Hy Labs (Rehovot, Israel) and data were analyzed using the ContigExpress software (Invitrogen, Carlsbad, CA). In all cases, the ORFs were completely sequenced from both strands and mismatches were rechecked by additional, independent PCR reactions and sequencing.

Phylogenetic and single-nucleotide polymorphism analyses. Full effector sequences (both amino acid and nucleotide) were aligned using the BioEdit software (Ibis Biosciences, Carlsbad, CA). Phylogenetic trees were generated by three different methods—maximum parsimony, maximum likelihood, and neighbor-joining—using MEGA5 software (34). Bootstrap values were derived from 1,000 replicates in each case to validate tree topology. Phylogenetic trees were generated for individual ORFs of effector genes as well as for concatenated sequences of all effector ORFs. The genetic dissimilarity of *A. citrulli* effector genes to their closest homologous sequences in the database was too large (>21%) to use the latter as suitable outgroups (31). Therefore, artificial outgroups were created by randomly generating nucleotide substitutions in strain AAC00-1 at a rate of 2 to 3% in positions conserved among the different strains (31). Phylogenetic trees were generated in a similar manner based on multilocus sequence analysis (MLSA) of seven housekeeping genes—*gltA*, *trpB*, *lepA*, *ugpB*, *gmc*, *phaC*, and *pilT*—of strain ZUM4000 (for which we have the draft genome sequence) and selected group I and II strains, whose sequences are available from the study of Feng et al. (10). In this case, outgroups consisted of ortholog sequences from the closely related *A. avenae* 30003, as reported by Feng et al. (10). Sequence polymorphism, GC content, and effective number of codons (ENC) were determined using DnaSP software (24).

Seedling virulence assays. Virulence of *A. citrulli* strains representative of each group (I, II, and ZUM strains) was evaluated on watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), and squash (*Cucurbita pepo*) seedlings, as previously described (14,38). Briefly, ‘Crimson Sweet’ watermelon, ‘Athena’ melon (cantaloupe type), and ‘Early Yellow Crookneck’ squash seedlings were grown in 250-ml plastic pots in a greenhouse (30 to 35°C, ≈80% relative humidity (RH), and ≈12 h of natural sunlight daily). Seedlings were cultivated in a 3:1 ratio of fine-grade composted pine bark and vermiculite. Two weeks after planting, seedlings were spray-inoculated with cell suspensions of ≈1 × 10⁶ CFU/ml of group I (M6, Tw76, AAC98-17, AAC203-57, and AACAU-9, 5), group II (AAC00-1, Tw16, Tw148, AAC94-21, AAC206-102, and 7a1), and ZUM (ZUM4000 and ZUM4001) strains. To prepare bacterial suspensions, –80°C freezer stock cultures of each strain were cultured on King’s B medium (18) at 28°C for 48 h. Subsequently, a single colony of each culture was used to inoculate 3 ml of NB, which was allowed to grow at 30°C for 12 h with agitation at 250 rpm in an incubator shaker (Innova, New Brunswick, NJ). Cells in 1 ml of each NB culture were pelleted by centrifugation at 10,000 × *g* for 2 min, rinsed once with 0.1 M phosphate buffered saline (PBS), and resuspended in 3 ml of 0.1 M PBS. The optical density of the cell suspensions was adjusted to 0.3 at a wavelength of 600 nm (≈0.5 × 10⁸ CFU/ml) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY), and 10-fold serial dilutions were performed to generate cell suspensions with ≈1 × 10⁶ CFU/ml. Using plastic spray bottles, cell suspensions of each strain were sprayed until run-off onto 2-week-old seedlings of each cucurbit species. Each seedling was incubated in a transparent plastic bag (≈100% RH) for 24 h and then the seedlings were incubated in a greenhouse as described above. Ten days after inoculation, seedlings were evaluated for BFB severity according to a previously described 1-to-9 rating scale (14). This experiment was repeated

three times and each experiment included four replicates (individual plants) for each strain–host combination. Each plant was evaluated by three independent evaluators. In some instances, plants were damaged due to sunscald during incubation inside the plastic bags (these plants were excluded from the data set).

