JOURNAL OF BACTERIOLOGY, Apr. 2003, p. 2374–2378 Vol. 185, No. 7
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.7.2374–2378.2003
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Biofilm Formation by Hyperpiliated Mutants of *Pseudomonas aeruginosa*

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Received 4 October 2002/Accepted 19 December 2002

Under static growth conditions, hyperpiliated, nontwitching *pilT* and *pilU* mutants of *Pseudomonas aeruginosa* formed dense biofilms, showing that adhesion, not twitching motility, is necessary for biofilm initiation. Under flow conditions, the *pilT* mutant formed mushroom-like structures larger than those of the wild type but the *pilU* mutant was defective in biofilm formation. Therefore, twitching motility affects the development of biofilm structure, possibly through modulation of detachment.

Type IV pili (TFP) are the major virulence-associated adhesins of *Pseudomonas aeruginosa* (8). TFP mediate binding to host cells and generate a form of surface translocation called twitching motility (15). *P. aeruginosa* is a model organism for the study of biofilms, attached communities of microorganisms that are medically significant due to their increased tolerance of antimicrobials (3). Recent studies have given contrasting evidence on the role of twitching motility in microcolony formation and biofilm differentiation. O'Toole and Kolter (12) showed that *pilB*, *pilC*, and *pilY1* mutants, which have been found by electron microscopy to lack TFP, did not form microcolonies, a deficit attributed to the loss of twitching-mediated cell aggregation in early (8-h) biofilms. Other adhesins (*cupA*) can promote biofilm formation under static growth conditions in the absence of TFP, suggesting that neither TFP-mediated adhesion nor motility is absolutely necessary (18). Heydorn et al. (9) showed that a TFP-dependent chemosensory mutant (Δ*pilHIJK*), which has aberrant twitching motility, formed dense microcolonies in a mature (98-h) biofilm. *P. aeruginosa* variants with enhanced biofilm-forming abilities have been noted to have a hyperpiliated phenotype (5), but the specific TFP genes involved were not identified. These hyperpiliated and hyperadhesive variants had a small rough colony phenotype similar to that recently described for drug-resistant *P. aeruginosa* variants with enhanced biofilm-forming ability isolated from cystic fibrosis patients undergoing antibiotic therapy (6).

In *P. aeruginosa*, both PilT and its homologue PilU are required for twitching motility (21, 22). PilT is thought to be a pilin depolymerase that disassembles TFP into pilin subunits, resulting in filament retraction (11), while the function of PilU is currently unknown. Mutations in either *pilT* or *pilU* result in a nontwitching, hyperpiliated phenotype (22). In this study, we used *pilT* and *pilU* mutants to examine the role of twitching motility in biofilm initiation and development under both static and flowing growth conditions.

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Adhesion, not twitching motility, is important for biofilm formation. Strains used in this study are listed in Table 1. pUCP20Gm was constructed by linearizing pUCP20 (10) at a unique *Sca*I site within the *bla* gene, followed by insertion of a gentamicin resistance cassette released from pUCGm (14) with *Sma*I. Primers *pilT*up (5′-GGGATCCGCTCATCCGGT GTTTTCCTT-3′) and *pilT*down (5′-GGGAAGCTTCTTGAA TCCTAGACGCAGTT-3′), based on the *Pseudomonas* genome sequence (17), were used to amplify *pilT* from strain PAK. The amplification product was digested with *Bam*HI and *Hin*dIII and ligated into the multiple cloning site (MCS) in pUCP20Gm to form pUCP20Gm-*pilT*. The construct was introduced into strain R364 by electroporation and selected on

TABLE 1. Strains and vectors used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
PAK	Wild type; piliated; twitching	7
PAK-NP	Tet^r cassette inserted into *pilA*; nonpiliated; nontwitching	19
R364	Tn*5*-B21 insertion in *pilT*; hyperpiliated; nontwitching	21
S34	Tn*5*-B21 insertion in *pilU*; hyperpiliated; nontwitching	22
R364+	R364 complemented with pUCP20Gm-*pilT*; twitching	This study
S34+	S34 complemented with pUCP20Gm-*pilU*; twitching	This study
Plasmids		
pUCP20	Shuttle vector encoding beta-lactam resistance	20
pUCGm	Source of gentamicin resistance cassette	14
pUCP20Gm	pUCP20 with *Sma*I-flanked Gm cassette inserted into unique *Sca*I site within *bla*	This study
pUCP20Gm-*pilT*	pUCP20Gm with *pilT* cloned into *Bam*HI-*Hin*dIII in MCS	This study
pUCP20Gm-*pilU*	pUCP20Gm with *pilU* cloned into *Hin*dIII in MCS	This study

