Type IV Pili Are Required for Virulence, Twitching Motility, and Biofilm Formation of *Acidovorax avenae* subsp. *citrulli*

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Acidovorax avenae subsp. *citrulli* is the causal agent of bacterial fruit blotch (BFB), a threatening disease of watermelon, melon, and other cucurbits. Despite the economic importance of BFB, relatively little is known about basic aspects of the pathogen's biology and the molecular basis of its interaction with host plants. To identify *A. avenae* subsp. *citrulli* genes associated with pathogenicity, we generated a transposon (Tn5) mutant library on the background of strain M6, a group I strain of *A. avenae* subsp. *citrulli*, and screened it for reduced virulence by seed-transmission assays with melon. Here, we report the identification of a Tn5 mutant with reduced virulence that is impaired in *pilM*, which encodes a protein involved in assembly of type IV pili (TFP). Further characterization of this mutant revealed that *A. avenae* subsp. *citrulli* requires TFP for twitching motility and wild-type levels of biofilm formation. Significant reductions in virulence and biofilm formation as well as abolishment of twitching were also observed in insertional mutants affected in other TFP genes. We also provide the first evidence that group I strains of *A. avenae* subsp. *citrulli* can colonize and move through host xylem vessels.

The gram-negative bacterium *Acidovorax avenae* subsp. *citrulli* (formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*) is the causal agent of bacterial fruit blotch (BFB) of cucurbits (Schaad et al. 1978; Willems et al. 1992). The disease gained importance in the late 1980s, following outbreaks in watermelon fields in several states in the United States. Since then, BFB has spread worldwide and has been reported in other cucurbits, such as melon, pumpkin, squash, and cucumber (Burdman et al. 2005; Isakeit et al. 1997; Langston et al. 1999; Martin and O'Brien 1999; O'Brien and Martin 1999; Palkovics et al. 2008; Rane and Latin 1992; Schaad et al. 2003; Somodi et al. 1991; Walcott et al. 2004; Wall and Santos 1988).

A. avenae subsp. *citrulli* is a seedborne pathogen with highly destructive potential. Under favorable conditions, the bacterium spreads rapidly throughout nurseries and in the field, leading to seedling blight or, at a later stage, fruit rot (Schaad et al. 2003). Strategies for managing BFB are limited and there are no reliable sources of BFB resistance. Therefore,

the disease represents a serious threat to the cucurbit industry worldwide. On the basis of DNA-fingerprinting profiles, whole-cell fatty-acid composition, carbon-source utilization, and pathogenicity assays, two genetically distinct groups of *A. avenae* subsp. *citrulli* have been identified: group I includes strains isolated mainly from nonwatermelon hosts while group II includes strains that are mainly associated with watermelon (Burdman et al. 2005; Walcott et al. 2000, 2004). Thus, *A. avenae* subsp. *citrulli*–host interactions make up a unique and interesting system in which a significant level of host specificity is distinguished among members of the same subspecies of a plant-pathogenic bacterium.

Despite the economic importance of BFB, very little is known about basic aspects of the biology of *A. avenae* subsp. *citrulli* and the molecular basis of BFB pathogenesis. Recently, the genome sequence of a group II *A. avenae* subsp. *citrulli* strain, AAC00-1, was released by the Joint Genome Institute (JGI), making a great contribution to the investigation of basic aspects of BFB. Most gram-negative biotrophic phytopathogenic bacteria depend on a functional type III secretion system (TTSS) encoded by hypersensitivity response and pathogenicity (*hrp*) genes to promote disease or to trigger a hypersensitive response (HR) in susceptible and resistant plants, respectively (Buttner and Bonas 2002). The presence of an *hrp* cluster as well as of genes encoding putative type III-secreted effectors was confirmed in *A. avenae* subsp. *citrulli* by sequencing of the AAC00-1 genome. The essential requirement of Hrp-TTSS for pathogenicity of *A. avenae* subsp. *citrulli* was confirmed in our lab following generation of *hrcV* mutants in the background of the group I and II strains M6 and W1, respectively. Neither mutant was able to cause disease in susceptible hosts (melon and watermelon) and they lost the ability to induce HR in the non-host plants tobacco and tomato (*unpublished results*). In agreement with our findings, an *hrcC* mutant, generated in the background of strain AAC00-1, lost its ability to trigger nonhost HR on *Nicotiana benthamiana* and to cause disease in watermelon (R. Walcott, *personal communication*).

However, the ability of pathogenic bacteria to infect plants involves more than their ability to form the Hrp-TTSS and transfer virulence effectors through it. Successful establishment of a pathogenic bacterium in the host tissue requires the coordinated activity of a plethora of genes, the identity and mode of action of many of which are still unidentified, even in the best-studied bacteria. To enrich our knowledge of the gene machinery required by *A. avenae* subsp. *citrulli* to conquer the host and cause disease, we generated a library of transposon mutants in the background of the group I strain M6, and screened for mutants affected in virulence. Here, we report the

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characterization of a Tn5 mutant impaired in *pilM*, as well as *pilA* and *pilT* mutants generated by insertional mutagenesis. These genes are involved in the synthesis and function of type IV pili (TFP). We show that these mutants are significantly affected in their ability to cause disease, as well as in biofilm formation and twitching motility. We also provide first evidence for the notion that group I strains of *A. avenae* subsp. *citrulli* possess the ability to spread systemically through and colonize the host xylem vessels.

RESULTS

Identification of an *A. avenae* subsp. *citrulli* *pilM* mutant with reduced virulence.

A transposon mutant library, generated on the background of the group I strain M6 (Table 1), was screened for reduced virulence on melon by seed-transmission assays. An interesting observation was recorded for one of the selected mutants, M6-223, when streaked on nutrient agar (NA) plates: whereas typical twitching motility haloes formed around wild-type colonies after several days (Fig. 1A and B), no haloes formed around the colonies of this mutant (Fig. 1C).

Cloning and sequence analysis of the mutated region revealed that the transposon cassette had been inserted into an operon of six genes encoding several proteins required for the synthe-

sis of TFP. More precisely, the cassette was inserted into the coding sequence of the first gene of this operon, *pilM*, which, according to the annotation of the AAC00-1 genome and BLAST analysis, encodes a TFP assembly protein. This mutant strain was further renamed M6-M (for *pilM* mutant). TFP-mediated twitching motility is a flagellum-independent translocation on solid surfaces, characterized by expansion of the colony by outward migration of bacteria rather than by cell-division-mediated growth. Indeed, twitching often leads to the formation of visible haloes around the bulk of the colonies (Henrichsen 1983).