DNA:DNA hybridization. Bacterial cells in suspensions of *A. citrulli* strains (ZUM4000, DSM17060, and AAC00-1) containing ≈10⁹ CFU/ml were disrupted using a French pressure cell (Thermo Scientific, Waltham, MA) and DNA in the crude lysate was purified by chromatography on hydroxyapatite, as described (6). DNA:DNA hybridization was carried out as described (8), with modifications (16), using a Bio UV/VIS-spectrophotometer (Varian, Palo Alto, CA) model Cary 100 equipped with a Peltier-thermostatted six-by-six multicell changer and a temperature controller with in situ temperature probe (Varian).

PFGE. DNA fingerprinting of *A. citrulli* strains was conducted by PFGE separation of whole-cell DNA digested with *SpeI*, as previously described (39). The strains analyzed in this experiment included representatives of groups I (Tw76, AAC98-17, AACAU-9, M1, M6, 5, and AAC 203-57) and II (AAC92-17, AAC00-1, W1, W2, W4, W6, Tw148, Tw16, AAC94-12, AAC94-21, AAC206-102, and 7a1). Attempts were also made to generate *SpeI* PFGE DNA fingerprints for strains ZUM4000 and ZUM4001. After electrophoresis, agarose gels were stained in an ethidium bromide solution of 0.5 µg/ml for 30 min and destained in deionized water for 15 min, and digital images were captured in tagged image file format (TIFF) using a Foto/UV 26 Transilluminator (FotoDyne Inc., Hartland, WI). DNA profiles were analyzed using Dice’s coefficient of analysis and the unweighted pairwise group method with arithmetic mean algorithm generated a dendrogram to visualize relationships among strains (9). Data analysis and dendrogram generation were conducted using the BioNumerics software package (V. 3.00; Applied Math, Sint-Martens-Latem, Belgium).

rep-PCR. Attempts to generate PFGE profiles with genomic DNA from strains ZUM4000 and ZUM4001 consistently failed; therefore, rep-PCR was used to compare ZUM strains with representative group I (AACAU-9, AAC98-17, AAC203-57, Tw76, 5, M1, and M6) and group II (AAC00-1, AAC94-21, AAC94-12, AAC92-17, AAC206-21, Tw16, Tw148, 7a1, W1, W2, W4, and W6) strains. rep-PCR was conducted using ≈2 ng of total DNA from each strain as template, the BOX-element primer, BOXA1R (5′-CTA CGG CAA GGC GAC GCT GAC-3′), and PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Cleveland, OH), as previously described (38). PCR amplicons were separated on a 1% agarose gel run at 70 V for 7 h at 5°C and stained with ethidium bromide. Gel images were captured as TIFF files and DNA fingerprint data were analyzed as described above for PFGE data.

GC-FAME. All strains were grown on trypticase soy broth agar (Difco) for 24 h at 37°C. Harvesting of the cells, saponification, methylation, and extraction were done precisely as recommended for taxonomic evaluation by the Sherlock Microbial Identification System (http://www.midi-inc.com/pdf/MIS_Technote_101.pdf). The samples were analyzed on Gas Chromatograph 6890N (Agilent Technologies, Santa Clara, CA). The raw data were presented as the percentage of a specific fatty acid among all fatty acids.

OmniLog GEN III phenotype microArray assays. The metabolic characteristics of *A. citrulli* strains were analyzed using GEN III Microplates (Biolog Inc., Bremen, Germany). This method analyzes the respiration kinetics of a microorganism based on 94 phenotypic tests, including 71 sole carbon sources and 23 chemical sensitivity assays, thus providing a phenotype fingerprint. The microplates were inoculated with cells grown on LB agar plates for ≈20 h to produce a cell suspension in IF-A inoculation fluid (Biolog Inc.) with a cell density of 95% transmission. Each strain was assayed in three independent repetitions. Purple coloration through the reduction of the tetrazolium redox

dye was indicative of increased respiration and metabolic activity. Briefly, the microtiter plates with substrates, dye, and bacterial cells were loaded into the reader (OmniLog), which provided the appropriate incubation conditions (at 28°C for 96 h) and automatically read the color intensity during tetrazolium reduction every 15 min. The optical density data were further analyzed with the opm package for R software (35,36), using its functionality for displaying the curve kinetics and aggregating curve parameters such as the maximum value (A) and the area under the curve by spline fitting algorithms.

