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Twitching Motility Contributes to the Role of Pili in Corneal Infection Caused by *Pseudomonas aeruginosa*

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Twitching motility is a form of surface-associated bacterial movement mediated by type IV pili of *Pseudomonas aeruginosa*. Others have shown that *pilT* and *pilU* mutants, which are piliated but defective in twitching motility, display reduced cytotoxic capacity towards epithelial cells in vitro. Although these mutants efficiently infected lungs in vivo, they were defective in dissemination to the liver. In this study the role of twitching motility in *P. aeruginosa* epithelial cell invasion and corneal disease pathogenesis was explored. *pilU* and *pilT* mutants of *P. aeruginosa* strain PAK were compared to a nonpiliated *pilA* mutant and to wild-type bacteria in their ability to associate with and to invade corneal epithelial cells in vitro and to cause disease in a murine model of corneal infection. As expected, the *pilA* mutant demonstrated reduced association and invasion of corneal epithelial cells ($P < 0.05$ in both cases). The *pilT* mutant, but not the *pilU* mutant, was less invasive than wild-type PAK was ($P < 0.05$ versus $P = 0.43$), while both *pilU* and *pilT* mutants exhibited association levels similar to those of the wild type ($P = 0.31$ and 0.52, respectively). In vivo, all mutants were markedly attenuated in virulence and showed reduced ability to colonize the cornea at 4 and 48 h (all P values < 0.02). Thus, twitching motility contributed to the role of pili in corneal disease but was not involved in the role of pili in adherence to or invasion of corneal epithelial cells.

Pseudomonas aeruginosa is an opportunistic bacterial pathogen (22) capable of causing sight-threatening corneal ulcers (9). It can also cause infections in immunocompromised people or in those suffering from burns or cystic fibrosis (2, 23).

Type IV pili are polar surface filamentous appendages expressed by many pathogenic bacteria, including *P. aeruginosa* (10, 24). In *P. aeruginosa*, type IV pili consist of a homologous polypeptide formed from pilin, a 15-kDa protein encoded by the *pilA* gene (20). The transcription of *pilA* is tightly regulated by a classical RpoN (σ^{54})-dependent two-component sensor-regulator pair, PilS and PilR (14). Pilus synthesis and assembly require at least 40 genes, which are located in six unlinked regions of the genome (15).

Some type IV pili serve as receptors for bacteriophages (3) and are thought to play a role in bacterial adherence to epithelial cells and mucosal surfaces (7). Nonpiliated isolates of *P. aeruginosa* have been shown to be less virulent in lung infection models than piliated isogenic variants, suggesting that pili contribute to disease pathogenesis (11). It is often assumed that their requirement for virulence relates directly to their role in adherence (7), even though published data on *P. aeruginosa* infection in the eye suggest that piliation does not always correlate with adherence or virulence (13).

Type IV pili are also involved in a form of surface movement termed twitching motility (4) or social gliding (29). Twitching motility involves the retraction and extension of pili (17), which allow bacteria to "walk" on infected surfaces (4). Twitching motility has been shown to be one of many factors required for the formation of biofilms on abiotic surfaces (18).

In *P. aeruginosa*, *pilT* and *pilU* encode proteins with putative nucleotide-binding domains that provide energy for type IV pilus retraction (27). Both *pilT* and *pilU* mutants appear hyperpiliated and are defective in twitching motility, probably because they are unable to retract their pili (4). While *pilU* mutation does not affect bacterial susceptibility to the bacteriophage PO4, *pilT* mutation causes bacteria to become resistant to normal killing activity of this phage (similar to a nonpiliated mutant) (28).

Other investigators have shown that *pilT* and *pilU* mutants of *P. aeruginosa* have reduced cytotoxic activity towards various types of epithelial cells in vitro (6). Although both mutants retained full virulence in the lung environment, they were each defective in their ability to disseminate from the lung to the liver. In this study we explored the role of twitching motility in *P. aeruginosa* epithelial cell invasion in vitro and in corneal disease pathogenesis in vivo.