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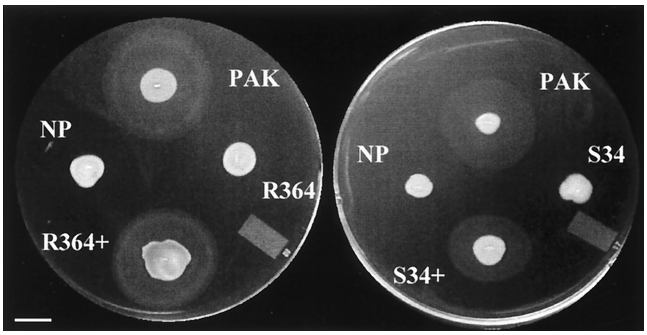

FIG. 1. Complementation of twitching motility in *pilT* and *pilU* mutants. Thin (3-mm) Luria-Bertani 1% agar plates were stab inoculated with a toothpick and incubated at 37°C for 48 h (15). Twitching motility is visualized as a halo between the agar and plastic plates, surrounding the point of inoculation. Both twitching and nontwitching strains form colonies on the agar surface. Scale bar, 10 mm. NP, mutant PAK-NP.

Luria-Bertani agar containing 200 μg of gentamicin/ml. Similarly, *pilU* was amplified with primers *pilU*up (5′-AAAAAGCTTCCCGAATCCCGGAACTGCGT-3′) and *pilU*down (5′-AAAAAGCTTCAGCACCCTGCAACTGGAAA-3′). The *pilU* amplicon was digested with *Hin*dIII, followed by ligation into the corresponding MCS in pUCP20Gm to construct pUCP20Gm-*pilU*.

The piliation and twitching motilities of all strains were examined by using transmission electron microscopy (TEM) and subsurface twitching motility assays (15), respectively. The wild-type PAK strain and complemented mutants R364+ and S34+ were positive for twitching motility, while the structural mutant PAK-NP and the functional mutants R364 and S34 exhibited no twitching zone (Fig. 1). TEM analysis showed that PAK-NP was nonpiliated but that R364 (Fig. 2) and S34 were hyperpiliated. The complemented mutants R364+ (Fig. 2) and S34+ were similar to the wild type.

Biofilm formation assays were conducted as described by O'Toole and Kolter (12), with modifications. Overnight cultures in Davis minimal media (Difco) supplemented with 0.1% glucose (DMM+) were washed and standardized to an optical density at 600 nm (OD_{600}) of 0.05 (~10^7 CFU/ml). Ten microliters of standardized culture was added to 190 μl of fresh medium in each of 10 wells, and the wells were incubated for 24 h at room temperature (RT). Planktonic cells were transferred to a new plate for measurement of the OD_{600} prior to rinsing and staining of biofilms with crystal violet.

As reported previously (12, 18), the lack of TFP (a characteristic of mutant PAK-NP) resulted in less biofilm formation than that of the wild type but the lack of twitching motility in the presence of hyperpiliation (a characteristic of mutants R364 and S34) resulted in the establishment of dense biofilms (Fig. 3). The growth rates of R364 and S34 were similar to that of the wild type (data not shown), showing that increased biofilm formation was not due to increased growth. The observed increase in biofilm formation by *pilT* and *pilU* mutants is consistent with the phenotypes reported for *P. aeruginosa* variants with increased biofilm-forming ability (5, 6), although such strains have not been demonstrated to have altered expression of *pilT* or *pilU*. The complemented strains R364+ and