Statistical analysis and data visualization. Statistical analysis and data visualization were performed using the R environment (29). The R package multcomp was utilized for multiple comparison of groups (13,15). The R package opm was used to analyze OmniLog MicroArray Phenotype data (35,36). The R package ggplot2 was used for data visualization (41). The R Sweave function (23) was used to document all analyses into Supplemental Appendix 1.

Deposit of sequences in GenBank. The sequence data for the T3S effector genes and the housekeeping gene *gltA* that were generated in this study are available under GenBank accession numbers KF944669 to KF944906. Partial sequences of the additional six housekeeping genes of strain ZUM4000 used for MLSA were deposited under GenBank accession numbers KJ584633 to KJ584638.

RESULTS

Clustering of *A. citrulli* strains based on T3S effector sequences concurs with previously described grouping scheme. We hypothesized that the repertoire of T3S effector genes in *A. citrulli* correlates with the distribution of strains into groups I and II. To assess this hypothesis, we cloned and sequenced 11 putative effector genes from 21 *A. citrulli* strains that were isolated from various cucurbit hosts in different countries (Table 1). The genes selected for sequencing were predicted to be T3S effector genes based on the genome annotation of the group II *A. citrulli* strain AAC00-1. These genes showed homology to known T3S effectors in other plant-pathogenic bacteria (Supplemental Table 3). For comparative analyses of effectors, we also included the sequenced strain AAC00-1; thus, our population included 22 *A. citrulli* strains. Also included was a housekeeping gene, *gltA*, which was found to possess the greatest variability from a list of seven multilocus sequence-typing (MLST) genes of *A. citrulli* (10).

Comparative analyses of effector genes revealed that, within each group, the strains exhibited a high level of similarity, show-

ing only a few single-nucleotide polymorphisms (SNPs) in certain strains. The group I strain AAC98-17 showed a single SNP in position 439 (of the coding sequence) of gene *Aave_1548*, while strain 5 showed a single SNP at position 406 of gene *Aave_3237* relative to the other group I strains. Regarding group II strains, a single SNP was found for strain Tw148 at position 1284 of gene *Aave_3237*. This strain also showed a single SNP in position 172 of the housekeeping gene *gltA* relative to the other group II strains. The information summarizing the number of SNP-based haplotypes in each group is provided in Table 2.

In contrast to the above picture, clear differences were detected between group I and II strains in their effector gene sequences. First, gene *Aave_2708* was present in all group II strains tested but it could not be amplified from all tested group I strains, despite attempts using various primer combinations from either the flanking regions or the internal regions of the gene. Importantly, the draft genome sequence of the group I strain M6 generated by our research group confirmed the absence of this gene (unpublished results). Second, base deletions leading to ORF shifts were detected in genes *Aave_2166* and *Aave_3062* of all group I strains relative to group II strains (Table 1). For the latter, a nucleotide at position 558 of the ORF was deleted in all group I strains relative to group II strains, causing a frame shift and the occurrence of an early stop codon ≈220 bp before its location in the group II intact gene. In the case of *Aave_2166*, all tested group I strains possessed a 123-bp sequence deletion spanning 72 bp upstream of the predicted start codon and 51 bp downstream of it. Additionally, for most other effectors, group I and II strains clustered separately, with a total of 75 SNPs distinguishing the two groups when combining the T3S effector gene sequences for all strains tested (Table 2).

Evaluation of the ENC and GC content of T3S effector genes. In the sequenced strain AAC00-1, the 11 putative effector genes are distributed across the genome, from gene *Aave_0277* until *Aave_4728* (out of 4,796 genes), without detectable pathogenicity islands containing a cluster of effectors genes. We determined the ENC values for the 11 effector genes, compared with a set of reference genes from MLST databases and housekeeping genes (10,11). The ENC value represents how far the codon usage of a gene departs from equal usage of synonymous codons, independently of gene length and amino acid composition (43). These values range from 20 to 61, where a value of 20 means that for every amino acid there is only one codon usage (extremely high bias), while a value of 61 means that the use of alternative synonymous codons is equally likely. Genes that are part of the core genome are more likely to have greater codon bias (ENC

TABLE 2. Single-nucleotide polymorphisms (SNPs) and amino acid substitutions in each type III secreted effector gene and *gltA* for group I and group II *Acidovorax citrulli* strains^a

