Association Between Loss of Type IV Pilus Synthesis Ability and Phenotypic Variation in the Cucurbit Pathogenic Bacterium *Acidovorax citrulli*

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Acidovorax citrulli is the causal agent of bacterial fruit blotch of cucurbits. We have shown that functional type IV pili (T4P) are required for full virulence of this bacterium. To identify *A. citrulli* genes required for T4P activity, we screened a library of about 10,000 transposon mutants of *A. citrulli* M6 for altered T4P-mediated twitching motility. This screen led to the identification of 50 mutants impaired in twitching ability due to transposon insertions into 20 different genes. Representative mutants with disruptions in these genes were further characterized. All mutants were compromised in their virulence in seed transmission and stem inoculation assays and had reduced biofilm formation ability relative to wild-type M6. When grown on nutrient agar, most mutants produced colonies with a translucent and fuzzy appearance, in contrast to the opaque and smooth appearance of wild-type colonies. The colony morphology of these mutants was identical to that of previously reported phenotypic variants of strain M6. The exceptions were M6 mutants disrupted in genes *tonB*, *pilT*, *pilW*, and *pilX* that exhibited typical wild-type colony morphology, although lacking twitching halos surrounding the colony. Transmission electron microscopy revealed that most mutants lacked the ability to produce T4P. The exceptions were mutants with disruptions in *tonB*, *pilT*, *pilW*, and *pilX* genes that were shown to produce these appendages. These findings support the idea that colony phenotypic variation in *A. citrulli* is determined by the lack of ability to synthesize T4P but not by lack of T4P functionality.

Acidovorax citrulli is a gram-negative bacterium that causes bacterial fruit blotch (BFB) of cucurbits (Schaad et al. 2008). Under favorable conditions, characterized by high relative humidity and intermediate to high temperatures, the bacterium is able to cause seedling blight and fruit rot of a range of cucurbitaceous plants (Burdman and Walcott 2012). BFB started to gain importance after devastating outbreaks in watermelon fields in the

Mariana Islands and in Florida in the late 1980s (Somodi et al. 1991; Wall and Santos 1988). Since then, the disease rapidly spread to many parts of the world and was reported to cause significant losses in several crops within the Cucurbitaceae family, mainly melon and watermelon (Burdman and Walcott 2012). Due to the destructive potential of the disease and the limited efficacy of BFB management strategies, *A. citrulli* is considered a serious threat to cucurbit production worldwide (Burdman and Walcott 2012).

A. citrulli strains belong to two major groups that can be clearly distinguished based on DNA fingerprinting techniques, carbon utilization profiles, fatty acid methyl ester analysis by gas chromatography-fatty acid methyl ester analysis, and host preferential association. Group I comprises strains isolated mainly from nonwatermelon hosts, while group II comprises strains isolated mainly from watermelon seedlings and fruits (Burdman et al. 2005; Walcott et al. 2000, 2004). Few genomes of *A. citrulli* strains are available to the scientific community. These include those of strains M6 and AAC00-1, which are considered the model group I and II strains, respectively (Eckstein-Levi et al. 2016).

Despite the economic importance of BFB, little is known about the pathogen's virulence mechanisms. Like many other gram-negative plant pathogenic bacteria, *A. citrulli* relies on a functional Hrp (for hypersensitivity response and pathogenicity) type III secretion system to promote disease on susceptible plants (Bahar et al. 2010; Johnson et al. 2011). Eckstein-Levi et al. (2014) recently showed that group I and II strains of *A. citrulli* can be clearly distinguished based on differences in their arsenal of type III-secreted effectors (T3Es) suggesting that, in this bacterium, differences in T3Es are critical in determining the patterns of aggressiveness on different cucurbit species.

Type IV pili (T4P) (also called type IV fimbriae) play an important role in virulence of *A. citrulli* (Bahar et al. 2009a). T4P are hair-like appendages found on the poles of the cell surface, where they mediate twitching motility, an efficient flagellar-independent form of bacterial surface motility (Jarrell and McBride 2008; Mattick 2002). T4P have been shown to be involved in surface adhesion and colonization, biofilm formation, genetic material transfer, and virulence (Craig et al. 2004; Nudleman and Kaiser 2004). In a previous study, we characterized *pilM* and *pilT* mutants of *A. citrulli* M6. In T4P-producing bacteria, *pilM* encodes an inner membrane protein that is needed for T4P assembly while *pilT* encodes a cytoplasm or inner membrane component that mediates pilus retraction and disassembly (Burdman et al. 2011; Nudleman and Kaiser 2004). Accordingly, the *A. citrulli pilM* and *pilT* mutants were impaired in T4P assembly and function, respectively, and both mutants lacked twitching motility and had reduced biofilm

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formation ability and virulence as compared with the wild-type strain (Bahar et al. 2009a; 2010). We also showed that *A. citrulli* is able to proliferate in the xylem vessels of melon seedlings (Bahar et al. 2009a). Further, Makizumi et al. (2011) showed that *A. citrulli* was able to colonize the xylem tissue of mature squash leaves, thus supporting the vascular colonization ability of this pathogen.

Recently, we reported the natural occurrence of phenotypic variation in group I (M6) and II (7a1) strains of *A. citrulli* (Shrestha et al. 2013). Phenotypic variation in these strains was shown to occur at a relatively high frequency and under various conditions. Phenotypic variants (PVs) can be easily detected on nutrient agar (NA), since they produce colonies with distinguished morphology compared with those of their parental strains. In contrast to the opaque, smooth, and regular appearance of typical *A. citrulli* colonies, PV colonies are bigger than those of the parental strains and are translucent, fuzzy, and have irregular borders. In addition, under tested conditions, PVs do not produce T4P nor perform twitching motility and possess reduced biofilm formation and virulence abilities compared with the parental strains (Shrestha et al. 2013). All the above phenotypes are also characteristic of the *A. citrulli* M6 *pilM* mutant, suggesting an association between loss of either T4P synthesis, functionality, or both and phenotypic variation in this bacterium.

With the aim of identifying new genes involved in twitching motility in *A. citrulli*, we screened a Tn5 mutant library of strain M6 for impairment in this ability. We identified fifty mutants that lacked twitching motility. A plasmid rescue approach applied to all identified mutants led to the identification of twenty genes, including new mutants impaired in *pilM* and *pilT*, different from those reported by Bahar et al. (2009a; 2010). As similar as for the previously characterized *pilM* and *pilT* mutants, the new twitching mutants had reduced biofilm formation ability and compromised virulence relative to wild-type M6. A detailed characterization of mutants' abilities to produce T4P and of their colony morphology strongly support that in *A. citrulli*, phenotypic variation is associated with the loss of the ability to synthesize T4P but not with the loss of T4P-dependent twitching ability.