TFP is required for twitching motility of *A. avenae* subsp. *citrulli*.

To verify the role of TFP in twitching motility of *A. avenae* subsp. *citrulli*, additional mutants in TFP-associated genes were generated by insertional mutagenesis on the background of strain M6 and of W1, a representative group II strain (Table 1). Genes selected for knockout were *pilA* and *pilT*, which encode the major TFP subunit pilin, and an ATPase responsible for TFP retraction that motors twitching motility, respectively. According to the published sequence of strain AAC00-1, and as later confirmed for strains M6 and W1, *pilA* and *pilT* are part of single-gene loci (i.e., in contrast to *pilM*, they are not part of multiple-gene operons).

Table 1. Strains and plasmids used in this study

Strain or plasmid ^a	Characteristics ^b	Reference or source
<i>Acidovorax avenae</i> subsp. <i>citrulli</i>		
M6	Ap ^r ; wild type, group I strain	Burdman et al. 2005
W1	Ap ^r ; wild type, group II strain	Burdman et al. 2005
M6-M (M6-223)	Ap ^r , Km ^r ; Tn5 mutant defective in <i>pilM</i>	This study
M6-T	Ap ^r , Km ^r ; M6 insertional mutant defective in <i>pilT</i>	This study
M6-Tcomp	Ap ^r , Km ^r , Gm ^r ; M6-T complemented with pMLpilT	This study
W1-A	Ap ^r , Km ^r ; W1 insertional mutant defective in <i>pilA</i>	This study
W1-Acomp	Ap ^r , Km ^r , Gm ^r ; W1-A complemented with pMLpilA	This study
W1-T	Ap ^r , Km ^r ; W1 insertional mutant defective in <i>pilT</i>	This study
W1-Tcomp	Ap ^r , Km ^r , Gm ^r ; W1-T complemented with pMLpilT	This study
Other strains		
<i>Escherichia coli</i> DH5α	F-Φ80dlacZΔM15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1, hsdR17</i> (rk ⁻ , mk ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	Invitrogen
<i>E. coli</i> S17-1 λ pir	λ Lysogenic S17-1 derivative producing π protein for replication of plasmids carrying <i>oriR6K</i> ; <i>recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 λ⁻ pir</i>	Simon et al. 1983
<i>E. coli</i> BW25141	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3), Δ(<i>phoB-phoR</i>)580, λ ⁻ , <i>galU95</i> , Δ <i>uidA3::pir⁺</i> , <i>recA1, endA9</i> (del-ins)::FRT, <i>rph-1</i> , Δ(<i>rhaD-rhaB</i>)568, <i>hsdR514</i>	Datsenko and Wanner 2000
<i>Pseudomonas lachrymans</i> AV1	Wild-type strain; isolated from a cucumber plant showing angular leaf spot symptoms in 2004	Lab collection
Plasmids		
pUC-4K	pUC4 derivative (pMB1 ori, Ap ^r), containing the Km ^r cassette from Tn903	Vieira and Messing 1982
pMOD-3<R6Kγori/MCS>	Ap ^r ; transposon construction vector	Epilcentre
pTZ57R/T	Ap ^r ; cloning vector	Fermentas Inc.
pML122	Gm ^r ; broad host expression vector	Labes et al. 1990
pJP5603	Km ^r ; R6K-based suicide vector; requires the <i>pir</i> -encoded π protein for replication	Penfold and Pemberton 1992
pTZpilAmut	Ap ^r ; pTZ57R/T containing a 303-bp internal fragment of <i>pilA</i> (pilAmut) from strain W1	This study
pJPpilAmut	Km ^r ; pJP5603 containing the pilAmut fragment from pTZpilAmut; used to create mutant W1-A	This study
pTZpilTmut (W/M)	Ap ^r ; pTZ57R/T containing a 367-bp internal fragment of <i>pilT</i> (pilTmut) from strains W1 or M6	This study
pJPpilTmut (W/M)	Km ^r ; pJP5603 containing the pilTmut fragment (from strain W1 or M6); used to create mutants W1-T and M6-T, respectively	This study
pTZpilA	Ap ^r ; pTZ57R/T containing a 1,134-bp fragment of <i>pilA</i> (including its promoter region and the entire open reading frame) from strain W1	This study
pMLpilA	Gm ^r ; pML122 vector containing the <i>pilA</i> gene fragment from strain W1, excised from pTZpilA; used to complement W1-A	This study
pTZpilT (W/M)	Ap ^r ; pTZ57R/T containing a 1,509-bp fragment of <i>pilT</i> (including its promoter region and the entire open reading frame) from strains W1 or M6	This study
pMLpilT (W/M)	Gm ^r ; pML122 vector containing the <i>pilT</i> gene fragment from W1 or M6, excised from the corresponding pTZpilT; used to complement W1-T and M6-T	This study

^a W/M indicates that vectors for mutagenesis and complementation of *pilT* were prepared with fragments from W1 and M6 strains and used correspondingly in each strain.

^b Km^r, Gm^r, and Ap^r indicate kanamycin, gentamicin, and ampicillin resistance, respectively.

pilT knockout strains M6-T and W1-T were generated on the backgrounds of strains M6 and W1, respectively. A *pilA* mutant, W1-A, was generated only for strain W1 because, despite numerous attempts, we failed to create such a mutant in strain M6. The polymerase chain reaction (PCR) primers for these genes were designed based on the sequence of the group II strain AAC00-1. It is possible that, because of this, we were not able to amplify any PCR product related to *pilA* of the group I strain M6. Also, we were not able to generate an M6 *pilA* mutant with the mutagenesis vector pJPpilAmut, prepared with a *pilA* fragment from strain W1.

All mutants were verified by Southern blot analyses that yielded bands of the expected sizes, based on the AAC00-1 annotation (not shown). Cloning and sequencing revealed that the coding regions of *pilA* and *pilT* genes of strain W1 are identical to those of AAC00-1, whereas a single T-to-C silent base substitution was found at nucleotide position 588 of the coding region of the M6 *pilT* gene relative to strains AAC00-1 and W1. The *pilT* sequence of strain M6 and the *pilT* and *pilA* sequences of strain W1 were submitted to GenBank under accession numbers FJ705134, FJ705133, and FJ705132, respectively.