<i>Aave</i> gene ^b	Length of open reading frame (nt)			Number of SNP-based haplotypes, nt (aa)				SNPs between different groups, nt (aa)		
	I	II	ZUM	I	II	ZUM	Σ	I/II	I/ZUM	II/ZUM
0277	1,554	1,554	1,554	1	1	1	3	3 (2)	2 (1)	3 (1)
1548	1,455	1,455	1,455	2 (2)	1	1	4 (4)	33 (15)	4 (2)	33 (15)
2166 ^c	1,026	1,077	nd	1	1	nd	2	3 (3)	nd	nd
2173	1,119	1,119	1,119	1	1	1	3	1 (1)	1 (1)	2 (2)
2708	nd	1,119	nd	nd	1	nd	1	nd	nd	nd
2876	591	591	591	1	1	1	3	7 (3)	5 (2)	2 (1)
3062 ^d	1,241	1,242	1,242	1	1	1	3	16 (7)	71 (37)	81 (38)
3237	1,410	1,410	nd	3 (2)		nd	3 (2)	0	nd	nd
3452	1,122	1,122	1,122	1	1	1	3	7 (2)	6 (2)	7 (4)
3462	1,527	1,527	1,527	1	1	1	3	5 (2)	5 (2)	6 (2)
4728 ^e	1,923	1,923	1,914	1		1	2	0	58 (35)	58 (35)
<i>gltA</i>	486	486	486	1	2 (2)	1	4 (2)	3 (0)	4 (0)	1 (0)

^a Polymorphism at the DNA (nt = nucleotide) and protein (aa = amino acid) levels of each effector and the housekeeping gene *gltA*; nd = not detected.

^b *Aave* gene number; according to AAC00-1 annotation.

^c *Aave_2166* has a deletion of 123 bp spanning 72 bp upstream of the predicted start codon and an additional 51 bp downstream of it in all tested group I strains.

^d *Aave_3062* has a 1-bp deletion at position 558 of the open reading frame in all tested group I strains.

^e *Aave_4728* has several indels in ZUM strains as compared with both group I and II strains.

values closer to 20) than genes that were acquired by LGT. The reference genes used in this study included eight representative genes from each of the 11 clusters of orthologous genes (COGs) (<http://www.mlst.net/misc/further.asp>, <http://pubmlst.org/data/>), giving a total of 88 genes.

We observed little to no difference among the various *A. citrulli* strains based on their ENC values for the various T3S effector genes. Therefore, ENC values for effector and reference genes were compared only for sequence data from AAC00-1. This comparison revealed significantly higher ENC values for effector over reference genes (Fig. 1), meaning that, in *A. citrulli*, there is less bias in codon usage for genes encoding T3S effectors than other genes. In addition, there was a tendency toward a larger range of GC content of effector as compared with reference genes (Supplemental Figure 2). Altogether, these findings, as well as the high similarity of the putative effector genes with effector genes from other plant-pathogenic bacteria, strongly suggest that the assessed effector genes of *A. citrulli* were acquired through LGT.

Comparative analyses of T3S effector genes suggest the existence of a third group of *A. citrulli* strains. Strains ZUM4000 and ZUM4001 were isolated from symptomatic watermelon seedlings in India in 2008. These strains were less virulent on watermelon than other *A. citrulli* strains and, in contrast to other strains, yielded negative reactions in certain serological tests and PCR reactions with some *A. citrulli* primers (B. Woudt, Syngenta Vegetable Seeds, personal communication). Similar to group I strains, the ZUM strains were found to lack gene *Aave_2708*. In contrast, the ZUM strains possessed a functional *Aave_3062* gene, without the single base deletion observed for

group I strains. On the other hand, although only 16 SNPs were found in gene *Aave_3062* between group I and II strains, 71 SNPs were detected when comparing group I and ZUM strains, and 81 SNPs were detected between group II and ZUM strains (Table 2). Overall, 158 and 192 SNPs were detected when comparing the entire sequences of all assessed T3S effector ORFs of ZUM strains relative to group I and group II strains, respectively (Table 2).

Furthermore, two genes, *Aave_2166* and *Aave_3237*, that were present in all tested group I and II strains, were not detected in the ZUM strains (Tables 1 and 2). Finally, although gene *Aave_4728* was highly conserved among group I and II strains (no SNPs were detected among strains of the two groups), 58 SNPs and 21 indels in two different regions (one 15-base deletion and one 6-base insertion) were observed in the ZUM strains (Table 2).