RESULTS

Identification of *Acidovorax citrulli* M6 mutants impaired in twitching motility.

A random mutant library in the background of *Acidovorax citrulli* M6 was constructed using the Epicenter EZ-Tn5 technology. The mutagenesis cassette consisted of a kanamycin resistance (Km^R) marker flanked by mosaic ends, which are recognized by the transposase provided with the kit. *A. citrulli* M6 competent cells were transformed with the cassette and the transposase and about 10,000 colonies were screened on NA plates containing Km for altered twitching motility as compared with the wild type. Importantly, twitching motility in *A. citrulli* can be easily detected on NA plates by the formation of twitching haloes surrounding the bacterial colony. These haloes are clearly visible by the naked eye after 72 h of growth (Bahar et al. 2009a).

A total of 50 mutants that lacked twitching motility were identified. Southern blot analyses of restricted DNA from the mutants, using the Km^R cassette as a probe, confirmed the effectiveness of the strategy for random mutagenesis of *A. citrulli* (Bahar et al. 2009a). In all mutants, the cassette appeared to be inserted only once and at varied positions in the bacterial chromosome (not shown). A plasmid rescue approach was used to identify the chromosomal location of the Tn5 cassette insertions in all mutants. Many mutants shared Tn5 cassette insertions in the same gene, leading to a total of 20 different genes (Table 1). Twenty mutants representing all identified genes were selected for further characterization. A

schematic representation of the Tn5 cassette insertion in these mutants is shown in Supplementary Figure S1. To verify the impairment of twitching ability as seen by the naked eye, mutant colonies were observed under a light microscope. All mutant colonies lacked the typical wild-type twitching-mediated halo around the colony perimeter (Fig. 1).

In most T4P-producing bacteria, most genes encoding T4P biosynthetic and regulatory components are named *pil* and *fim* genes (for pilus and fimbriae, respectively) (Burdman et al. 2011; Mattick 2002). Therefore, it was not surprising that most genes detected in this study are homologs of *pil/fim* genes from other T4P-producing bacteria like the plant-pathogenic bacterium *Xylella fastidiosa* and the well-investigated bacteria in terms of T4P research, *Pseudomonas aeruginosa* and *Neisseria* spp. (Carbonnelle et al. 2006; Craig and Li 2008; Mattick 2002). They included genes *pilA* (*pilE* in *Neisseria* spp.), *pilB*, *pilE*, *pilM*, *pilN*, *pilO*, *pilP*, *pilQ*, *pilT*, *pilU*, *pilW* (*pilF* in *P. aeruginosa*), *pilX*, *pilY1*, *fimV*, and *fimT* (Table 2).

Three mutants were disrupted in genes that belong to the same operon and encode components of chemosensory systems that regulate different types of motility in bacteria (Wadhams and Armitage 2004). The mutated genes encode proteins that are homologs of a methyl-accepting chemotaxis protein (MCP) (*PilJ*), a CheW-like coupling protein (named *PilI* in *X. fastidiosa* and *ChpC* in *P. aeruginosa*), and a CheA-like histidine kinase (*PilL* in *X. fastidiosa* and *ChpA* in *P. aeruginosa*) (Table 2). Hereafter, we refer to these genes using the names designated for *X. fastidiosa*.

An additional gene that was detected in our screen was *tonB*, encoding a cytoplasmic membrane proton-motive force protein. TonB proteins have been shown to be required for active transport of various substrates via the outer membrane in several gram-negative bacteria (Noinaj et al. 2010). Last, a twitching-deficient mutant was detected in gene *APS58_04405*, which encodes a histidine kinase located upstream of a gene encoding a putative transcriptional regulator that belongs to the *LytR/AlgR* family (Table 2).

Biofilm formation ability is compromised in the twitching motility mutants.

T4P have been shown to be involved in biofilm formation of various bacterial species (Craig et al. 2004; Nudleman and Kaiser 2004). Moreover, we showed that *pilM* and *pilT* mutants of *A. citrulli* M6 were compromised in this ability (Bahar et al. 2009a; 2010). In the present study, all mutants were significantly ($P < 0.05$) impaired in biofilm formation ability relative to wild-type M6 (Fig. 2). While most mutants showed a strong reduction in biofilm formation ability, with biofilm levels ranging from 15 to 40% of that of the wild type, few mutants (e.g., mutants in genes *tonB*, *pilJ*, *pilX*, and *APS58_04405*) showed a slighter but still significant reduction in this ability (Fig. 2).

Twitching mutants have reduced virulence relative to wild-type M6.

Bahar et al. (2009a) showed that *pilM* and *pilT* mutants of *A. citrulli* M6 are compromised in their virulence relative to wild-type M6. We performed stem inoculation and seed transmission assays to characterize the newly identified mutants. In stem inoculation assays, two independent experiments with similar results revealed higher percentages of wilted seedlings following inoculation with wild-type M6 in comparison with the twitching motility mutants. Data from a representative experiment is shown in Figure 3. While inoculation with strain M6 led to about 95% of dead seedlings at 7 days after inoculation (dai), the average percentage of dead seedlings among those inoculated with the mutants ranged from approximately 15 to 50% (Fig. 3). The *pilM* mutant strain characterized by Bahar et al. (2009a) was also used in these experiments as an additional control (Fig. 3, strain M6-M)

and showed a similar effect on seedling death as that observed for the newly identified *pilM* mutant (Fig. 3A).

In seed transmission assays, virulence was evaluated by measurement of foliage weight and stem length of seedlings emerging from inoculated seeds at 10 dai. We have shown that, in these assays, these parameters correlate negatively with disease severity (Bahar et al. 2009b). Three seed transmission assays were performed that showed similar results. Most (18 of 20) twitching motility mutants exhibited significant ($P < 0.05$) reduction in virulence (e.g., higher stem length and foliage

weight values) relative to wild-type M6 (Fig. 4; Supplementary Fig. S2). With that said, two of the mutants, deficient in genes *pilJ* and *APS58_04405*, did not differ significantly from wild-type M6 in these experiments, although higher stem length and foliage weight averages were measured in plants inoculated with these mutants relative to M6-inoculated plants. Regarding the *pilJ* mutant, further seed transmission experiments that were performed (under different conditions, discussed below) to assess complementation of selected mutants showed that the *pilJ* mutant had significantly reduced virulence than the wild type. In addition,