When grown on NA, *pilT* and *pilA* mutants exhibited a phenotype similar to that of M6-M (i.e., no twitching-typical haloes were formed around the colonies, shown for W1-A in Figure 1E). Complementation of *pilA* and *pilT* mutants using the broad host vector pML122 expressing the corresponding genes (pMLpilA and pMLpilT, respectively) (Table 1) restored twitching motility (Fig. 1D and F, wild-type W1 and W1-Acomp, respectively).

Transmission electron microscopy confirmed the presence of TFP in *A. avenae* subsp. *citrulli*.

Transmission electron microscopy (TEM) observations confirmed the presence of TFP in wild-type strains of *A. avenae* subsp. *citrulli* from both groups (Fig. 2A and D). In contrast, TFP were completely absent in mutants M6-M (Fig. 2E) and

W1-A (Fig. 2B). As expected, hyperpiliated cells were observed in *pilT* mutants W1-T and M6-T (Fig. 2C and F, respectively), due to the fact that *pilT* mutants lack the ATPase activity required for TFP degradation. Complementation of the mutants with the corresponding genes expressed in pML122 restored the wild-type phenotypes (not shown).

Confirmation of the role of TFP in virulence of *A. avenae* subsp. *citrulli*.

To verify the involvement of TFP in virulence of *A. avenae* subsp. *citrulli*, the TFP mutants were examined in detailed seed-transmission assays. Burdman and associates (2005) reported that, although strain M6 and other group I strains are very aggressive in seed-transmission assays, group II strains do not cause consistent seedling blight symptoms on either watermelon or melon under the tested conditions. Therefore, our comparisons focused on strain M6 and its mutants.

In seed-transmission assays with melon, mutants M6-M and M6-T exhibited a significant reduction in virulence relative to wild-type M6. However, whereas M6-M caused mild disease symptoms in most of the inoculated seedlings, M6-T was unable to do so: seedlings inoculated with the latter exhibited a phenotype similar to that of noninoculated controls. Complementation of M6-T with pMLpilT (strain M6-Tcomp) restored the virulence phenotype (Fig. 3A). Importantly, the TFP mutants generated in this study did not differ in their ability to grow in rich (nutrient broth [NB]) and minimal (M9) media relative to their parental strains (not shown), thus indicating that the reduced virulence in the TFP mutants is not due to reduced growth ability.

Because TFP are associated with surface adherence, we speculated that the aforementioned differences between mutant and wild-type strains could be due, at least in part, to a higher initial inoculum adhering to the seed in the wild type relative to the mutants. To verify this possibility, we determined the amount of bacteria that adhered to the seed following the inoculation procedure used for the seed-transmission assays. In three

Fig. 1. Images showing the occurrence or absence of twitching motility of *Acidovorax avenae* subsp. *citrulli* strains as seen by **A**, the naked eye or **B** through **F**, light microscopy after 96 h of growth on nutrient agar plates. Typical haloes surrounding the bulk colonies (indicated by arrows) are formed by bacteria migrating via twitching. **A** and **B**, Wild-type M6. **C**, M6-M (*pilM* mutant). **D**, Wild-type W1. **E**, W1-A (*pilA* mutant). **F**, W1-Acomp (complemented W1-A).

independent experiments, no significant differences in CFU per seed were observed between the wild type and the TFP mutants (Fig. 3B, data from one experiment).

We suspected that these results might be deceptive because, if the wild type possesses higher seed-adhesion ability than the mutants, it might be harder to extract the former from the seed. This would down-bias the counts of the wild type rela-

tive to the mutants. Therefore, we collected the seed debris remaining after bacterial extraction and transferred them into new tubes to which an aggressive treatment consisting of vortexing, sonication, and boiling was applied. The resulting suspensions were then subjected to DNA extraction, and PCR using *A. avenae* subsp. *citrulli*-specific primers (BX-S primer set) (Bahar et al. 2008) was performed on serial dilutions of

Fig. 2. Transmission electron microscopy of wild-type and mutant strains of *Acidovorax avenae* subsp. *citrulli* following growth for 48 h on nutrient agar plates. A, W1. B, W1-A. C, W1-T. D, M6. E, M6-M. F, M6-T. Solid and dashed arrows indicate type IV pili and polar flagellum, respectively. Bars = 0.5 μ m.

Fig. 3. A, Seed-transmission assays and **B,** seed adhesion on melon with wild-type M6 and its type IV pili mutants. **A,** Emerging seedlings following seed transmission. Pictures were taken 8 days after sowing. **B,** Quantification of the amount of bacteria extracted from seed following inoculation of seed for seed-transmission assays. Differences among treatments were not significant at $P = 0.05$.

these suspensions. In these assays, bacteria from all treatments were only detected in the first serial dilution (not shown). The used primers are highly sensitive (able to detect one cell per PCR) (Bahar et al. 2008); therefore, these results indicated that very few bacterial cells, on the order of 10 to 100, were left on the seed debris following our bacterial extraction method. Because these amounts are insignificant compared with the determined counts of CFU per seed, we concluded that the wild type and TFP mutants do not significantly differ in their ability to adhere to melon seed in our procedure.

Assessment of TFP mutants in stem-inoculation assays.

The role of TFP in plant colonization and virulence of phytopathogenic bacteria has been studied mainly in the vascular pathogens *Ralstonia solanacearum* and *Xylella fastidiosa* (Kang et al. 2002; Liu et al. 2001; Meng et al. 2005). Until now, it has been generally assumed that *A. avenae* subsp. *citrulli* is not a vascular pathogen; however, this issue has never been thoroughly investigated. To assess this question, we performed stem-inoculation assays with TFP mutants and wild-type strains. As with the seed-transmission assays, preliminary stem-inoculation experiments revealed that, whereas strain M6 was highly virulent on both melon and watermelon seedlings, strain W1 was weakly virulent on both of these species. Therefore, only M6 wild-type and TFP mutant strains were assessed in further assays on melon, whereas strain W1 was used as a control along with *P. lachrymans*, a nonvascular pathogen of melon.