In terms of sequence comparison, phylogenetic trees generated by the maximum parsimony method revealed that the majority of T3S effector genes from the ZUM strains clustered separately but closer to group I than group II strains (Fig. 2A, gene *Aave_3452*; Supplemental Figure 3, most other effectors). Only in the case of one effector gene, *Aave_2876*, did the ZUM strains cluster more closely to group II than group I strains (Fig. 2B). Another scenario was that the ZUM strains clustered as a third, completely distinguishable group (Fig. 2C, *Aave_3062*; *Aave_2173* and *Aave_4728*). Overall, by comparative analyses of the combined sequences of all ($n = 11$) assessed T3S effector genes, the ZUM strains clustered separately from groups I and II (Fig. 2D). This observation is independent of inclusion of the outgroup taxon in the trees (Supplemental Figure 4A and B). In addition, a similar observation was obtained when the trees were generated using maximum likelihood and neighbor-joining methods.

Fig. 1. Effective number of codons (ENC) usage of type III secreted effector genes relative to housekeeping genes in *Acidovorax citrulli* AAC00-1. ENC values of effectors genes were determined using DnaSP software. The obtained values were compared with ENC values of 88 housekeeping genes from 11 different clusters of orthologous genes (8 genes per category). ENC values range from 20 to 61, with 20 corresponding to high codon bias (for every amino acid, only one codon take place) and 61 corresponding to no codon bias (the use of synonymous codons is equally likely). Overall, the ENC values of effector genes were found to be significantly higher than those of housekeeping genes. The boxplots underlying the dots indicate minimum, first quartile, median, third quartile, and maximum values.

We recently sequenced the genome of one of the ZUM strains, ZUM4000 (draft sequence; unpublished data). We performed a MLSA of seven conserved genes, using the sequences from ZUM4000 and selected group I and II strains, available from the report of Feng et al. (10). Despite the lower level of variability of conserved genes relative to effector genes, these phylogenetic analyses also led to a clustering into three different groups, with the ZUM strain clustering separately from group I and II strains (Supplemental Figure 5). These results strengthened the hypothesis that there are at least three different groups of *A. citrulli* strains, and variability among T3S effector genes reflects divergence of this species into three distinct lineages.

ZUM strains are less virulent than group I and II strains. The virulence of selected *A. citrulli* strains (representing different PFGE-based haplotypes from previous characterizations) (Table 1) was compared on melon, watermelon, and squash seedlings. A high variability in virulence was observed among strains belonging to the same group, and also between replicates of the

same strain. Nevertheless, in terms of comparison of group I and II strains, results from virulence assays were in accordance with previous reports (3,38,39). Group I and II strains did not differ significantly in their virulence on watermelon, while group I strains induced the highest degrees of disease severity on melon and squash (Fig. 3; statistical analyses). Exceptions were the strains isolated in Taiwan, Tw76 (group I), Tw16, and Tw148 (group II), which, in contrast to typical group I and II strains characterized in this and other studies, showed very low levels of virulence on the three tested hosts (Fig. 3). This could be due to loss of virulence of these strains during storage. The fact that the three strains were obtained from the same collection supports this assumption. Also, as previously reported (38), squash plants were less susceptible than melon and watermelon. In agreement with preliminary information (B. Woudt, personal communication), the ZUM strains were significantly less virulent than group I and II strains on watermelon but also on melon and squash (Fig. 3).

Fig. 2. Phylogenetic trees of representative type III secreted effector gene sequences of *Acidovorax citrulli*. Phylogenetic trees based on the nucleotide sequences of some effector genes using the maximum parsimony method with 1,000 replicates bootstrap test. GI = group I and GII = group II. **A**, Tree based on *Aave_3452* sequence data; the ZUM strains cluster closer to group I strains. **B**, Tree based on *Aave_2876* sequence data; the ZUM strains cluster closer to group II strains. **C**, Tree based on *Aave_3062* sequence data; the ZUM strains cluster as a third distinguished group. **D**, Tree based on sequences of all (11) effector genes combined; the ZUM strains cluster as a third group.