Table 1. Strains and plasmids used in this study

Strains/plasmids	Characteristics ^a	Reference or source	Locus tag in M6 (and in AAC00-1) ^b
<i>Acidovorax citrullii</i>			
M6	Ap ^R , wild type, group I strain	Burdman et al. 2005	
M6-M	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilM</i>	Bahar et al. 2009a	
M6 <i>pilA::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilA</i>	This study	N.A. ^c (<i>Aave_4679</i>)
M6 <i>pilB::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilB</i>	This study	<i>APS58_08770</i> (<i>Aave_3680</i>)
M6 <i>pilE::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilE</i>	This study	<i>APS58_05050</i> (<i>Aave_3550</i>)
M6 <i>pilI::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilI</i>	This study	<i>APS58_00910</i> (<i>Aave_0903</i>)
M6 <i>pilJ::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilJ</i>	This study	<i>APS58_00905</i> (<i>Aave_0904</i>)
M6 <i>pilL::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilL</i>	This study	<i>APS58_00900</i> (<i>Aave_0905</i>)
M6 <i>pilM::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilM</i>	This study	<i>APS58_00440</i> (<i>Aave_0996</i>)
M6 <i>pilN::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilN</i>	This study	<i>APS58_00435</i> (<i>Aave_0997</i>)
M6 <i>pilO::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilO</i>	This study	<i>APS58_00430</i> (<i>Aave_0998</i>)
M6 <i>pilP::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilP</i>	This study	<i>APS58_00425</i> (<i>Aave_0999</i>)
M6 <i>pilQ::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilQ</i>	This study	<i>APS58_00420</i> (<i>Aave_1000</i>)
M6 <i>pilT::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilT</i>	This study	<i>APS58_10505</i> (<i>Aave_0637</i>)
M6 <i>pilU::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilU</i>	This study	<i>APS58_10515</i> (<i>Aave_0635</i>)
M6 <i>pilW::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilW</i>	This study	<i>APS58_13315</i> (<i>Aave_1422</i>)
M6 <i>pilX::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilX</i>	This study	<i>APS58_05060</i> (<i>Aave_3552</i>)
M6 <i>pilY1::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>pilY1</i>	This study	<i>APS58_05055</i> (<i>Aave_3551</i>)
M6 <i>fimT::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>fimT</i>	This study	<i>APS58_05045</i> (<i>Aave_3549</i>)
M6 <i>fimV::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>fimV</i>	This study	<i>APS58_12055</i> (<i>Aave_1218</i>)
M6 <i>tonB::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>tonB</i>	This study	<i>APS58_01195</i> (<i>Aave_0842</i>)
M6 <i>APS58_04405::Tn5</i>	Ap ^R , Km ^R , Tn5 mutant defective in <i>APS58_04405</i>	This study	<i>APS58_04405</i> (<i>Aave_1948</i>)
M6 <i>tonB</i> -comp	Ap ^R , Km ^R , Gm ^R tonB complemented strain (carrying plasmid pBBRtonB)	This study	
M6 <i>pilI</i> -comp	Ap ^R , Km ^R , Gm ^R , <i>pilI</i> complemented strain (carrying plasmid pBBRpilI)	This study	
M6 <i>pilJ</i> -comp	Ap ^R , Km ^R , Gm ^R , <i>pilJ</i> complemented strain (carrying plasmid pBBRpilJ)	This study	
M6 <i>pilL</i> -comp	Ap ^R , Km ^R , Gm ^R , <i>pilL</i> complemented strain (carrying plasmid pBBRpilL)	This study	
<i>Escherichia coli</i>			
BW25141	F ⁻ , Δ (<i>araD-araB</i>) 567, Δ (<i>lacZ4787::rrnB-3</i>), Δ (<i>phoB-phoR</i>)580, λ^- , <i>galU</i> 95, <i>AuidA</i> 3::pir ⁺ , <i>recA</i> 1, <i>endA</i> 9(Δ del-ins)::FRT, <i>rph</i> -1, Δ (<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	Datsenko and Wanner 2000	
S17-1 λ pir	λ lysogenic S17-1 derivative producing π protein for replication of plasmids carrying <i>oriR6K</i> ; <i>recA pro hsdR</i> RP4-2-Tc::Mu-Km::Tn7 λ^- pir	Simon et al. 1983	
Plasmids			
pUC-4K	pUC4 derivative (pMB1 ori, Ap ^R), containing the Km ^R cassette from Tn903	Vieira and Messing 1982	
pMOxD-3 < R6K γ ori/MCS >	Ap ^R ; transposon construction vector	Epicenter	
pBBR1-MCS-5	Gm ^R ; broad host range vector	Kovach et al. 1995	
pBBRprom-tonB	Gm ^R ; pBBR1-MCS-5 containing a 406-bp fragment upstream of the coding sequence of <i>tonB</i> gene	This study	
pBBRprom-pil	Gm ^R ; pBBR1-MCS-5 containing a 456-bp fragment upstream of the <i>APS58_00920-APS58_0900</i> operon that contains genes <i>pilI</i> , <i>pilJ</i> , and <i>pilL</i>	This study	
pBBRtonB	Gm ^R ; pBBR1-MCS-5 containing the the <i>tonB</i> gene with its 406-bp upstream region	This study	
pBBRpilI	Gm ^R ; pBBR1-MCS-5 containing the <i>pilI</i> gene, the upstream region of operon <i>APS58_00920-APS58_0900</i>	This study	
pBBRpilJ	Gm ^R ; pBBR1-MCS-5 containing the <i>pilJ</i> gene, the upstream region of operon <i>APS58_00920-APS58_0900</i>	This study	
pBBRpilL	Gm ^R ; pBBR1-MCS-5 containing the <i>pilL</i> gene, the upstream region of operon <i>APS58_00920-APS58_0900</i>	This study	

^a Km^R, Ap^R, and Gm^R indicate kanamycin (50 μ g/ml), ampicillin (100 μ g/ml), and gentamicin (10 μ g/ml) resistance, respectively.

^b Locus numbers of mutants identified in our screen for twitching-deficient mutants. *APS58_XXXXX* are the locus numbers in strain M6 (GenBank accession LKUW00000000). *Aave_XXXX* (shown between parentheses) are the ortholog loci in strain AAC00-1 (GenBank accession NC_008752).

^c N.A. = not applicable.

as indicated above, both *pilJ* and *APS58_04405* mutants induced substantially lower levels of seedling death than the wild type in stem-inoculation experiments (Fig. 3). Therefore, we are confident that all twitching-deficient mutants assessed in this study are compromised in virulence relative to the wild-type strain.

As similar as the previously characterized *pilM* and *pilT* mutants (Bahar et al. 2009a), the twitching motility mutants characterized in this study did not differ in their ability to grow in nutrient broth (NB) relative to strain M6 (not shown), thus indicating that the reduced virulence of the twitching motility mutants is not due to reduced growth ability.

Fig. 1. The occurrence or absence of twitching motility of *Acidovorax citrulli* wild-type strain M6 and Tn5 mutants, respectively, as seen by light microscopy after 48 h of growth on nutrient agar plates at 28°C. Typical haloes surrounding the bulk colonies (indicated by the black line in strain M6) are formed by bacteria migrating via twitching. Pictures were taken using an Axio Scope light microscope equipped with a DXM1200F digital camera.