Three independent experiments with similar results clearly revealed substantially higher percentages of wilted seedlings following inoculation with the wild type versus the TFP mutants. Data from a representative experiment is shown in Figure 4. Although inoculation with strain M6 led to 90 to 100% dead seedlings by the end of the three experiments, the average percentage of dead seedlings among those inoculated with the mutants was 30 to 50 and 20 to 25% for strains M6-M and M6-T, respectively. Prior to seedling collapse and wilt, we generally observed a brown necrotic line stretching from the point of inoculation up to the cotyledons, causing typical water-soaked lesions on the underside of leaves, which eventually turned necrotic. In all experiments, approximately 80% of the seedlings inoculated with strain W1 did not wilt and hardly developed disease symptoms, while treatment with *P. lachrymans* was not different from the water treatment and did not cause any seedling death (Fig. 4).

To examine the downward migration ability of these strains inside the plant tissue, stem inoculation was performed at the stem-cotyledon junction. At 1 day after inoculation, all examined strains could already be detected in the lowest segment (8 cm below the inoculation point). Despite a relatively high variability between replicates, strain M6 was detected in higher amounts than strains W1 and M6-T in all examined segments, on all tested days, and these differences were significant ($P = 0.05$) at most samplings (not shown). In most samplings, no significant differences were found between wild-type M6 and the M6-M mutant.

Detection of *A. avenae* subsp. *citrulli* in xylem vessels of melon and watermelon seedlings.

The results from the stem-inoculation assays supported the idea that *A. avenae* subsp. *citrulli* M6 can be systemically transmitted in the plant. However, in those assays, the bacteria were introduced directly into the plant stem. Therefore, we performed further seed-transmission assays to confirm the bacterium's ability to colonize the xylem vessels by electron microscopy. In a way, this assay mimics natural infection by *A. avenae* subsp. *citrulli* because it has been shown that, in both naturally and artificially infected seed, the pathogen can be extracted from both the seed coat and the embryo (Rane and Latin 1992).

Following seed inoculation, seedlings were allowed to grow for 4 to 5 days and were then collected, usually before symptom appearance. Then, longitudinally and latitudinally cut samples of the inoculated stems were prepared and specimens were examined under a scanning electron microscope for the presence of bacteria colonizing the xylem vessels. Although bacterial cells were readily found inside xylem vessels of M6-inoculated seedlings (Fig. 5), we were unable to detect bacteria in samples from seedlings inoculated with strains M6-M, M6-T, or W1. Taken together, these results support the notion that *A. avenae* subsp. *citrulli* M6 possesses vascular colonization ability and that TFP significantly contribute to this characteristic.

TFP are involved in biofilm formation of *A. avenae* subsp. *citrulli* M6.

TFP are involved in biofilm formation in various bacterial species (O'Toole and Kolter 1998). Moreover, in mammalian pathogens, formation of biofilm on host epithelial cells is crucial for disease development (Merz et al. 1999; Pujol et al. 1999). Because biofilm formation ability may influence *A. avenae* subsp. *citrulli* colonization in the seed or in xylem ves-

Fig. 4. Stem-inoculation assay on 8-day-old melon seedlings. Seedlings were inoculated in the hypocotyl with different strains, with the help of a needle, and the percentage of dead seedlings was recorded every day for a period of 8 days. Data from one experiment out of three with similar results are shown.

sels, we asked whether TFP play a role in biofilm formation by this bacterium. Biofilm assays were conducted on two different surfaces: polystyrene culture plates and microscope glass slides. Preliminary experiments on both surfaces revealed that, whereas strain M6 and other group I strains form clear biofilm fringes on both surfaces, strain W1, as well as other group II strains, fail to do so (not shown). Therefore, in these assays as well, comparisons among the wild type and TFP mutants were performed only with strain M6.

On polystyrene plates, although wild-type M6 formed a clear biofilm, a very weak biofilm was formed by mutant M6-M, and mutant M6-T failed to form any biofilm at all (Fig. 6A). Quantification of biofilm following staining with methyl violet and washing with ethanol confirmed the above qualitative observations, showing that biofilm formation by M6 was significantly ($P = 0.05$) higher than that by each of the two

mutants. In addition, biofilm formation by mutant M6-M was significantly ($P = 0.05$) higher than that by the M6-T mutant, for which optical density (OD) measurements were practically zero (Fig. 6B). Partial restoration of biofilm-formation ability was observed in the complemented strain M6-Tcomp (Fig. 6). Similar results were observed in biofilm assays on microscope glass slides (not shown).

During growth of strains in liquid media and in biofilm assays, we observed pronounced aggregation of M6-T cells. A quantitative assay was conducted to evaluate aggregation ability of M6 wild-type and mutant strains. In agreement with the qualitative observations, the M6-T mutant showed a significantly ($P = 0.05$) higher rate of cell aggregation than wild-type and M6-M strains (Fig. 7). Complementation of the M6-T mutant with pMLpilT partially restored the wild-type phenotype. In contrast to the M6-T mutant, the *pilT* mutant on the back-

Fig. 5. Scanning electron microscopy of melon xylem vessels following seed-transmission assays with *Acidovorax avenae* subsp. *citrulli* M6. M6 cells are seen colonizing the xylem vessels. Size bars: 20 and 10 µm for A and B, respectively.

Fig. 6. Biofilm formation of *Acidovorax avenae* subsp. *citrulli* M6 compared with mutants M6-M, M6-T, and M6-Tcomp. **A**, Images of biofilm formed in polystyrene culture plates characterized by stained methyl violet fringes around the well (indicated by arrows in the wild type and in M6-Tcomp). **B**, Biofilm quantification following washing of methyl violet stain with ethanol and optical density measurements at 590 nm. Data represent averages and standard error of one experiment out of three with similar results. Different letters indicate significant differences ($P = 0.05$) among treatments.

ground of strain W1 (W1-T) did not form any visible aggregates and showed aggregation percentages similar to those of its wild-type parent (not shown).