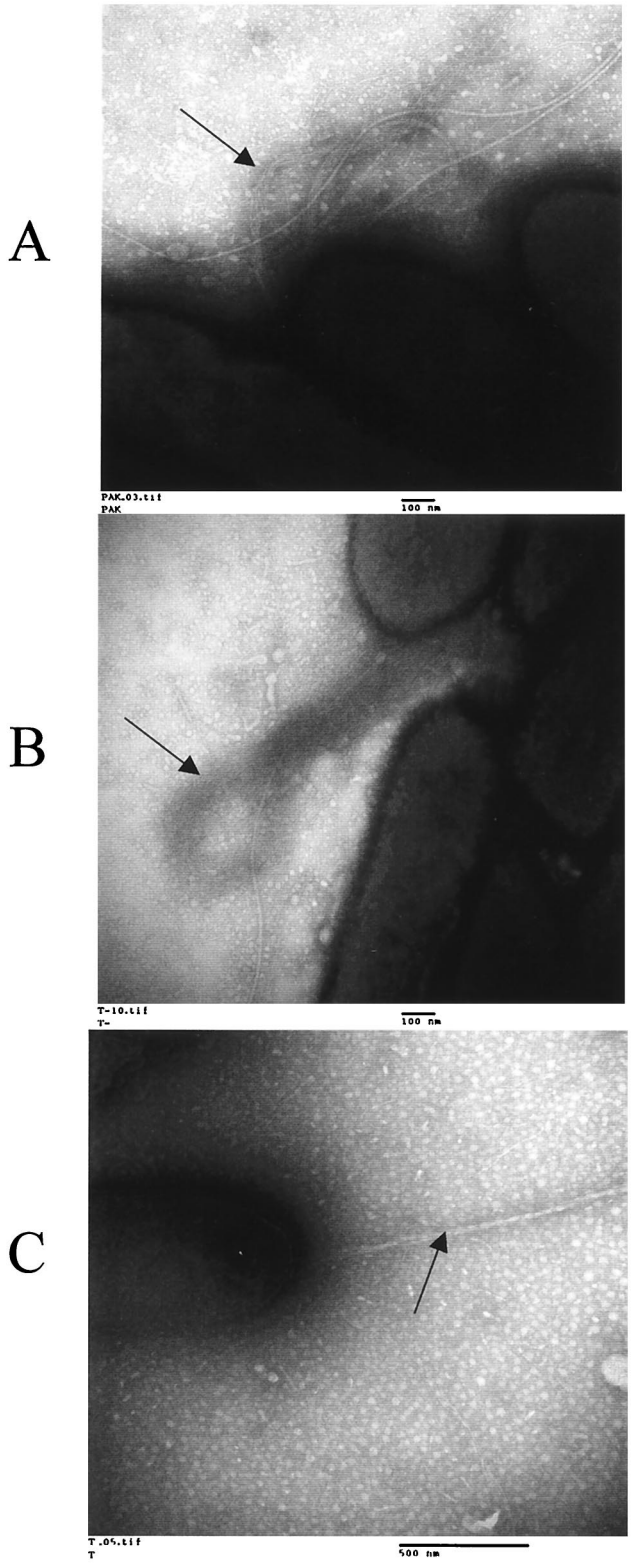

FIG. 2. Transmission electron micrographs of negatively stained (phosphotungstic acid) PAK and the *pilT* and complemented *pilT* mutants. (A) Wild-type PAK strain; (B) *pilT* mutant R364; (C) complemented *pilT* mutant R364+. Images were acquired with a JEM 1230 electron microscope (JEOL, Peabody, Mass.) equipped with a charge-coupled device camera (Amount, Denver, Mass.). The arrows indicate pili. Size bars are shown on the bottom of each panel.