Table 2. Genes that were shown to be required for twitching motility in *Acidovorax citrulli* M6 and their homologs in *Pseudomonas aeruginosa* and *Xylella fastidiosa*, based on BlastN results

Mutated gene ^a	Homolog in <i>P. aeruginosa</i>	Homolog in <i>X. fastidiosa</i>	Gene function/product	Category ^b
<i>pilA</i> (2)	<i>pilA</i>	<i>pilA</i>	Major pilin	A
<i>pilB</i> (3)	<i>pilB</i>	<i>pilB</i>	ATPase that mediates pilus assembly and extension	C
<i>pilE</i> (3)	<i>pilE</i>	<i>pilE</i>	Minor pilin	A
<i>pilI</i> (3)	<i>chpC</i>	<i>pilI</i>	CheW-like coupling proteins	B
<i>pilJ</i> (4)	<i>pilJ</i>	<i>pilJ</i>	Methyl accepting chemotaxis protein	B
<i>pilL</i> (3)	<i>chpA</i>	<i>pilL</i>	CheA-like histidine kinases	B
<i>pilM</i> (3)	<i>pilM</i>	<i>pilM</i>	T4P assembly protein	A
<i>pilN</i> (2)	<i>pilN</i>	<i>pilN</i>	Accessory protein that aids PilQ secretin activity	A
<i>pilO</i> (1)	<i>pilO</i>	<i>pilO</i>	Accessory protein that aids PilQ secretin activity	A
<i>pilP</i> (2)	<i>pilP</i>	<i>pilP</i>	Accessory protein in the outer membrane	A
<i>pilQ</i> (4)	<i>pilQ</i>	<i>pilQ</i>	Outer membrane secretin	D
<i>pilT</i> (3)	<i>pilT</i>	<i>pilT</i>	ATPase that mediate pilus retraction and disassembly	C
<i>pilU</i> (1)	<i>pilU</i>	<i>pilU</i>	Protein with nucleotide binding domain	C
<i>pilW</i> (2)	<i>pilF</i>	<i>pilW</i>	Required for T4P biogenesis/stability	A
<i>pilY1</i> (5)	<i>pilY1</i>	<i>pilY1</i>	Adhesin	B
<i>pilX</i> (1)	<i>pilX</i>	<i>pilX</i>	Pilus-associated protein	A
<i>fimT</i> (3)	<i>fimT</i>	<i>fimT</i>	Pilus assembly protein	A
<i>fimV</i> (3)	<i>fimV</i>	<i>fimV</i>	Putative peptidoglycan-binding domain	B
<i>tonB</i> (1)	<i>tonB</i>	<i>tonB</i>	Cytoplasmic membrane proton-motive force protein	B
<i>APS58_04405</i> (1)	<i>algZ</i>	<i>yesM</i>	Histidine kinase of a two-component regulatory system	B

^a In *A. citrulli* M6. The number of mutants isolated for each gene in this study is indicated in parentheses.

^b Categories are based on Carbone et al. (2006): A, pilus assembly; B, functional maturation and regulation; C, retraction; and D, emergence on the cell surface.

Mutants impaired in the ability to produce T4P form colonies with identical morphology to those of PVs.

We have recently characterized the phenomenon of phenotypic variation in *A. citrulli*. PVs of strain M6 resemble several phenotypes of a *pilM* mutant of this strain that does not produce T4P (Bahar et al. 2009a). Both PVs and the *pilM* mutant produce colonies with a translucent and fuzzy appearance, in contrast to the opaque and smooth appearance of M6 colonies (Shrestha et al. 2013) (Fig. 5). In addition, both are impaired in twitching motility and have reduced virulence and biofilm formation ability (Bahar et al. 2009a; Shrestha et al. 2013). A different mutant of strain M6, with a disruption in the *pilT* gene, also lacks twitching motility and possesses reduced virulence and biofilm formation ability (Bahar et al. 2009a), but its colony morphology is similar to that of wild-type M6 (Fig. 5). In contrast to the *pilM* mutant that does not produce T4P, the *pilT* not only produces them, it is hyperpiliated (Bahar et al. 2009a). These observations suggested that phenotypic variation in *A. citrulli* is determined by the lack of the ability to produce T4P rather than by injury in T4P function. To assess this hypothesis, we performed detailed observations of colonies of the new twitching-deficient mutants under a binocular and assessed, by transmission electron microscopy (TEM), whether the mutants possess the ability to produce T4P under the same growth conditions.

Most of the mutants characterized in this study showed a colony morphology type identical to those of PVs (V1 to V4) of strain M6 (Fig. 5). The exceptions were mutants with disruptions in genes *tonB*, *pilJ*, *pilT*, *pilW*, *pilX*, and *APS58_04405*, which had a colony morphology similar to that of wild-type M6 (Fig. 5). In agreement with the aforementioned hypothesis, TEM observations revealed that these six mutants, indeed, produce T4P while T4P could not be detected in the other mutants, under tested conditions (Fig. 6, showing some of the mutants; Supplementary Figs. S3 and S4).

Complementation of selected mutants fully or partially restored T4P-associated phenotypes.

Four twitching-deficient mutants, disrupted in *tonB*, *pilI*, *pilJ*, and *pilL* genes, were selected for complementation. This was done by transforming the mutants with plasmid pBBR1-MCS-5 carrying the corresponding genes from wild-type M6 (Table 1). As mentioned above, the *tonB* and *pilJ* mutants produce T4P but do not twitch and possess a wild type-like colony appearance (Fig. 5). In contrast, the *pilI* and *pilL* mutants

do not synthesize T4P and possess a PV-like colony morphology (Fig. 5). Among the four mutants, we were able to detect restoration of twitching motility only for *tonB* and *pilJ* complemented mutants (Supplementary Fig. S5A). In terms of colony morphology, the *tonB* and *pilJ* complemented strains did not differ from the corresponding mutants and wild-type M6 (not shown). This result was expected, since all these strains synthesize T4P. Interestingly, the *pilI* and *pilL* mutants carrying the complementation plasmids showed an intermediate phenotype between wild-type M6 (Fig. 5) and the corresponding mutants in terms of colony appearance; although these strains were not restored for twitching motility, their colony edges were more round-shaped in comparison with their corresponding mutants. In contrast to the *pilI* and *pilL* mutants that do not produce T4P, we were able to detect pili in the complemented strains, which could explain the aforementioned differences in colony morphology.