Confirmation of twitching motility characteristics. To confirm that the *pilA*, *pilT*, and *pilU* mutants (Table 1) were defective in their ability to travel across a solid surface in our hands, we examined their twitching motility qualitatively by examination of colony morphology. Bacteria were point inoculated with a sterile toothpick onto the surface of a slab of Luria-Bertani agar (1%). Plates were incubated for 16 h at 37°C and then for 72 h at room temperature. With this method, wild-type PAK produced large spreading colonies with rough serrated edges. In contrast, all three mutants formed small, smooth, and clear-edged colonies, indicating a lack of twitching motility (4).

Effect of twitching motility on *P. aeruginosa* adherence and

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TABLE 1. *P. aeruginosa* strains and mutants used

Strain	Relevant characteristics	Source or reference
PAK	Wild type; pili$^+$ and flagella$^+$	J. S. Mattick
PAK*pilA*::Tc^r	Tetracycline resistance cassette inserted into *pilA*; nonpiliated	26
PAK*pilT*::Tn*5*	Tn*5* insertion in *pilT* (mutant R364); hyperpiliated, phage resistant	28
PAK*pilU*::Tn*5*	Tn*5* insertion in *pilU* (mutant S34); hyperpiliated, phage sensitive	28

invasion of corneal epithelial cells in vitro. Immortalized rabbit corneal epithelial cells (S. Okamoto, M. Oji, J. R. Hassell, R. A. Thoft, and J. M. Pipas, abstract from the Association for Research in Vision and Ophthalmology annual meeting 1993, Investig. Ophthalmol. Vis. Sci. **34:**S1010, 1993) were grown in 24-well tissue culture plates or 3.5-cm-diameter tissue culture dishes (Corning, New York, N.Y.) or on 0.4-μm-pore-size, 12-mm-diameter semipermeable Transwell filters (Corning Costar Corp., Cambridge, Mass.). Cells were fed with SHEM (16) and used in experiments 3 to 6 days after passaging (cells used in these experiments were between passages 6 and 11).

Adherence of strain PAK to corneal epithelial cells was compared to that of the *pilA*, *pilT*, and *pilU* mutants. The *pilA* mutant is twitching motility negative because it lacks pili and thus was used as a control to distinguish the contributions of pili and their twitching motility function. Cells were grown to confluence on Transwell filters (confluence was confirmed by measuring transepithelial resistance across the monolayer with an EVOM meter [World Precision Instruments, Sarasota, Fla.]). Cells were then inoculated with 2×10^5 CFU of bacteria in 200 μl of buffered minimal essential medium (MEM) at 37°C. At least three filters of confluent cells were used for each strain. After 3 h of incubation with bacteria, cells were washed three times with MEM to remove nonadherent bacteria. Each filter was then cut away from its plastic holder to exclude bacteria adherent to the sides of the tissue culture insert. Cells were then lysed with 0.25% (vol/vol) Triton X-100 in MEM, and bacteria in the homogenate were enumerated by viable counts. All experiments were repeated at least twice. The *pilA* mutant exhibited a reduced ability to adhere to the corneal cells ($P = 0.001$, *t* test) (Table 2), but the *pilT* and *pilU* mutations had no effect on *P. aeruginosa* adherence ($P = 0.5$ and 0.3, respectively; *t* test) (Table 2).

Previous studies have shown that pili contribute to *P. aeruginosa* adherence to tracheal epithelia (31) and are also involved in *P. aeruginosa* adherence to human pneumocytes (5, 8). However, Hazlett and coworkers showed that the relationship between pili and *P. aeruginosa* adherence to corneal epithelia is complex (13). For example, a nonpiliated PAK/PR11 mutant (equivalent to a *pilA* mutant) adhered as efficiently as did wild-type PAK to whole corneas in organ culture. Our data showed that the effects of pili on *P. aeruginosa* adherence to corneal epithelial cells in culture were small and variable. Indeed, in some experiments, differences were not statistically significant. Thus, nonpiliated mutants remained able to adhere in significant numbers to the corneal epithelial cells, showing that nonpilus adhesins (21) also play a role in *P. aeruginosa* adherence to these cells. The reason for the difference between airway and corneal epithelial cells in the apparent contribution of pili to *P. aeruginosa* adherence is not known.