Because the polar flagellum is known to contribute to biofilm formation and colonization processes in other bacteria, we speculated that the abundance of TFP on the cell surface of the hyperpiliated mutant M6-T or its enhanced cell aggregation might have a negative effect on flagellum-mediated motility. Indeed, microscopy observations revealed that swimming motility is compromised in this mutant relative to wild-type M6 and the M6-M mutant. In agreement with these, swimming-motility assays on soft NA revealed that, whereas strains M6 and M6-M were able to expand from the center of the plate outward, the M6-T mutant as well as strain W1 were unable to do so and formed regular colonies (Fig. 8A). Swimming-motility haloes began to appear in M6 and M6-M after 5 to 6 h of incubation and rapidly expanded with time. Complementation fully restored the swimming ability of M6-T. Measure-

ments of the expansion diameter revealed significant ($P = 0.05$) differences between wild-type strains M6 and W1, as well as among the M6 wild-type and mutant M6-T (Fig. 8B). Differences between strains M6 and W1 were consistent with differences among other tested wild-type strains from the two groups (not shown).

DISCUSSION

To identify virulence-associated genes of *A. avenae* subsp. *citrulli*, we generated a library of transposon mutants on the background of the group I strain M6. Coupled with a suitable pathogenicity screen on melon, this allowed us to identify several mutants with compromised virulence. Sequence analyses of the cassette-insertion region of some mutants revealed several interesting genes likely contributing to the pathogen's virulence. These include, among others, the flagellin biosynthesis gene *fliR*; a two-component system, histidine kinase gene homologous to *cheA*; a general secretion pathway protein G gene; and the TFP assembly gene *pilM* (genes Aave_4383, 4378, 0925, and 0996, respectively, in the sequenced strain AAC00-1; GenBank accession NC_008752). Characterization of the latter mutant, named M6-M, as well as of additional TFP mutants generated by insertional mutagenesis, are the subject of this report.

TFP are involved in many processes in bacteria, including twitching motility, adherence to surfaces and colonization, biofilm formation, DNA uptake, bacteriophage sensitivity, and virulence (Craig et al. 2004; Mattick 2002; Nudleman and Kaiser 2004). In contrast to the well-established role of TFP in the pathogenicity of animal-pathogenic bacteria, their role in plant-pathogenic bacteria is poorly understood. The involvement of TFP in the interaction between pathogenic bacteria and plants has been studied mainly with regard to vascular bacterial pathogens. It is assumed that TFP might contribute to the optimal establishment, colonization, and spread of these pathogens via the plant xylem vessels. TFP were demonstrated to contribute to the virulence of *R. solanacearum* as well as to

Fig. 7. Aggregation ability of *Acidovorax avenae* subsp. *citrulli* M6 compared with mutants M6-M, M6-T, and a M6-Tcomp. Data represent averages and standard error of one experiment out of two with similar results. Different letters indicate significant differences ($P = 0.05$) among treatments.

Fig. 8. Motility assay in soft (0.3%) agar. **A**, Representative plates containing nutrient soft agar medium, 48 h after being stab inoculated with the indicated strains. Whereas wild type W1 and mutant M6-T, impaired in flagellar motility, remain in the center of the plate and form regular colonies, strains M6, M6-Tcomp, and M6-M exhibit flagellar motility, leading to the formation of swimming haloes that expand from the center of the plate outward. **B**, Diameter measurements of the haloes formed by the different strains after 48 h. Different letters indicate significant differences ($P = 0.05$) among strains.

this bacterium's twitching motility and polar adhesion to tobacco suspension-cultured cells and to tomato roots (Kang et al. 2002; Liu et al. 2001). TFP are also important for twitching motility, biofilm formation, cell aggregation, and upstream migration of the xylem-residing pathogen *X. fastidiosa* (De La Fuente et al. 2007, 2008; Li et al. 2007; Meng et al. 2005). Although these reports suggest an important role for TFP in *X. fastidiosa* pathogenicity, this assumption was not directly demonstrated by pathogenicity assays.

Regarding nonvascular plant pathogens, TFP were suggested to contribute to fitness and survival on the leaf surface of *P. syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria*. However, under tested conditions, TFP mutants of these pathogens did not differ from their wild types in their disease symptom induction abilities (Ojanen-Reuhs et al. 1997; Roine et al. 1998). In contrast, Romantschuk and Bamford (1986) showed that TFP-minus mutants of *P. syringae* pv. *phaseolicola* failed to induce disease symptoms on bean following spray inoculation. Recently, TFP have been reported to be involved in the virulence of the nonvascular rice pathogen *X. oryzae* pv. *oryzicola* (Wang et al. 2007).

Our initial findings showed that the M6 *pilM* mutant possesses significantly reduced virulence and biofilm-formation ability, as well as abolished twitching motility, relative to the wild type. To verify the role of TFP in the above features in this bacterium, we generated knockout mutants of *pilT* in strain M6 and *pilT* and *pilA* in the group II strain W1. *pilT* encodes an ATPase responsible for the TFP retraction that drives twitching motility while *pilA* encodes the major TFP subunit pilin (Nudleman and Kaiser 2004).

As expected, twitching motility was also abolished by the *pilA* and *pilT* mutations. TEM confirmed the absence of TFP in *pilM* and *pilA* mutants, as well as the expected hyperpilated phenotype of the *pilT* mutants. Significant differences were observed between M6 wild-type and TFP mutants in biofilm-formation ability and in pathogenicity assays performed in this study. The ability to form biofilm was significantly reduced in the *pilM* mutant relative to the wild type but was nevertheless retained. In contrast, the hyperpilated M6 *pilT* mutant did not form any biofilm at all. These results are in contrast to findings with *P. aeruginosa*, in which twitching-minus, hyperpilated *pilT* mutants formed higher amounts of biofilm than the wild-type parent (Chiang and Burrows 2003). On the other hand, similar to our findings, a recent study with a *pilT* mutant of a different *P. aeruginosa* strain showed the opposite picture; namely, reduced biofilm formation relative to the wild type (Jenkins et al. 2005).

In our study, the inability of the *pilT* mutant to form biofilm could be related, at least in part, to its high cell aggregation ability, which resulted in the settling of cell aggregates at the bottom of the well. Similarly, hyperpilated *pilT* mutants of *P. aeruginosa* and *Neisseria meningitidis* have been reported to form aggregates to a much higher extent than their wild-type parents (Carbonnelle et al. 2005; Helaine et al. 2005). The opposite effects of the *pilT* mutation on biofilm formation versus cell aggregation in *A. avenae* subsp. *citrulli* suggest that, in this bacterium, different extracellular components, or differential interactions between them, are involved in different adhesion processes.