The complemented strains were also assessed in seed transmission assays. For these experiments, we had a different commercial soil that was less conducive to disease; therefore, 10^8 -CFU/ml suspensions were used for seed inoculation (rather than 5×10^6 CFU/ml, as in previous seed transmission assays). In contrast to the first set of experiments shown in Figure 4, in these experiments, the *pilJ* mutant induced lower disease severity than wild-type M6, as reflected in significantly ($P < 0.05$) higher foliage weight of inoculated plants (Supplementary Fig. S6). This was also the case for the other mutants. In these assays, the averages of foliage weight recorded for plants treated with the complemented strains were lower than those inoculated with the mutants, but these differences were not statistically significant. On the other hand, the four complemented strains but not the mutants significantly ($P < 0.05$) differed from non-inoculated controls. Moreover, while foliage weight averages of plants treated with the complemented strains had higher values than those treated with wild-type M6, no significant differences were found between the latter and the *tonB* and *pilJ* complemented strains. Interestingly, these were the only two strains that were complemented for twitching motility.

DISCUSSION

The role of T4P in pathogenicity of animal-pathogenic bacteria has been widely investigated (Alm and Mattick 1997; Collyn et al. 2002; Craig et al. 2004; Zahavi et al. 2011). In contrast, relatively less attention has been given to the contribution of T4P

Fig. 2. Biofilm formation of *Acidovorax citrulli* M6 and twitching-deficient mutants. Bacteria were grown in M9 minimal media at 28°C for 48 h without agitation. The cultures were removed, the tubes were washed gently and biofilms formed on the tube surface were stained with 0.1% methyl violet. Stained biofilms were solubilized with 95% ethanol and biofilm formation was quantified by optical density measurements at 595 nm. Data represent averages and standard errors of one experiment out of three with similar results (three replicates per strain in each experiment). Different letters indicate significant differences ($P < 0.05$) among treatments by Tukey-Kramer and analysis of variance.

to virulence and fitness of plant-pathogenic bacteria. With that said, in the last 15 years, several studies have contributed to the understanding of the involvement of these surface appendages in pathogenicity of some plant-pathogenic bacterial species (Burdman et al. 2011). In this regard, a clear contribution of T4P to virulence has been mainly attributed to vascular pathogenic bacteria, e.g., those bacterial pathogens that can colonize and spread through the plant xylem. These include *Ralstonia solanacearum* (Kang et al. 2002; Liu et al. 2001), *Xylella fastidiosa* (De La Fuente et al. 2007, 2008; Li et al. 2007; Meng et al. 2005), *Xanthomonas oryzae* pv. *oryzae* (Lim et al. 2008; Wang et al. 2008), *Xanthomonas campestris* pv. *campestris* (McCarthy et al. 2008), and *Acidovorax citrulli* (Bahar et al. 2009a). One exception is the nonvascular pathogen *Pseudomonas syringae* pv. *tabaci*, for which T4P were shown to contribute to virulence (Nguyen et al. 2012). It has been hypothesized that, in the case of vascular plant-pathogenic bacteria, T4P contribute to colonization and spread in the xylem vessels by promoting attachment to and biofilm formation on the vessel surfaces as well as twitching motility in this niche (Burdman et al. 2011).

To enrich our understanding of the gene machinery used by *A. citrulli* to produce and regulate T4P activity and to further investigate the contribution of T4P to the virulence of this bacterium, we screened a library of *A. citrulli* M6 mutants for impairment in twitching motility. Sequence analyses of the mutated genes in the twitching-deficient mutants led to identification of 20 genes (Table 2). As expected, most of the detected mutants had disruptions in genes that, in other bacteria, are known to be directly connected with T4P assembly or function and are named *pil* or *fim* genes (Burdman et al. 2011; Mattick 2002). In agreement with findings in these and other bacteria, our screen revealed *A. citrulli* twitching-deficient mutants in genes that encode components of chemosensory systems that regulate motility in bacteria. In *X. fastidiosa*, these genes have been designated *pilI*, encoding a CheW-like coupling protein, *pilJ*, encoding a MCP, and *pilL*, encoding a CheA-like histidine kinase. Similarly as in *X. fastidiosa* and in *P. aeruginosa* (Cursino et al. 2011; Fulcher et al. 2010), in *A. citrulli*, these genes are organized in a so-called Pil-Chp operon that controls twitching motility.

Fig. 3. Stem inoculation of melon seedlings with *Acidovorax citrulli* M6 and twitching-deficient mutants. Six-day-old melon cv. Ophir seedlings were inoculated by placing a 5- μ l droplet containing bacteria (10^8 CFU/ml) at the base of the hypocotyl and passing a needle through the droplet and the stem. The seedlings were maintained in a greenhouse (26 to 28°C) and the percentage of dead seedlings was recorded every day. Data represent averages from one experiment (15 replicates per strain) of two with similar results. Data in both graphs are from the same experiment, but the strains were split into two graphs to allow a better visualization of each strain. For comparative purposes, wild-type strain M6, mutant M6-M (*pilM* mutant characterized by Bahar et al. [2009a]), and control (noninoculated seedlings) are shown in both graphs.

According to a general model for chemotaxis, bacterial cells express transmembrane chemoreceptors that associate with CheA-like histidine kinases via CheW-coupling proteins. Ligand binding to the chemoreceptor alters the phosphorylation state of the histidine kinase, which, in turn, phosphorylates a

CheY-like response regulator. Upon phosphorylation, the response regulator interacts with the flagellum motor or with T4P to mediate swimming or twitching motility, respectively (Bertrand et al. 2010; Hazelbauer et al. 2008). In *X. fastidiosa*, the Pil-Chp operon was shown to be required for twitching motility. Disruption

Fig. 4. Seed transmission assays of melon with *Acidovorax citrulli* M6 and twitching-deficient mutants. Melon cv. Ophir seeds were inoculated with bacterial strains at 5×10^6 CFU/ml, sown in a peat-based commercial soil mixture and maintained in the greenhouse at 26 to 28°C for 10 days. At the end of this period, the weight of the above parts of the seedlings was measured, as this parameter negatively correlates with disease severity (Bahar et al. 2009b). For comparative purposes, mutant M6-M deficient in *pilM* (Bahar et al. 2009a) was included. Controls were plants emerging from noninoculated seeds. Data represent averages and standard errors from one experiment (15 replicates per strain) of three with similar results. Different letters indicate significant differences ($P < 0.05$) among treatments by Tukey-Kramer and analysis of variance.

Fig. 5. Colony morphology of *Acidovorax citrulli* M6, twitching-deficient mutants and M6 phenotypic variants V1, V2, V3, and V4. The strains were grown on nutrient agar at 28°C for 48 h. Colonies were observed with a SMZ1500 binocular equipped with a DS-Ri1 color digital camera.

of the *cheA* homolog *pilL* led to loss of twitching motility although T4P formation was retained (Cursino et al. 2011). The authors concluded that, in this bacterium, *pilL* is involved in T4P function and regulation but not in T4P biogenesis. In contrast, disruption of *chpA*, the *pilL* homolog in *P. aeruginosa*, abolished the ability of this bacterium to produce T4P (Whitchurch et al. 2004). In our study, knockouts of *pilL* and *pilI* resulted in loss of both twitching and T4P formation; however, disruption of a third gene in this operon, *pilJ*, led to loss of twitching motility but did not abolish T4P synthesis.