DNA:DNA hybridization confirms that the ZUM strains belong to species *A. citrulli*. Based on phylogenetic analyses of effector and conserved genes and other features, the ZUM strains appear to represent a distinct group, relative to the previously characterized groups I and II of *A. citrulli*. Recently, Makizumi et al. (25) isolated *Acidovorax* strains from infected cucurbits that were different from *A. citrulli* based on 16S ribosomal DNA sequences, indicating that other *Acidovorax* spp. may infect cucurbit plants and induce weak to mild symptoms. Taking this study into consideration, we sought to determine the identity of a representative ZUM strain using DNA:DNA hybridization, which is the “gold standard” for defining bacterial species (33). ZUM4000 was hybridized with both the sequenced strain AAC00-1 and the type strain DSM17060. The percentages of hybridization between DNA of ZUM4000 and DSM17060 or AAC00-1 were 87.5 and 78.6%, respectively. As a control comparison, the percentage of hybridization between AAC00-1 and DSM17060 was 84.8%. Because a threshold value of >70% DNA-DNA similarity meets the criteria for strains belonging to the same species (40), these results confirmed that strain ZUM4000 belongs to species *A. citrulli*. This is also likely for ZUM4001 that was shown to be identical to ZUM4000 based on T3S effector sequences. In fact,

because ZUM4000 and ZUM4001 were identical or highly similar in several other features (see below), and were isolated from the same batch of infected seedlings, it is likely that these strains are clonal.

Characterization of ZUM strains by DNA fingerprinting, carbon utilization, and GC-FAME. Thus far, T3S effector sequences and virulence data indicated that group I and II of *A. citrulli* strains are distinguishable based on their effectors' repertoire and that the ZUM strains represent a third, previously uncharacterized group. An additional objective of this study was to determine whether the ZUM strains can be considered as a third group in terms of characteristics that are not directly associated with pathogenicity. To accomplish this objective, strains used in this study were evaluated by DNA fingerprinting (PFGE and rep-PCR), carbon substrate utilization assays (OmniLog PM), and composition of fatty acids by GC-FAME.

As expected from previous reports (3,38,39), PFGE of *SpeI*-digested DNA fragments clearly distinguished the representative group I and II strains (Supplemental Figure 6). However, in several attempts, we failed to generate PFGE profiles for the ZUM strains, probably due to DNA degradation during sample preparation (19,37). This was also the case when several other

Fig. 3. Disease severity induced by selected *Acidovorax citrulli* strains on different cucurbit seedlings. Fourteen-day-old seedlings of three different hosts (melon, watermelon, and squash) were spray inoculated with suspensions of 14 representative *A. citrulli* strains at $\approx 10^6$ CFU/ml or phosphate-buffered saline buffer (as a negative control). Seedlings were evaluated after 10 days after inoculation using a previously described 1-to-9 scale (14). Each treatment (plant-strain combination) was composed of up to four replicates (every dot represents a different repetition) and the experiment was repeated three times (Exp_1, Exp_2, and Exp_3). Different letters indicate significant differences ($P = 0.05$) among different groups (group I, group II, ZUM strains, and control) in each host plant.

restriction enzymes were used (data not shown). Therefore, strains were assessed by rep-PCR using the BOX primer, which was also shown to effectively differentiate group I and II strains (3,38). BOX-PCR analysis not only differentiated group I and II strains but also clustered the ZUM strains as a third group (Fig. 4).

In contrast, GC-FAME and carbon utilization assays did not clearly differentiate the ZUM strains as a third group. However, these assays were also less able to discriminate between group I and II strains compared with T3S effector gene analyses and DNA fingerprinting. For example, based on GC-FAME analysis, the group I strain M1 clustered with some group II strains, while the group II strain AAC00-1 was closer to some group I strains (Fig. 5). Despite this, detailed observations of several individual fatty acids such as 18:1 w7c and 14:0 revealed clear differences between the ZUM strains and the others (Supplemental Figure 7). Regarding carbon utilization data, the group II strain AAC92-17 clustered far away from the rest of the group II strains, and the same pattern was observed for the group I strain Tw76 relative to the rest of group I strains (Fig. 6).