Biofilm formation has been associated with virulence of pathogenic bacteria. In animal hosts, biofilm formation was shown to be important for virulence of *P. aeruginosa*, in part through the high tolerance conferred by the condensed structure of the biofilm to the host immune response and antimicrobial treatments (O'Toole and Kolter 1998; Stewart and Costerton 2001). In plants, it is speculated that biofilms formed by bacterial pathogens contribute to virulence in several ways. During

the epiphytic phase on plant surfaces, bacteria present in biofilms are considered to be more resistant to environmental stresses than planktonic cells (Kang et al. 2002; Ojanen-Reuhs et al. 1997). Inside plant tissues, biofilms may be involved in protection against antimicrobial agents released by the plant. In vascular pathogens, however, biofilms are thought to contribute to virulence in a more direct fashion, by blocking sap flow in the xylem vessels and promoting plant wilt (Meng et al. 2005). However, direct evidence for the roles of biofilm in pathogenicity of plant pathogens is still lacking.

The inability of the tested group II strains to form biofilm was somewhat surprising, because both group I and II strains possess functional TFP. It is possible that differences in other biofilm-associated extracellular components between strains from the two groups are responsible for the observed differences in biofilm formation. For example, bacterial flagella play an important role in the attachment and adhesion processes of many bacteria (Moens and Vanderleyden 1996). In *P. aeruginosa*, in addition to TFP, polar flagella are required for biofilm formation (Barken et al. 2008; Kang et al. 2002; O'Toole and Kolter 1998). Interestingly, the M6 *pilT* mutant, which was also affected in its polar flagellum-mediated motility, showed significantly reduced biofilm formation ability and virulence in seed-transmission assays than the M6 *pilM* mutant. We have shown that all tested group II strains have significantly lower swimming ability and are less virulent in seed-transmission assays than group I strains (Burdman et al. 2005). In a recent study, we also showed that group II strains possess lower adhesion ability than group I strains to melon seed (Bahar et al. 2009). Here, whereas polar flagella were clearly observed in M6 cells by TEM, they were barely seen in W1 cells. This evidence suggest that, in addition to TFP, polar flagella may play an important role in adhesion and biofilm formation of *A. avenae* subsp. *citrulli*, and that these features could contribute to the pathogen's virulence under certain conditions. A role of polar flagellum in virulence of this bacterium is also supported by the fact that, as mentioned, one of the mutants identified in our virulence screens was affected in the flagellin biosynthesis gene *flhR*.

Considering the well-known role of TFP in adhesion to both inert and plant surfaces, we speculated that, in seed-transmission assays, a higher amount of wild-type versus TFP mutant cells could adhere and attach to the seed. However, no significant differences were observed in seed adhesion between M6 wild type and *pilM* and *pilT* mutants. Similarly, *pilT* mutants of *P. aeruginosa* have been shown to adhere to different surfaces at similar or higher extents than the wild type (Chiang and Burrows 2003). These results suggest that the role of TFP in virulence in seed-transmission assays comes into play at a later stage of the pathogen-host interaction. However, bacterial distribution in the seed may also be important. It has been shown that, following natural or artificial seed infestation, *A. avenae* subsp. *citrulli* can be extracted from both the seed coat and the embryo (Rane and Latin 1992). Thus, the possibility exists that the wild-type strain gains entrance to the embryo more efficiently than the TFP mutants.

As mentioned, microscopic observations and swimming-motility assays revealed that the hyperpilated M6 *pilT* mutant is affected in its polar flagellum-mediated motility. This could be due, at least in part, to the enhanced cell aggregation of this mutant. To the best of our knowledge, this is one of the first reports showing a negative effect of hyperpilation on swimming motility; we were only able to find a single abstract reporting a similar result with a *pilT* mutant of *P. aeruginosa* (Evans et al. 2003).

Stem inoculations confirmed the role played by TFP in *A. avenae* subsp. *citrulli* virulence and strengthened the impor-

tance of TFP for successful infection of the host plant beyond the stage of seed adhesion and colonization by the bacterium. As mentioned, this might involve efficient access to, colonization of, and migration through xylem vessels. Evaluation of downward migration following stem inoculation revealed that both the wild type and TFP mutants can migrate against the sap flow to some extent. However, these assays revealed that the wild type is able to migrate and proliferate in the xylem more successfully than the *pilT* mutant. These results indicate that, whereas functional TFP may not be fully required for downward migration, they do contribute to the bacterial ability to induce seedling collapse.

Altogether, this study demonstrates that *A. avenae* subsp. *citrulli* requires functional TFP for twitching motility and for wild-type levels of biofilm formation and virulence. TFP appear to contribute to virulence in both the early stages of seed infection and germination and in later stages of the pathogen–host interaction. Importantly, our findings from stem inoculations and scanning electron microscopy (SEM) observations also provide, for the first time, strong evidence for the notion that at least group I strains of *A. avenae* subsp. *citrulli* possess vascular infection ability.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids.

Strains and plasmids used in this study are listed in Table 1. *A. avenae* subsp. *citrulli* strains were generally grown in NB (Difco Laboratories, Detroit) or NA (NB containing agar at 15 g/liter) at 28°C. In some experiments, the strains were grown in M9 minimal medium (Sambrook et al. 1989) amended with sodium citrate and casamino acids at a final concentration of 0.75%. For inoculation, strains were grown on NA for 48 h, resuspended from plates in sterile distilled water (SDW), adjusted to an OD at 600 nm (OD₆₀₀) of 0.6 (approximately 10⁸ CFU/ml) using a Helios Gamma spectrophotometer (Thermo Electron Corp., Rochester, NY, U.S.A.), and then diluted to 10⁷ or 10⁶ CFU/ml. *P. lachrymans* AV1 was grown in King's B medium at 28°C for 48 h and inoculation suspensions were obtained as for *A. avenae* subsp. *citrulli*. *Escherichia coli* strains were cultured in Luria-Bertani (LB) at 37°C. Antibiotics used in this study, according to the resistances described in Table 1, were ampicillin, 100 µg/ml; kanamycin (Km), 50 µg/ml; and gentamicin (Gm), 20 µg/ml.

Molecular manipulations.