Another twitching-deficient mutant detected in our screen had a disruption in *tonB*. *tonB* genes encode cytoplasmic membrane proton-motive force proteins that are required for active transport of several substrates across the outer membrane of gram-negative bacteria, including iron-siderophore complexes, vitamin B12,

metals, sugars, and oligosaccharides (Noinaj et al. 2010; Postle and Kadner 2003; Schauer et al. 2008). Takase et al. (2000) showed that a *tonB1* mutant of *P. aeruginosa* was affected in iron acquisition and host infection. Further, Abbas et al. (2007) reported that disruption of *tonB1* in this bacterium also compromises biofilm formation and led to reduced twitching and swarming motilities compared with the wild-type strain. This mutant also produced lower levels of the quorum sensing (QS) signal molecule 3-oxo-C12 acyl homoserine lactone (AHL). Treatment with exogenous AHL molecules restored biofilm and motility phenotypes in the mutant, thus linking between TonB1 activity and QS (Abbas et al. 2007). Huang et al. (2004) showed that a different copy of *tonB*, i.e., *tonB3*, is also required for wild-type levels of T4P assembly and twitching motility in *P. aeruginosa*. Similar results were reported for

Fig. 6. Transmission electron microscopy of *Acidovorax citrulli* M6 and twitching-deficient mutants in genes *tonB*, *pilW*, *pilJ*, *pilI*, and *pilL*. The strains were grown on nutrient agar at 28°C for 48 h. Bacterial samples were prepared and observed in a FEI Tecnai-12 electron microscope. Solid and dashed arrows indicate polar flagellum and type IV pili (T4P), respectively. Among these strains, T4P could be detected in wild-type M6 and mutants in *tonB*, *pilW*, and *pilJ* but not in mutants deficient in *pilI* and *pilL*.

X. fastidiosa, in which disruption of *tonB1* resulted in loss of twitching motility and significantly reduced biofilm formation and virulence (Cursino et al. 2009). Since the *X. fastidiosa tonB1* mutant formed T4P, it was hypothesized that, in this bacterium, *tonB1* is responsible for providing the energy necessary for cellular import of molecules involved in twitching motility but not for T4P assembly (Cursino et al. 2009). In line with this background, the *A. citrulli* M6 Tn5 mutant deficient in *tonB* produced T4P but failed to perform twitching motility. It also had reduced levels of biofilm formation and virulence on melon seedlings, as compared with the wild-type strain. The genomes of *A. citrulli* M6 and AAC00-1 carry two additional *tonB* genes, and Blast analysis revealed that the one detected in our screen is more similar to *tonB1* of other bacteria. It would be interesting to generate and characterize mutants deficient in the two other *tonB* copies of *A. citrulli* to assess whether they are also involved in T4P activity.

Our screen also identified a gene, *APS58_04405*, that encodes a histidine kinase protein. This gene is upstream of a *lytR/algR* transcriptional regulator–encoding gene and is likely in the same operon. The *LytR/AlgR* family of transcriptional regulators commonly regulate expression of virulence factors in a variety of bacterial species (Nikolskaya and Galperin 2002). Among the best-studied transcriptional regulators of this family are *Escherichia coli* YehT (Kraxenberger et al. 2012), *Staphylococcus aureus* AgrA (Koenig et al. 2004), and *Enterococcus faecalis* FsrA (Del Papa and Perego 2011). The *AlgR* transcriptional regulator of *P. aeruginosa* also belongs to this family of transcriptional regulators and forms a two-component regulatory system with its concomitant histidine kinase *AlgZ*. In fact, the *AlgZR* two-component system is one of the global regulatory systems in *P. aeruginosa*, being important for both chronic and acute infections (Okkotsu et al. 2014). Among the phenotypes regulated by the *AlgZR* system in *P. aeruginosa* are alginate production (Darzins and Chakrabarty 1984) and twitching motility (Lizewski et al. 2004; Whitchurch et al. 1996). However, global transcriptome analyses support the idea that this two-component system is involved in regulation of additional traits, including QS, type III secretion, and anaerobic metabolism (Okkotsu et al. 2014). It will be interesting to further characterize the role of this two-component system in virulence and other traits of *A. citrulli*.

As previously shown for *pilM* and *pilT* mutants of *A. citrulli* M6 (Bahar et al. 2009a; 2010), all twitching-deficient mutants characterized in this study had significantly reduced biofilm formation ability and virulence on melon seedlings. Nevertheless, mutants in genes *pilJ*, *pilX*, *tonB*, and *APS58_04405* showed biofilm formation levels that were significantly higher than the other twitching-deficient mutants (Fig. 2). Despite lacking twitching ability under tested conditions, these mutants do synthesize T4P. We speculate that, in these mutants, T4P are not fully functional in order to perform twitching motility, but their presence still contributes to some level of biofilm formation, probably because of the adhesive characteristics of T4P. In this regard, the exceptions were the *pilW* and *pilT* mutants that also produce T4P but possess biofilm formation levels that are comparable to those of the T4P synthesis-deficient mutants (Fig. 2). *pilW* genes (named *pilF* in *P. aeruginosa* and *tgl* in *Myxococcus xanthus*) encode outer membrane proteins that assist in the assembly of the outer membrane secretin *PilQ* (Carbonnelle et al. 2005; Koo et al. 2008; Rodriguez-Soto and Kaiser 1997). The *pilW* orthologs in *P. aeruginosa* and *M. xanthus* are required for T4P formation (Rodriguez-Soto and Kaiser 1997; Watson et al. 1996). In contrast, in *Neisseria meningitidis*, *pilW* contributes to stability and functionality of the pilus fibers but is not required for their assembly (Carbonnelle et al. 2005). It seems that, in *A. citrulli*, disruption of *pilW* does not abolish pilus

assembly but renders nonstable pili that are not able to contribute to the levels of biofilm formation seen in the T4P-producing mutants deficient in *pilJ*, *pilX*, *tonB*, or *APS58_04405*. Regarding *pilT*, we have previously shown that disruption of this gene in *A. citrulli* leads to hyperpiliation (Bahar et al. 2009a). Consequently, probably because of the adhesive character of T4P, in liquid suspension or media, *pilT* mutants form aggregates that sink to the bottom (Bahar et al. 2009a), thus explaining the low levels of biofilm formation of *pilT* mutants under tested conditions.