DISCUSSION

Like other gram-negative plant-pathogenic bacteria, *A. citrulli* requires a functional T3SS to cause disease (1,17). Annotation of the *A. citrulli* group II strain AAC00-1 revealed several genes encoding putative T3S effectors, based on their similarities to validated effectors from other plant-pathogenic bacteria. Based on gene organization, sequence analysis, and regulation, *hrp* clusters are divided into two classes: class I contains *Pseudomonas syringae* and enteric plant-pathogenic bacteria, while class II contains *Xanthomonas* spp. and *Ralstonia solanacearum* (2,5). Based on sequence analysis and cluster organization, the *A. citrulli* *hrp*

Fig. 5. Heat map based on gas chromatography fatty acid methyl ester analysis. Heat map represents color-coded abundance of short chain fatty acids in different *Acidovorax citrulli* strains. The rectangle at the left indicates the group of each strain: pale blue = group I, brown-yellow = group II, and purple = ZUM strains (group III). Values of each strain sum up to 100. However, fatty acids that could not be unequivocally identified are not shown. The two ZUM strains are more closely related to group II strains. The main difference between the ZUM strains and the others are in fatty acid 18:1 w7c.

Fig. 4. Repetitive polymerase chain reaction (rep-PCR) profiles of selected *Acidovorax citrulli* strains. rep-PCR profiles were performed using the BOXA1R primer (BOX-PCR). Profiles were analyzed using Dice’s coefficient of analysis and the unweighted pairwise group method with arithmetic mean algorithm generated a dendrogram to visualize relationships among strains.

cluster belongs to class II (4). Therefore, it is not surprising that most putative T3S effector genes detected in the AAC00-1 genome are closely related to effector genes from *Xanthomonas* spp. and *R. solanacearum*.

The fact that the 11 effector genes investigated in this study were detected based on their similarity to effectors of other plant-pathogenic bacteria strongly suggests that these effector genes were assimilated by LGT. LGT is generally reflected by differences in GC content and codon usage bias relative to the core genome (7,22). Indeed, a comparison between the 11 effector genes and 88 housekeeping genes from strain AAC00-1 repre-

sending various COG classes and spread throughout the genome revealed that the ENC was significantly higher in the effector genes (Fig. 1), which also showed a more scattered profile of GC content. These data support the notion that the effector genes assessed in this study were acquired by *A. citrulli* by LGT.

To date, there have been no reports on experimental and functional characterization of *A. citrulli* T3S effectors. In the present study, we cloned and sequenced the ORFs of 11 putative T3S effectors from a collection of *A. citrulli* strains. Our hypothesis was that, due to the crucial role of T3S effectors in pathogenicity, the effector repertoire of *A. citrulli* strains may

Fig. 6. Heat map based on carbon substrate utilization data (OmniLog Phenotype MicroArray). Heat map representing color-coded capability of the different *Acidovorax citrulli* strains to utilize various carbon sources. The rectangle at the left indicates the group each strain belongs to: pale blue = group I, brown-yellow = group II, and purple = ZUM strains (group III). Values represent the area under the curve of each well. The two ZUM strains are more closely related to group I strains. Note that some of the group II strains are clustered together with group I strains and vice-versa.

correlate with the preferential host association observed for group I and II strains (3,38,39). In support of this hypothesis, we found that group I and II strains of *A. citrulli* cluster separately based on effector sequences (Table 1; Fig. 2). First, one effector present in all assessed group II strains, *Aave_2708* (according to the AAC00-1 annotation), was absent in all group I strains. Second, two effectors, *Aave_2166* and *Aave_3062*, had base deletions in all group I strains, likely affecting their functionality. Third, although a high degree of conservation was found among effector sequences within each group, several SNPs (often leading to amino acid substitutions) were detected for most effector genes when comparing strains among groups (Table 2). In contrast, few SNPs with no amino acid substitutions were observed for the housekeeping gene *gltA* between the groups (Table 2) which, according to a recent MLST study, was shown to be the most variable housekeeping gene among the ones tested (10). This suggests a rather strong degree of divergence in the effector genes between groups I and II compared with housekeeping genes. In agreement with previous reports (3,38,39), virulence assays on various cucurbit hosts (Fig. 3), DNA-fingerprinting by BOX-PCR (Fig. 4), and PFGE all distinguished between strains of both groups.