Routine molecular manipulations were carried out using standard procedures (Sambrook et al. 1989). T4 DNA ligase

and restriction enzymes were purchased from Fermentas (Ontario, Canada). Kits for plasmid and PCR product purification were purchased from Real Biotech Corporation (Taipei, Taiwan). Genomic DNA from *A. avenae* subsp. *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.). PCR primers (Table 2) were purchased from Hy Laboratories (Rehovot, Israel) and PCR assays 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.

Transformation of *A. avenae* subsp. *citrulli* cells.

A. avenae subsp. *citrulli* cells were transformed by electroporation or conjugation by biparental mating. Electrocompetent cells were prepared using standard procedures (Sambrook et al. 1989). Electroporations were carried out in an Eppendorf 2510 electroporator, at 1,800 V and a time constant of approximately 5 ms, according to the manufacturer's instructions. Following electroporation, cells were transferred to 1 ml of NB for 2 h (28°C, 200 rpm), and then plated on NA with suitable antibiotics for isolation of putative transformants. For conjugations, *A. avenae* subsp. *citrulli* and *E. coli* S17-1 harboring the desired vector were grown on NB and LB, respectively, containing the appropriate antibiotics. Cultures were grown to an OD₆₀₀ of 0.3 to 0.5 and then mixed at a ratio of 5:1 (*A. avenae* subsp. *citrulli*:*E. coli*). Bacterial mixtures were then vortexed, centrifuged at 2,000 × *g* to pellet the bacteria, resuspended in 100 µl of SDW, and plated on NA without antibiotics for 24 h at 28°C. Afterward, cells were resuspended in SDW and plated on NA plates with appropriate antibiotics for selection. Transformants were verified by PCR, Southern blot, or plasmid preparation, as appropriate.

Construction of a random transposon library of *A. avenae* subsp. *citrulli* and identification of mutated genes.

A random transposon library was generated on the background of strain M6 using the Ez-Tn5 kit (Epicentre, Madison, WI, U.S.A.). Briefly, a Km-resistant (Km^r) gene was excised from pUC-4K with *Bam*HI and subsequently cloned into the multiple cloning site (MCS) of the transposon construction vector pMOD-3<R6K γ ori/MCS> which was cut with the same enzyme. This vector was then digested with *Pvu*II to excise the mutagenesis cassette, which included the Km^r gene and

Table 2. Description of oligonucleotide polymerase chain reaction (PCR) primers used in this study

Primer set	Primer sequence (5'–3') ^a	Description of PCR product
<i>pilA</i> -mut-F	GCGAATTCGATTACACCAAGAAAGCCAA (E)	...
<i>pilA</i> -mut-R	GCGAATTCATTGCTAGCAGCATGTCATGG (E)	303-bp internal fragment of <i>pilA</i> ; used to create W1-A
<i>pilA</i> -comp-F	GCCGAATTCCTCCATAGTGATTAGGCACCA (E)	...
<i>pilA</i> -comp-R	GCCGGATCCGCCGCTGAACGCCCATCC (B)	1,134-bp fragment of <i>pilA</i> ; used for complementation of W1-A
<i>pilT</i> -mut-F	GCCGAATTCCTCGCGCCATGGTGAACCACTCAACGA (E)	...
<i>pilT</i> -mut-R	GCCGGATCCAGCATGGCGCGACCATTTCTTTTCCT (B)	367-bp internal fragment of <i>pilT</i> ; used to generate <i>pilT</i> mutants
<i>pilT</i> -comp-F	GCCGGATCCTGTGTTGGGGGATGGGTGAACGG (B)	...
<i>pilT</i> -comp-R	GCCTCTAGATCCGGCCGCTGCTGCGACAGG (X)	1,509-bp fragment of <i>pilT</i> ; used for complementation of <i>pilT</i> mutants
pMOD<MCS>F	GCCAACGACTACGCACTAGCCAAC	Used for sequencing of the insertion site of the Tn5 cassette (forward primer, Epicentre)
pMOD<MCS>R	GAGCCAATATGCGAGAACACCCGAGAA	Used for sequencing of the insertion site of the Tn5 cassette (reverse primer, Epicentre)

^a Underlined nucleotides in some of the primers represent restriction sites of enzymes indicated in parentheses (E = *Eco*RI; B = *Bam*HI; X = *Xba*I). The corresponding enzymes were used to excise the obtained PCR products from the cloning vector pTZ57R/T and clone them into pJP5603 or pML122 for insertional mutagenesis or complementation, respectively.

R6K γ ori, flanked by 20-bp-long mosaic ends (ME) on both sides. The cassette, along with the EZ-Tn5 transposase, was electroporated into M6 cells, and Km^r mutants were stored in 20% glycerol at -80°C. A random set of 35 mutants was verified by Southern blot, using the cassette as a probe. In all tested mutants, the cassette appeared to be inserted only once and at different positions in the bacterial chromosome (not shown).

Mutants were screened for altered virulence relative to the wild type by seed-transmission assays (Bahar et al. 2009). Briefly, a bacterial suspension of approximately 10⁸ CFU/ml of each mutant was used to inoculate 8 to 10 seeds of melon (*Cucumis melo*) cv. Ophir (Zeraim Gedera, Israel) at room temperature for 2 h with gentle agitation. Seeds were then sown in 600-ml pots filled with sand. At 7 to 10 days after inoculation, mutants showing reduced virulence (based on the severity of seedling blight symptom) were selected. Selected mutants were verified in detailed seed-transmission assays (see below). To identify the mutated region of a selected mutant, a rescue clone procedure was applied. The genomic DNA of the mutant was purified and digested with an enzyme that does 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 *E. coli* BW25141 cells. Km^r transformants were used for plasmid preparation and sequencing of the flanking regions surrounding the insertion site of the mutagenesis cassette using pMOD<MCS> primers (Table 2).

Generation of *pilA* and *pilT* knockout mutants and complementation.

To generate knockout mutants on the background of strains M6 and W1, internal fragments of *pilT* and *pilA* (which do not span the 3' and 5' ends of these genes) were PCR amplified with suitable primers (Table 2) using DNA from the corresponding strain as template. The PCR products were cloned into pTZ57R/T, verified by sequencing, excised with appropriate restriction enzymes (as indicated in Table 2), and then cloned into pJP5603 to yield pJPilTmut and pJPilAmut. Insertional mutagenesis of target genes in strains M6 and W1 was performed by electroporation or biparental mating as described above, to yield mutants M6-T, W1-T, and W1-A. Gene interruption was confirmed by Southern blot, PCR, and phenotypic analysis. For complementation, *pilT* and *pilA* open reading frames were PCR amplified from each wild type, along with 403 or 466 bp upstream of their start codon, respectively, in order to include the native promoter of each gene. Suitable primers (Table 2) were designed based on the sequence of strain AAC00-1, and the PCR products were cloned into pTZ57R/T, verified by sequencing, and then cloned into pML122. Complementation vectors (Table 1) were introduced into the mutants by conjugation as described above and complemented strains were selected by Gm resistance.