TABLE 2. Bacterial association with cultured rabbit corneal epithelial cells[a]

Strain	Infecting dose (CFU/ml)	Adherent bacteria (CFU/ml)
Wild-type PAK	2.80×10^6	$2.15 \times 10^5 \pm 1.99 \times 10^4$
PAK*pilA*::Tc^r	2.75×10^6	$0.63 \times 10^5 \pm 2.34 \times 10^4$[b]
PAK*pilT*::Tn*5*	3.25×10^6	$2.23 \times 10^5 \pm 1.02 \times 10^4$
PAK*pilU*::Tn*5*	3.50×10^6	$2.01 \times 10^5 \pm 3.51 \times 10^3$

[a] Cells were exposed to 2×10^5 CFU of bacteria for 3 h, and viable bacteria adherent to cells were quantified. Data represent the means and standard deviations of the results.

[b] Significant difference compared to wild-type PAK ($P < 0.05$, *t* test).

Mutations in *pilU* of *Neisseria gonorrhoeae* have been shown to increase bacterial adherence to epithelial cells (19), as has mutation of the *bfpF* nucleotide-binding domain of enteropathogenic *Escherichia coli*, which abolishes twitching motility while increasing piliation. Thus, it was not altogether surprising that inactivation of *pilU* in *P. aeruginosa* did not reduce bacterial adherence to corneal epithelial cells. However, our results differed somewhat from the recent findings of Comolli and coworkers (6), who showed decreased adherence of *pilT* and *pilU* mutants to three different epithelial cell lines, MDCK, A549, and HeLa cells. Differences between their study and ours might relate to differences between the epithelial cell types studied. Otherwise, they may be due to differences in methodology. For example, Comolli and coworkers used conditions that allowed wild-type *P. aeruginosa* to damage epithelial cells. Since *P. aeruginosa* binds avidly to damaged mammalian cells (21) and twitching motility mutants were less cytotoxic than wild-type bacteria were, their adherence results may be complicated by variations in epithelial cell damage. In contrast, our methods involved measurement of adherence under conditions that did not induce cell damage.

Strain PAK was also compared to the *pilA*, *pilT*, and *pilU* mutants for the ability to invade corneal epithelial cells in vitro by gentamicin survival assays (11). Cells grown on 24-well tissue culture plates were inoculated as described for adhesion assays At least four wells were assigned for each strain. Following 3 h of incubation at 37°C, bacterial growth was enumerated by viable counts of the cell supernatant to ensure that mutants survived and grew as efficiently as the wild type did in the presence of epithelial cells. Cells were then washed with phosphate-buffered saline prior to incubation with 200 μg of gentamicin (Sigma)/ml in MEM for 1 h to kill extracellular bacteria. After being washed twice with phosphate-buffered saline (500 μl) to remove antibiotic, cells were lysed with Triton X-100 in MEM (0.25% [vol/vol], 15 min). A viable count was performed on each well to quantify survivors (intracellular bacteria). All experiments were repeated twice.

The nonpiliated *pilA* mutant exhibited significantly reduced ability to invade corneal epithelial cells compared to that of wild-type bacteria ($P < 0.05$, *t* test) (Table 3). In contrast, invasion levels demonstrated by the *pilU* mutant did not significantly differ from those of wild-type PAK ($P = 0.43$, *t* test) (Table 3), while those of the *pilT* mutant were significantly lower than those of the wild type ($P < 0.0001$, *t* test). Both twitching motility mutants, *pilU* and *pilT*, invaded more effi-

TABLE 3. Bacterial invasion of cultured rabbit corneal epithelial cells[a]

Strain	Infecting dose (CFU/ml)	Invading bacteria (CFU/ml)
Wild-type PAK	2.55×10^6	$2.28 \times 10^4 \pm 2.21 \times 10^3$
PAK*pilA*::Tc^r	2.45×10^6	$3.33 \times 10^3 \pm 4.60 \times 10^{2b}$
PAK*pilT*::Tn*5*	2.85×10^6	$6.32 \times 10^3 \pm 3.66 \times 10^{2b}$
PAK*pilU*::Tn*5*	3.00×10^6	$2.13 \times 10^4 \pm 2.84 \times 10^3$

[a] Viable intracellular bacteria in corneal cells were quantified by gentamicin survival assays. Data represent the means and standard deviations of the results.
[b] Significant difference compared to wild-type PAK ($P < 0.05$, t test).