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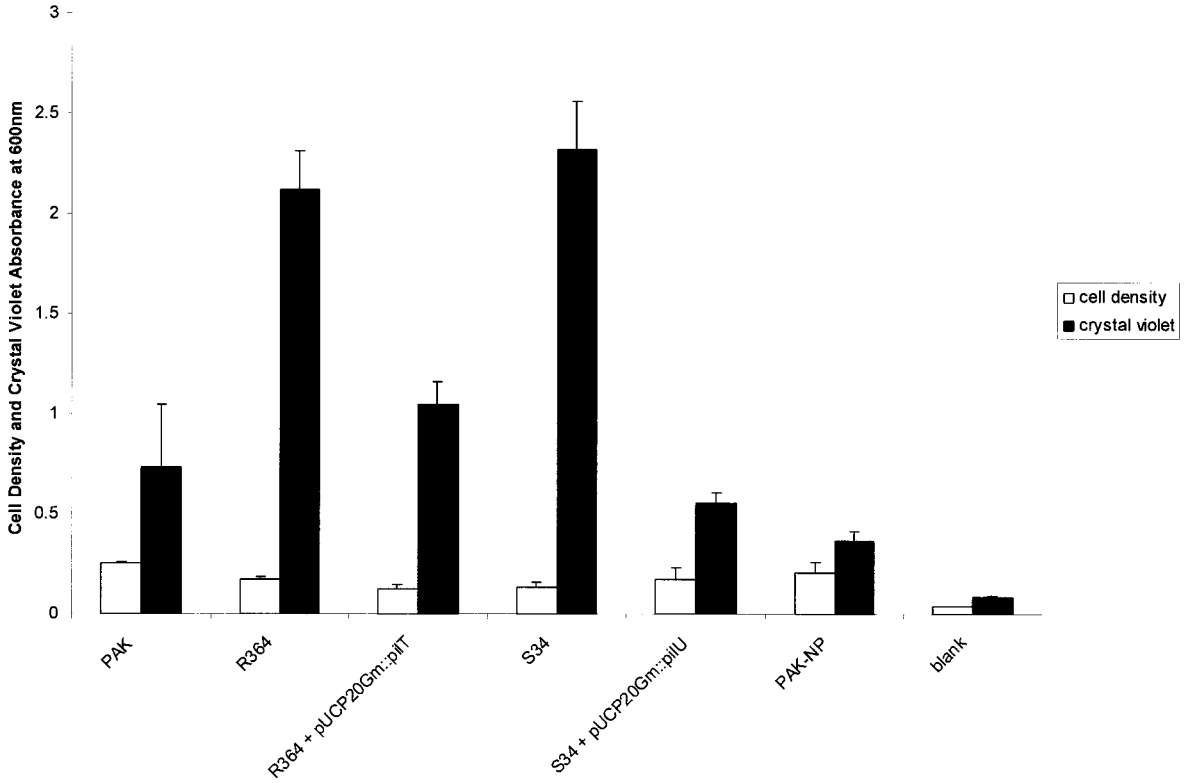

FIG. 3. Biofilm formation assay under static conditions. Standardized cultures were inoculated into DMM+ in microtiter plates and incubated at RT for 24 h. The OD_{600} of the planktonic culture in each well was measured to control for growth inconsistencies. The level of biofilm formation was quantitated indirectly by crystal violet staining (12). The results shown are the averages of results from five separate experiments.

S34+ formed levels of biofilm slightly less than or comparable to that of the wild type. Singh et al. (16) recently reported that sequestration of iron by lactoferrin increased the twitching motility of *P. aeruginosa*, with concomitant reduction in microcolony formation and subsequent biofilm formation. Taken together, these findings indicate that TFP may promote the initiation of biofilm formation through cell-substratum and cell-cell adhesion rather than twitching. This finding is in contrast to that of the contribution of flagella to biofilm formation, since the absence of flagellar motor activity, even in the presence of intact flagella, reduces biofilm formation (18).

Twitching motility is involved in generating normal biofilm morphology. Confocal scanning laser microscopy (CSLM) was used to examine biofilm formation by hyperpiliated mutants under flow conditions. Glass flow cells (Stovall) were modified by splicing a rubber injection port (Continu-Flo; Baxter) into the tubing immediately upstream of the flow cell. One milliliter of washed, standardized culture (10^7 CFU/ml) was injected into the flow cell, which was incubated without irrigation for 1 h at RT to allow attachment. Flow cells were irrigated at 30 ml/h with DMM+ at RT for 24 to 96 h by using peristaltic pumps (Cole Parmer). Biofilms were stained with BacLight Live/Dead stain (Molecular Probes) as suggested by the manufacturer and imaged with a Zeiss CSLM and LSM510 software (Zeiss).