In the present study, we included two strains, ZUM4000 and ZUM4001 (ZUM strains), which were isolated from symptomatic watermelon seedlings in India. We included these strains because previous unpublished information suggested that they behaved differently than typical *A. citrulli* strains. Briefly, the ZUM strains appeared to be less virulent than other strains on watermelon, reacted negatively with Agdia and Envirologix immunostrips diagnostic for *A. citrulli*, and did not yield PCR products for certain *A. citrulli* primers (B. Woudt, personal communication). Overall, this information suggested that these strains could constitute a new group of *A. citrulli* strains. Indeed, comparative analyses of effector genes as well as housekeeping genes supported that the ZUM strains represent a third, previously unreported *A. citrulli* group. In contrast with group I and II strains, genes *Aave_2166* and *Aave_3237* could not be amplified in these strains. In addition, a much higher number of SNPs were detected when comparing effector genes from ZUM strains with those of group I and II than when comparing between group I and II strains (Table 2). The maximum parsimony method was preferred for constructing phylogenetic trees because the amount of variation in each sequence was relatively small (26); however, results with this method were confirmed by maximum likelihood and neighbor-joining methods. A phylogenetic tree based on all effector sequences confirmed that the ZUM strains form a third, unique clade (Fig. 2D). The picture emerging from phylogenetic analysis of effector genes was confirmed by MLSA of seven housekeeping genes and by BOX-PCR profiling (Fig. 4), in which the ZUM strains clustered separately from group I and II. DNA:DNA hybridization assays performed with ZUM4000, confirmed that this strain belongs to the *A. citrulli* species. The same is likely true for ZUM4001 because previous evidence and results from this study indicate that these strains are clonal.

In agreement with previous preliminary observations, the ZUM strains were consistently less virulent than group I and II strains on watermelon but also on melon and squash seedlings (Fig. 3). We hypothesize that the lack of some effector genes in these strains, as well as the high number of base substitutions in other effectors relative to group I and II strains, contribute, at least partially, to the observed reduced virulence of these strains. However, this hypothesis should be further verified by experimental characterization of different T3S effector genes. The reduced virulence of these strains and, consequently, their likely reduced epidemiological potential could also explain why strains belonging to the new group have not been commonly isolated from BFB outbreaks. In contrast to comparative analyses of effector genes, virulence assays, and BOX-PCR, GC-FAME and

carbon substrate utilization assays failed to clearly discriminate the ZUM strains from group I and II strains. However, these methods were also less sensitive than the other analyses for discriminating between group I and II strains (Figs. 5 and 6).

Yan et al. (44) recently characterized the genetic diversity of *A. citrulli* in China using MLST and PFGE. The authors reported that, based on the MLST analysis, one of the strains (Fc380, isolated in the United States) did not cluster with group I or II strains. However, this separated singleton was visible only when performing eBURST analysis, whereas phylogenetic analyses and PFGE clearly positioned this strain within group II. In addition, further verification of the sequence data of strain Fc380 revealed a high number of base insertions and deletions causing frame shifts in some of the assessed housekeeping genes (three of five genes that showed variability among the different strains). Because the number of indels leading to frame shifts is unlikely for housekeeping genes, this suggests the occurrence of sequencing errors for this strain. Therefore, it is unlikely that the singleton reported by Yan et al. (44) represents a new distinguished group of *A. citrulli*.

In conclusion, this study showed that group I and II strains of *A. citrulli* can be clearly distinguished based on their effector repertoire. It is possible that the difference in the arsenal of effectors contributes to host preference association of the groups. In this regard, host selection pressure (e.g., watermelon versus non-watermelon cucurbits) could have contributed to shaping differences in T3S effectors, because different hosts may carry different sets of pathogenicity targets or resistance proteins that evolved independently. We also report, for the first time, the occurrence of a third, clearly distinguished group of *A. citrulli*. It is important to stress that the current number of predicted T3S effectors in *A. citrulli*, which was based on similarity searches, is likely underestimated. Moreover, this study was based on predicted effectors from the annotated group II strain AAC00-1. As we found that some AAC00-1 or group II effectors are not present or are not functional in group I and ZUM strains, it is also possible that group I or ZUM strains carry functional, group-specific effectors. We are currently searching for new, putative T3S effectors in a range of *A. citrulli* genomes. In addition, we are generating mutant strains impaired in selected effectors or carrying swapped effectors from other groups to assess the contribution of individual effectors to virulence and host preferential association. Effectors showing significant contribution to virulence or host preference could be used in the future screens for sources of BFB disease resistance, which are lacking to date.

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