Assessment of twitching motility.

Bacteria were grown on NA for at least 72 h, and twitching motility was assessed by the naked eye or by light microscopy on an Axioscope microscope (Carl Zeiss, Jena, Germany) equipped with a DXM1200F digital camera (Nikon, Tokyo). Colonies exhibiting twitching motility were characterized by the formation of a thin, light halo around the bulk colony.

Electron microscopy.

TEM was used to visualize TFP. SDW drops (20 μ l) were placed on 48-h-old colonies for 2 min; then, a 200-mesh Formvar-coated copper grid was placed on top of each water drop for 1 min. Copper grids were negatively stained using 1% phosphotungstic acid for 30 s and examined using a Fei Tecnai

G2 Spirit transmission electron microscope (Fei, Hillsboro, OR, U.S.A.). SEM was used to visualize the colonization of plant xylem vessels by *A. avenae* subsp. *citrulli* following seed-transmission assays. Specimens were prepared by chemical fixation of plant stems, cut both vertically and horizontally, in 5% glutaraldehyde for 2 h. Then, specimens were rinsed, dehydrated using increasing amounts of ethanol, critical-point dried using Bio-Rad C.P.D 750 (Hercules, CA, U.S.A.), and sputter coated with gold. Samples were visualized using a Jeol JSM-5410LV scanning electron microscope (Jeol Ltd., Tokyo).

Pathogenicity assays.

Seed-transmission assays were performed as described for the library screens, with minor modifications. Here, 24 melon (cv. Ophir) seeds were placed inside a 50-ml Falcon tube containing 10 ml of bacterial suspension at approximately 10⁶ CFU/ml. Seeds were incubated with gentle agitation for 2 h; then, suspensions were discarded and seeds were rinsed briefly and dried overnight. Seeds were then sown in 600-ml pots containing sand (six seeds per pot) and grown in a greenhouse at 25 to 28°C for 10 to 14 days. For determination of CFU/seed following this inoculation procedure, eight seeds per strain were treated as described above and then ground with a mortar and pestle. Serial dilutions were plated onto NA with appropriate antibiotics for bacterial counts. To assess the effectiveness of this method, the resultant seed debris were resuspended in SDW, vortexed for 30 s, sonicated for 5 min, and boiled for 10 min. Following centrifugation for 5 min at 600 $\times$ g to remove seed debris, DNA was purified from extracts, serially diluted, and evaluated by PCR with the *A. avenae* subsp. *citrulli*-specific primer set BX-S as described (Bahar et al. 2008). Seed-transmission assays and adhesion tests were performed at least three times for each tested strain.

Stem-inoculation experiments were performed on 20 8-day-old melon cv. Ophir or watermelon (*Citrullus lanatus*) cv. Malali (Hazera Genetics Co., Israel) seedlings for each evaluated strain. Seedlings were inoculated by placing a 5- μ l drop of 10⁸ CFU/ml suspensions on the hypocotyls (approximately 1 cm from the site of emergence). Then, a 25-gauge needle was used to stab the stem through the drop. Seedlings were kept in the greenhouse (25 to 28°C) for 8 days and the percentage of dead seedlings was recorded daily. This experiment was repeated three times. To examine downward bacterial migration ability, stem inoculations were performed in seedlings at the stem-cotyledon junction. In these assays (repeated twice), bacterial migration was estimated by cutting the stem into 2-cm segments below the inoculation point, crushing them, and plating serial dilutions. Counts were performed daily until 4 days after inoculation.

Biofilm assays.

Biofilm assays were performed using microscope glass slides or polystyrene 24-well multidishes (Nunc, Denmark) as described in O'Toole and associates (1999), with a few modifications. Briefly, sterile microscope slides were placed inside 50-ml Falcon tubes containing 15 ml of M9 medium with appropriate antibiotics. The tubes were then inoculated with a 1:100 dilution of an overnight bacterial culture. Multidishes were filled with 1 ml of M9 with appropriate antibiotics and then inoculated in the same manner as the Falcon tubes. Both tubes and polystyrene multidishes were incubated at 28°C for 48 h without agitation. Then cultures were poured out and wells or slides were rinsed with SDW. Following fixation at 80°C for 20 min, biofilms on both surfaces were stained with 0.1% methyl violet for 30 min. Microscope slides were then washed with distilled water and biofilms formed on glass were analyzed qualitatively by visualization. Multidishes were rinsed

with distilled water and analyzed both qualitatively and quantitatively, by solubilizing the stained biofilms with 95% ethanol for 2 h and measuring OD₅₉₀ of the stained suspension in a spectrophotometer.

Cell-cell aggregation.

Aggregation assays were performed based on the protocol used by Burdman and associates (1998) with a few modifications. *A. avenae* subsp. *citrulli* strains were grown overnight inside 15-ml glass tubes containing 4 ml of NB with appropriate antibiotics. Then, cultures were kept at room temperature for 2 h to allow aggregates to settle to the bottom of the tubes. Turbidity (OD_s) was measured using a spectrophotometer at OD₆₀₀. The cultures were then dispersed by vortex for 10 s, and turbidity was measured again (OD_t). The percentage of aggregation was calculated as $(OD_t - OD_s) \times 100/OD_t$.

Flagellar motility assay.

Wild-type and mutant strains were streaked on NA plates with appropriate antibiotics for 48 h. Then, cells from single colonies were transferred to the center of a soft (0.3% agar) NA plate (12 plates per strain) using a toothpick. The plates were incubated at 28°C and the haloes formed by migrating bacteria were measured after 48 h. This experiment was repeated three times.

Statistical analysis.

All quantitative assays were analyzed using the Tukey-Kramer highly significant difference test for mean comparison using JMP software (SAS Institute Inc., Cary, NC, U.S.A.).

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