CSLM imaging of 24-h biofilms revealed that PAK-NP did not adhere to glass under our experimental conditions (data not shown) but that the hyperpiliated R364 (*pilT*) mutant formed a dense cell mat (Fig. 4). TFP are thus the major adhesins for initial attachment to glass surfaces under flow conditions. At 24 h, R364+ exhibited the beginnings of three-dimensional heterogeneity, but R364 was still relatively undifferentiated (Fig. 4). After 96 h, R364 formed larger, more numerous, and more tightly packed three-dimensional mushroom-like structures than either the wild-type PAK strain or R364+, which resembled the wild type (Fig. 4). These results demonstrate that twitching motility mediated by PilT is necessary for the establishment of normal biofilm morphology and that in its absence there is an increase in the accumulation of cell mass. Our data indicate that twitching-mediated cell dis-

FIG. 4. Confocal scanning laser micrographs of *P. aeruginosa* biofilms grown under flow conditions. Biofilms were stained with BacLight Live/Dead stain. Sagittal (X-Z) sections through the biofilms are presented at the top and right of each image, with arrows showing the image planes. Biofilm development at 24 h is shown for the wild type (A), R364 (C), R364+ (E), and S34 (G). Biofilm morphology at 96 h is shown for PAK (B), R364 (D), R364+ (F), and S34 (H). S34+ biofilms (not shown) resembled those of R364+. PAK-NP did not adhere to the glass (data not shown). The results shown are representative of results from three separate experiments. Scale bar, 100 μm.

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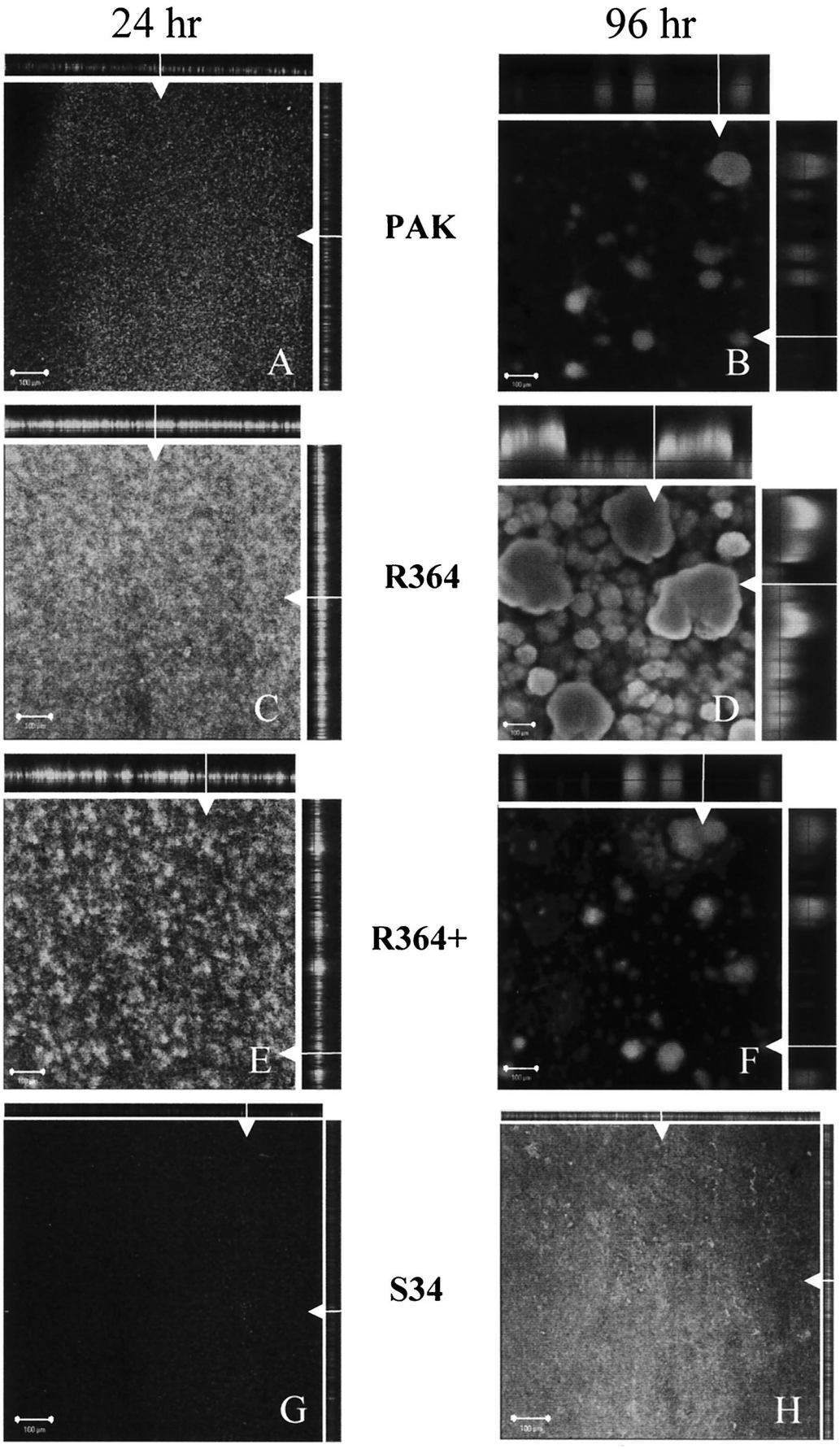