Importantly, we cannot exclude the possibility that some of the phenotypes observed for some of the mutants characterized in this study are due to polar effects. Here, we assessed complementation of mutants disrupted in genes *tonB*, *pilI*, *pilJ*, and *pilL*. While complementation vectors restored twitching motility in the *tonB* and *pilJ* mutants, we could not detect such phenotype restoration in the *pilI* and *pilL* mutants. With that said, in contrast to the *pilI* and *pilL* mutants that lack T4P synthesis ability, their corresponding complemented strains did produce T4P. These results as well as results from seed transmission assays suggest that the complementation vectors partially restored T4P deficiency in the four mutants. Partial phenotype restoration could be due to either nonoptimal expression of the complemented genes, to unsolved polar effects, or both, particularly in the cases of *pilI*, *pilJ*, and *pilL* that are part of an operon.

We have recently reported the phenomenon of phenotypic variation in *A. citrulli* (Shrestha et al. 2013). PVs of *A. citrulli* M6 were shown to possess similar phenotypes of those of a M6 *pilM* mutant, including loss of the ability to produce T4P and perform twitching, reduced biofilm formation and virulence abilities, and formation of clearly distinguished colonies relative to wild-type M6 (Shrestha et al. 2013). The association between phase or phenotypic variation and loss of T4P synthesis has been widely studied in human and animal pathogens. Since modulation of piliation may represent a mechanism to evade host immune response, the molecular basis of phase variation involving loss of T4P is of considerable interest (Mattick 2002). In *Neisseria gonorrhoeae* and *Moraxella bovis*, phenotypic variation typically involves genomic recombination or mutagenic events that directly affect pilin synthesis or assembly (Marrs et al. 1988; Segal et al. 1985). In *Eikenella corrodens*, it has been suggested that posttranslational events are involved in alteration of pilin export and assembly, leading to phenotypic variation (Villar et al. 1999). In the present study, identification and characterization of 20 *A. citrulli* M6 mutants deficient in twitching motility allowed us to conclude that, in this bacterium, phenotypic variation is connected to the loss of the ability to synthesize T4P but not to their function in terms of twitching motility. While mutants that lacked the ability to produce T4P showed colony morphology typical of naturally occurring PVs of strain M6, mutants deficient in twitching motility but that retained the ability to produce T4P (e.g., *tonB*, *pilJ*, *pilT*, *pilW*, *pilX*, and *APS58_04405*) had a colony morphology that was identical to the wild type (Fig. 5).

DNA-fingerprinting analyses by pulse-field gel electrophoresis and repetitive polymerase chain reaction (PCR) suggested that no major genomic rearrangements are involved in phenotypic variation of *A. citrulli* M6 (Shrestha et al. 2013). We have recently sequenced four PVs of strain M6, V1 to V4, by Illumina MiSeq. Comparative sequence analyses revealed many putative genetic differences in terms of nucleotide substitutions and insertions or deletions between the PVs and the parental strain (T. Rosenberg and S. Burdman unpublished results). However, we cannot exclude the possibility that many of the differences found in these analyses are, in fact, artifacts due to sequencing mistakes or errors in assembly. Importantly, we

were not able to detect mutations shared by the four variants and no mutations were detected in coding as well as promoter regions of known T4P-related genes. Based on these results and on the high frequency of occurrence of phenotypic variation in *A. citrulli* M6 (approximately 10^{-3} under a variety of conditions [Shrestha et al. 2013]), we hypothesize that disruption of any gene required for pilin synthesis or T4P assembly results in phenotypic variation in this bacterium.

Due to the importance of BFB and the high frequency of phenotypic variation in *A. citrulli*, the implication of this phenomenon to bacterial fitness is of considerable interest. We have shown that PVs of *A. citrulli* M6 are less virulent than the parental strain but possess higher swimming motility and produce colonies with significantly higher bacterial numbers than the parental strain (Shrestha et al. 2013). However, more investigation is needed to assess the implication of these findings to the interaction of the pathogen with its hosts and to its fitness in different environments.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 1. Unless stated otherwise, *Acidovorax citrulli* strains were grown in NB (Difco Laboratories, Detroit) or NA (NB containing 15 g of agar per liter) at 28°C for 48 h. For biofilm experiments, *A. citrulli* strains were grown in M9 minimal medium (Sambrook et al. 1989) with 0.3% casamino acid and 20% glucose. For inoculation experiments, *A. citrulli* strains were grown on NA for 48 h, were resuspended from plates with sterilized distilled water (SDW), were adjusted to an optical density at 600 nm (OD_{600}) of 0.2 (about 10^8 CFU/ml) using a Helios Gamma spectrophotometer (Thermo Electron Corp., Rochester, NY), and were then diluted (or not) to the desired concentration (discussed below). Inoculum concentrations were verified by dilution plating. *E. coli* strains were cultured in Luria-Bertani (LB) (Difco Laboratories) at 37°C. Antibiotics were added to *A. citrulli* and *E. coli* cultures according to the resistances described in Table 1, i.e., ampicillin, 100 µg/ml and Km, 50 µg/ml.

Molecular manipulations.

Routine molecular manipulations and cloning were carried out using standard procedures (Sambrook et al. 1989). T4 DNA ligase and restriction enzymes were purchased from Fermentas (Burlington, Canada). Kits for plasmid and PCR product extraction and purification were purchased from BioNeer Corporation (Daejeon, Republic of Korea). Genomic DNA from *A. citrulli* strains was prepared using the GenElute bacterial genomic DNA kit (Sigma-Aldrich, Saint Louis). Southern blot hybridization was performed using the ECL direct nucleic acid labeling and detection system from Amersham Biosciences (Buckinghamshire, U.K.), according to the manufacturer's instructions. DNA sequencing was performed with the BigDye terminator cycle sequencing kit from Applied Biosystem, Inc. (Foster City, CA, U.S.A.), using an automated sequencer at Hy Laboratories (Rehovot, Israel). PCR primers were purchased from Hy Laboratories and PCR reactions were performed, with the Readymix Red Taq PCR reactive mix (Sigma-Aldrich) or with Phusion high-fidelity DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.), using an Eppendorf (Hamburg, Germany) thermal cycler.

Construction of a random transposon library of *A. citrulli* M6 and screening for impaired twitching motility.