TABLE 5. Comparison of colonization by wild-type PAK, nonpiliated PAK*pilA*::Tc^r, and the twitching motility mutants PAK*pilT*::Tn*5* and PAK*pilU*::Tn*5* in mouse eyes at 4 h postinoculation

Strain	Infecting dose (CFU)	Median final viable count (CFU/ml) (lower quartile–upper quartile)
Wild-type PAK	4.5×10^7	2.5×10^5 (2.20×10^5–3.50×10^5)
PAK*pilA*::Tc^r	5.5×10^7	2.7×10^3 (1.11×10^2–3.70×10^3)
PAK*pilT*::Tn*5*	5.5×10^7	6.4×10^4 (1.80×10^3–1.15×10^5)
PAK*pilU*::Tn*5*	5.0×10^7	5.0×10^4 (4.40×10^4–5.80×10^4)

ciently than the nonpiliated *pilA* mutant did ($P < 0.0001$, t test). Those results showed that pili were involved in corneal epithelial cell invasion, consistent with the involvement of pili in *P. aeruginosa* invasion of various other epithelial cell types (5, 6). They also showed that *P. aeruginosa* invasion required the physical presence of pili, rather than their twitching motility function. This differs from the role of pili in *P. aeruginosa* cytotoxicity, which involves twitching motility function (6).

pilU and *pilT* are both required for full virulence of *P. aeruginosa* in the cornea in vivo. The contribution of *pilU* and *pilT* to the virulence of *P. aeruginosa* in the cornea was tested in vivo using a murine model of infection. Six-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) were used, and all procedures were conducted in accordance with the policies established by the Association for Research in Vision and Ophthalmology. Following anesthesia, three full-thickness epithelial abrasions were produced on the left cornea with a sterile 26-gauge needle. The cornea was then inoculated with 5 μl of bacterial suspension (~10^7 CFU). Five mice were assigned to each sample group. Infected eyes were graded 1, 2, 4, and 7 days postinoculation by a masked investigator using a stereomicroscope; photographs were also taken. Corneal disease was graded as previously described (1): grade 0, eye macroscopically identical to the uninfected contralateral control eye; grade 1, faint opacity partially covering the pupil; grade 2, dense opacity covering the pupil; grade 3, dense opacity covering the entire anterior segment; grade 4, perforation of the cornea and/or phthisis bulbi (shrinkage of the eyeball following inflammatory disease).

Corneal virulence of the *pilU* and *pilT* mutants was compared to that of wild-type bacteria and the *pilA* mutant. All three mutants exhibited reduced virulence in the cornea (Table 4), showing that at least part of the contribution of pili to *P. aeruginosa* corneal infection involves their twitching motility function.

A previous study by Hazlett and coworkers (13) showed that a hyperpiliated mutant of PAK (PAK/PR1) was avirulent in the cornea. Although the exact details of the mutation in PAK/PR1 were not described, its hyperpiliated state was suggestive of a twitching motility defect. Interestingly, they also reported that a nonpiliated *pilA* mutant of PAK retained full virulence. Differences in the roles of pili in *P. aeruginosa* corneal virulence between that study and ours might relate to the different mouse species used, since they used Swiss Webster mice, which are designated as "resistant" to *P. aeruginosa* corneal infection because they clear the corneal disease in 4 to 6 weeks. The C57BL/6 mice used in our study are "susceptible," i.e., their corneas do not clear. "Resistant" and "susceptible" mice have recently been shown to have different immunological responses to *P. aeruginosa* corneal infection (12).

***pilU* and *pilT* are involved in *P. aeruginosa* colonization of the cornea in vivo.** To begin to determine the mechanism by which twitching motility might contribute to corneal infection, we examined the effects of the various mutations on the ability of *P. aeruginosa* to colonize the cornea. Corneas of C57BL/6 mice were infected with mutant or wild-type *P. aeruginosa* as previously described, and the number of bacteria colonizing the infected corneas was determined by viable counts of homogenized eyes at 4 (Table 5) and 48 (Table 6) h postinfection. Data were expressed as median values with lower and upper quartiles. The *pilA* mutant showed a marked decrease in its ability to colonize the cornea 4 h after inoculation compared to wild-type PAK ($P = 0.009$, Mann-Whitney test, Table 5). Despite their ability to adhere to and invade corneal epithelial cells in vitro, in vivo colonization levels of the twitching motility mutants (*pilU* and *pilT*) were also reduced compared to that of the wild type ($P = 0.01$, Mann-Whitney test). Both twitching motility mutants colonized significantly more effectively than the *pilA* mutant did ($P = 0.009$, Mann-Whitney test). Thus, twitching motility-dependent and -independent