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persal may be involved in maintaining the structure of a wild-type biofilm. Quorum sensing has also been implicated in biofilm differentiation in *P. aeruginosa* (4), but this effect is separate from that of twitching motility since the systems were shown recently to be unlinked (1). In addition, mutations in the TFP-dependent chemosensory system which result in aberrant twitching motility were reported to result in the development of large, well-defined, mushroom-like structures after 98 h, in contrast to the more dense and uniform biofilms formed in that system by the wild-type strain (PAO1) (9). Twitching motility appeared to be important for the colonization of the entire substratum, and without it, similar to our results with PAK, there was exaggerated localized development of oversized microcolonies.

The hyperpiliated *pilU* mutant S34 is weakly adherent to glass under flow conditions. Unexpectedly, the hyperpiliated *pilU* (S34) mutant did not form dense biofilms like R364 under flow conditions. Compared to PAK, S34 adhered poorly to glass (Fig. 4) and did not form typical biofilm structures, even after 96 h. The complemented mutant, S34+, adhered slightly better at 24 h but was still less adherent than the wild type (data not shown). These results are consistent with those observed in the assay with static conditions (Fig. 3). After 96 h of incubation under flow conditions, S34+ formed biofilm structures resembling those of the complemented *pilT* mutant and the wild type (data not shown). Using TEM analysis, Whitchurch and Mattick (22) observed that the TFP of another *pilU* mutant, S40, appeared fragile and often presented as clumps separated from the cell. We saw similar amounts of pili detached from the wild-type and mutant strains (data not shown); however, it remains possible that the integrity of the TFP in S34 is compromised in some manner that could result in the poor adherence of *pilU* mutants when subjected to shearing forces under flow conditions.

Other differences between *pilT* and *pilU* mutants have been reported, suggesting that the two gene products have separate functions despite their homology to one another. *pilT* and *pilU* mutants have been shown to exhibit differences in sensitivity to pilus-specific bacteriophages (22). In addition, *pilT* mutants were less adherent to epithelial cells than *pilU* mutants in two of three mammalian cell lines tested (2). Recent studies of PilU in *Neisseria* suggest that PilT and PilU may interact in a complex to provide normal pilus function (13). The differences between the phenotypes of R364 and S34 may be due to differences in pilus strength or integrity. Biochemical and biophysical analyses of pili from the *pilU* mutant will be the focus of future studies to shed light on the function of PilU in twitching motility.

In summary, we have shown that a lack of twitching motility does not preclude biofilm formation but that it appears to be important for the normal course of biofilm development, which includes cycles of attachment, microcolony formation and maturation, and cell dispersal. In the absence of twitching motility, biofilm structure is more dense, suggesting that it plays a key role in the latter step of biofilm development.

We are grateful to J. Boyd for providing bacterial strains. We thank C. Gallant for construction of pUCP20Gm and its derivatives and Y. M. Heng and M. Woodside for assistance with TEM and CSLM, respectively.

This work was supported by Canadian Institutes of Health Research grant no. MOP-49577 to L.L.B. P.C. is the recipient of a studentship from the Canadian Cystic Fibrosis Foundation.

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