A random transposon library formed by about 10,000 mutants was generated on the background of *A. citrulli* M6, using the EZ-Tn5 kit (Epicentre, Madison, WI, U.S.A.), as described

(Bahar et al. 2009a). Briefly, the Km^R gene from plasmid pUC-4K was cloned into the multiple cloning site of the transposon construction vector pMOD-3 < R6K γ ori/MCS > following restriction of both with *Bam*HI. The resulting vector was digested with *Pvu*II to excise the mutagenesis cassette, which included the Km^R gene and the R6K γ ori, flanked by 20-bp-long mosaic ends on both sides. The cassette, along with the EZ-Tn5 transposase were electroporated into *A. citrulli* M6 electrocompetent cells using an Eppendorf 2510 electroporator, and putative transformants were selected on NA with Km as described (Bahar et al. 2009a). The putative Tn5 mutants were replicated onto new NA and Km plates and were stored in 20% glycerol at -80°C. Mutants deficient in twitching motility were directly screened on the NA and Km selection plates and were verified by microscope observations of the colonies, using an Axio Scope light microscope (Carl Zeiss, Jena, Germany) equipped with a DXM1200F digital camera (Nikon, Tokyo). The selected mutants were tested by Southern blot to verify that they carry a single insertion of the Tn5 cassette. A plasmid rescue procedure was used to identify the mutated region of each mutant as described (Bahar et al. 2009a). Briefly, genomic DNA was purified and digested with restriction enzymes that do not cut inside the mutagenesis cassette, keeping the Km^R gene and the R6K γ ori fragment intact. Digestion products were then autoligated and electroporated into electrocompetent *E. coli* BW25141 cells. Km^R transformants were isolated in LB and Km plates and were used for plasmid preparation and sequencing of the flanking regions surrounding the insertion site of the mutagenesis cassette with primers pMOD < MCS > F (5'-GCCAACGACTACGCACTAGCCAAAC-3') and pMOD < MCS > R (5'-GAGCCAATATGCGAGAACACCCGAGAA-3').

Construction of complemented strains.

To generate complemented strains for mutants disrupted in *tonB*, *pilI*, *pilJ*, and *pilL* genes, suitable plasmids were constructed using pBBR1-MCS-5 by the restriction free (RF) cloning procedure (Restriction Free Cloning database). All primers were designed according to the *A. citrulli* M6 genome (GenBank NZ_LKUW000000000.1) and are detailed in Supplementary Table S1. Briefly, the *tonB* gene was cloned with its promoter region (406 bp) and each of the genes *pilI*, *pilJ*, and *pilL* were cloned with a region (456 bp) containing the promoter of the *APS58_00920-APS58_0900* operon, in which these genes are located. All RF-PCR amplifications were done using high-fidelity DNA polymerase Phusion (New England Biolabs). The resulting plasmids were transformed into *E. coli* S-17-1 λ pir by electroporation as described (Bahar et al. 2009a). Plasmids were extracted using the BioNeer AccuPrep plasmid mini extraction kit and were sent for sequencing at Hy Laboratories Ltd., before conjugation with the corresponding M6 mutant as described (Bahar et al. 2009a). The transformants were selected by gentamicin resistance and were used for phenotypic characterization.

Biofilm formation assays.

Biofilm assays were performed using 14-ml polystyrene round-bottom tubes (Thermo Fisher Scientific, Waltham, MA, U.S.A.), using a protocol modified from O'Toole et al. (1999). Briefly, each tube was filled with 1 ml of M9 minimal medium as described above and was then inoculated with 10 µl of a 3-ml NB overnight bacterial culture ($OD_{600nm} = 0.2$). The tubes were incubated at 28°C for 48 h without agitation. Then cultures were poured out and the tubes were rinsed with SDW. Following fixation at 80°C for 20 min, biofilms formed on the surfaces were stained with 0.1% methyl violet for 30 min. Tubes were rinsed with distilled water and were analyzed quantitatively by solubilizing the stained biofilms with 95% ethanol for 2 h and measuring

the OD₅₉₅ of the stained suspension in a Helios Gamma spectrophotometer. This experiment was carried out three times with three replicates per strain in each experiment.

Virulence assays.

Stem-inoculation experiments were performed as described by Bahar et al. (2011) with few modifications. Briefly, melon (*Cucumis melo*) cv. Ophir (Zeraim Gedera, Kibbutz Revadim, Israel) seedlings were grown individually in 11-cm diameter poly pots (Tefen, Nahsholim, Israel) containing a peat-based commercial soil mixture (Shacham Givat Ada, Israel), in a greenhouse at 26 to 28°C. At 6 days after sowing, each seedling was inoculated by placing a 5-μl droplet from a 10⁸-CFU/ml bacterial suspension at the base of the hypocotyl (about 1 cm from the soil). Then, a 25-gauge needle was passed through the droplet and the stem and immediately taken out. The droplets were immediately removed, and seedlings were kept in the greenhouse. The percentage of dead seedlings was evaluated every day until 7 dai. This experiment was carried out twice and 15 replicates per mutant or wild-type strain were used in each experiment.

Seed transmission assays were performed as described by Bahar et al. (2009b) with minor modifications. Melon cv. Ophir seeds were placed inside a 50-ml Falcon tube containing 10 ml of bacterial suspensions at 5 × 10⁶ CFU/ml. Seeds were incubated at room temperature with gentle agitation for 2 h. The bacterial suspensions were then discarded, and the seeds were washed briefly with SDW and were dried under a laminar flow hood. Seeds were then sown individually in 11-cm diameter poly pots (Tefen), containing a peat-based commercial soil mixture (Shacham Givat Ada), in a greenhouse (26 to 28°C). At 10 dai, the fresh weight of seedling above parts (foliage) and the length of the stems were measured. These parameters were shown to negatively correlate with disease severity in seed transmission assays in the *A. citrulli*-melon pathosystem (Bahar et al. 2009b). This experiment was carried out three times and 15 replicates per mutant or wild-type strain were used in each experiment. Further, two additional seed transmission assays were carried out to test *tonB*, *pill*, *pilJ*, and *pilL* complemented strains in comparison with their corresponding mutants and wild-type M6. For these experiments, we had a different commercial soil (Garden Mix; Peka, Rehovot, Israel) that was less conducive to disease, thus 10⁸-CFU/ml suspensions were used for seed inoculation. The other conditions were as described above. Two experiments, each with 15 replicates per treatment, were carried out.

Binocular and TEM observations.

Morphological observations of bacterial colonies were done using a SMZ1500 stereomicroscope (binocular) equipped with a DS-Ri1 color digital camera (both from Nikon). Transmission electron microscopy (TEM) was used to visualize T4P in wild-type and twitching-deficient mutant strains of *A. citrulli*. Samples from 48-h-old, NA-grown colonies were prepared. A 5-μl sterile water drop was dropped on a selected colony for 2 min, then, a 300-mesh carbon grid was placed on top of each colony for 30 s and the grid was negatively stained in a 1% uranyl drop for 10 s. The grids were examined using a FEI Tecnai-12 electron microscope (FEI, Eindhoven, The Netherlands) equipped with a F224HD 2k × 2k CCD camera (TVIPS, Gauting, Germany).

Statistical analysis.

All quantitative assays were analyzed using the Tukey-Kramer test for mean comparison in conjunction with analysis of variance using JMP software (SAS Institute Inc., Cary, NC, U.S.A.).

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