TABLE 4. Comparison of *P. aeruginosa* infection course in mouse eyes challenged with wild-type PAK, nonpiliated PAK*pilA*::Tc^r, and the twitching motility mutants PAK*pilT*::Tn*5* and PAK*pilU*::Tn*5*

Strain	Infecting dose (CFU)	Ocular scores[a]			
		Day 1	Day 2	Day 4	Day 7
Wild-type PAK	1.15×10^7	3, 3, 3, 3, 3	3, 3, 3, 3, 3	3, 3, 3, 4, 4	3, 3, 3, 4, 4
PAK*pilA*::Tc^r	1.35×10^7	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 1
PAK*pilT*::Tn*5*	1.02×10^7	0, 0, 1, 1, 1	1, 1, 1, 1, 1	0, 1, 1, 1, 1	0, 1, 1, 1, 1
PAK*pilU*::Tn*5*	1.27×10^7	0, 1, 1, 1, 2	1, 1, 1, 2, 2	1, 1, 1, 1, 2	1, 1, 1, 1, 2

[a] Ocular grading scores are defined in the text.

TABLE 6. Comparison of colonization by wild-type PAK, nonpiliated PAK*pilA*::Tc^r, and the twitching motility mutants PAK*pilT*::Tn*5* and PAK*pilU*::Tn*5* in mouse eyes at 48 h postinoculation

Strain	Infecting dose (CFU)	Median final viable count (CFU/ml) (lower quartile–upper quartile)
Wild-type PAK	6.7×10^7	700 (1.15×10^2–9.5×10^5)
PAK*pilA*::Tc^r	7.0×10^7	10 (10–35)
PAK*pilT*::Tn*5*	7.6×10^7	0 (0–0)
PAK*pilU*::Tn*5*	7.5×10^7	0 (0–0)

mechanisms contributed to the involvement of pili in early corneal colonization by *P. aeruginosa*.

The 48-h colonization results (Table 6) showed a bigger defect in colonization than at 4 h for the twitching mutants ($P < 0.02$ for all mutants compared to wild type, Mann-Whitney test). At this later time point, there was no longer a significant difference between either of the twitching motility mutants and the *pilA* mutant, showing that without twitching motility other roles of pili were no longer advantageous for colonization.

Both the in vitro and the in vivo results presented in this study suggested that the role of twitching motility in corneal virulence might be most important after early epithelial interactions have already occurred. This could involve twitching motility-dependent epithelial cell killing (6). However, since the invasive strain PAK does not encode ExoU, which cytotoxic *P. aeruginosa* strains use to kill epithelial cells, the mechanism for twitching motility-dependent cell killing by invasive strains is yet to be elucidated. Other possible roles for twitching motility in corneal virulence could relate directly to this method of locomotion. For example, twitching motility could allow bacteria to more easily assess nutrients, as social gliding motility does for *Myxococcus xanthus* (25). Or it might allow bacteria to travel to sites where they are safer. Otherwise, the role of twitching motility could be indirect. For example, twitching motility has been shown to be important in biofilm formation, which supports tissue colonization and also protects bacteria against killing (18).

It has been reported that the virulence of *P. aeruginosa* in immunosuppressed mice depends on the adherence capability of the strain for epithelial cells (11). However, the results presented in this and other studies (30) suggest that the relationship between *P. aeruginosa* adherence and virulence in the cornea is not straightforward.

In summary, the data presented in this report suggest that pili play at least two roles in *P. aeruginosa* pathogenesis. The first role is independent of twitching motility function and contributes to early interactions with corneal epithelial cells. In vivo, that role translated to a significant difference in 4-h corneal colonization levels between twitching motility and *pilA* mutants. A second role for pili was found to be dependent on their twitching motility function and was critical to corneal disease pathogenesis in these mice, but it was not involved in initial adherence to cells or in cellular invasion. Studies aimed at developing a better understanding of how twitching motility contributes to *P. aeruginosa* disease pathogenesis may yield important new insights into *P. aeruginosa* virulence strategies.

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