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Role of Cyclic Di-GMP and Exopolysaccharide in Type IV Pilus Dynamics

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ABSTRACT For *Pseudomonas aeruginosa*, levels of cyclic di-GMP (c-di-GMP) govern the transition from the planktonic state to biofilm formation. Type IV pili (T4P) are crucial determinants of biofilm structure and dynamics, but it is unknown how levels of c-di-GMP affect pilus dynamics. Here, we scrutinized how c-di-GMP affects molecular motor properties and adhesive behavior of T4P. By means of retraction, T4P generated forces of ~30 pN. Deletion mutants in the proteins with known roles in biofilm formation, swarming motility, and exopolysaccharide (EPS) production (specifically, the diguanylate cyclases *sadC* and *roeA* or the c-di-GMP phosphodiesterase *bifA*) showed only modest effects on velocity or force of T4P retraction. At high levels of c-di-GMP, the production of exopolysaccharides, particularly of Pel, is upregulated. We found that Pel production strongly enhances T4P-mediated surface adhesion of *P. aeruginosa*, suggesting that T4P-matrix interactions may be involved in biofilm formation by *P. aeruginosa*. Finally, our data support the previously proposed model of slingshot-like “twitching” motility of *P. aeruginosa*.

IMPORTANCE Type IV pili (T4P) play various important roles in the transition of bacteria from the planktonic state to the biofilm state, including surface attachment and surface sensing. Here, we investigate adhesion, dynamics, and force generation of T4P after bacteria engage a surface. Our studies showed that two critical components of biofilm formation by *Pseudomonas aeruginosa*, T4P and exopolysaccharides, contribute to enhanced T4P-mediated force generation by attached bacteria. These data indicate a crucial role for the coordinated impact of multiple biofilm-promoting factors during the early stages of attachment to a surface. Our data are also consistent with a previous model explaining why pilus-mediated motility in *P. aeruginosa* results in characteristic “twitching” behavior.

KEYWORDS *Pseudomonas aeruginosa*, pili, biofilm, cyclic di-GMP, exopolysaccharide, molecular motor

Type IV pili (T4P) are crucial factors in bacterial biofilm formation, as they mediate initial surface attachment and govern the structure of biofilms (1). For *Pseudomonas aeruginosa*, a role for T4P as surface sensors is emerging (2); in particular, active T4P retraction has been proposed to be required (3, 4). It is currently unclear, however, how T4P dynamics affect the earliest stages of biofilm formation.

Bacterial T4P are extracellular polymeric cell appendages required for initial attachment to biotic and abiotic surfaces, host cell signaling, microcolony formation, and natural transformation. The polymer incorporates major pilins (PilA) and a variety of minor pilins (5). The pilus polymer is anchored within the cell envelope by a membrane-spanning complex (6). Importantly, the length of the T4P is dynamic; elongation of T4P requires a cytoplasmic ATPase formed by PilB, and retraction requires its antagonist PilT (7). For *Neisseria gonorrhoeae* and *Myxococcus xanthus*, retraction of T4P generates a

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high mechanical force exceeding 100 pN (8, 9). Considering that T4P-related surface motility (also known as twitching motility) requires force generation (10, 11) and that *P. aeruginosa* is capable of twitching motility (12), it is reasonable to assume that the T4P of *P. aeruginosa* generate mechanical force by retraction. However, the motor properties in the species have not been addressed.

For *P. aeruginosa*, the current understanding of transition from the planktonic lifestyle to life within biofilms involves surface sensing and subsequent upregulation of second messengers (2). When grown in liquid culture, the T4P level is low compared to that of its growth on solid surfaces (13–15). The T4P participate in an uncharacterized surface engagement process that upregulates cyclic AMP (cAMP) in response to surface contact through the Pil-Chp chemosensory system (3, 4, 16, 17). As a consequence, the T4P-associated protein PilY1 is upregulated, leading to a subsequent increase in the level of the second messenger cyclic di-GMP (c-di-GMP) (3, 18). C-di-GMP, in turn, mediates the transition to biofilm formation, including downregulation of flagellar machinery and the production of exopolysaccharides (EPS) (19–23). The levels of c-di-GMP depend on diguanylate cyclases and phosphodiesterases. Different diguanylate cyclases govern different functions; for example, RoeA promotes the production of EPS, whereas SadC controls flagellar motility (21). Deletion of either *sadC* or *roeA* reduces the c-di-GMP level approximately 2-fold. The phosphodiesterase BifA modulates Pel production and flagellar motility (23), and deleting the gene that codes for BifA increases the c-di-GMP level approximately 5- to 10-fold. Thus, while there is evidence that T4P retraction induces upregulation of c-di-GMP, the effect of c-di-GMP levels on T4P dynamics remains elusive.

Various roles of EPS in biofilm initiation have been proposed. For *P. aeruginosa* strain PA14, used in this study, Pel is the dominant EPS, while *P. aeruginosa* strain PAO1 produces Psl (24–27). EPS is required for stable attachment (27). Interestingly, EPS secretion favors microcolony formation through a positive feedback loop; *P. aeruginosa* PAO1 cells secrete trails of EPS, and other cells tend to follow these tracks (28), which suggests that EPS enhances surface adhesion (29).

T4P are involved in biofilm initiation through mediating surface attachment, surface motility, and ultimately surface sensing. Here, we address the question of how T4P dynamics evolve once bacteria have committed themselves to a surface. Since motor properties are best analyzed at the single-molecule level, we used mutants with different levels of c-di-GMP and distinct defects in early biofilm formation that resulted from the deletion of two diguanylate cyclases (SadC and RoeA) and a phosphodiesterase (BifA) with known roles in early biofilm formation (21–23). Using laser tweezers to examine these mutant strains, we found no strong dependence of c-di-GMP on T4P velocity and force generation. Finally, our data are consistent with those from a model wherein T4P, together with Pel (an exopolysaccharide controlled by the diguanylate cyclase RoeA), enhance surface adhesion.

RESULTS

***P. aeruginosa* T4P retraction generates force.** Type IV pili can generate considerable mechanical force by retraction (10). For example, the force generated by a single T4P of *Neisseria gonorrhoeae* or *Myxococcus xanthus* amounts to more than 100 pN under normal laboratory conditions (8, 9). Since T4P are responsible for the twitching motility of *P. aeruginosa*, it was conceivable that the T4P of *P. aeruginosa* likewise generate force. Here, we confirmed this assumption. To this end, the *P. aeruginosa* PA14 Δ *flgK* strain was inoculated on glass coverslides, and polystyrene beads were placed close to the bacterial cell poles using laser tweezers. When T4P bind to the bead and retract, they deflect the bead from the center of the trap (Fig. 1a). We used the force clamp mode to measure the velocity of T4P retraction at various forces. In this mode, the force is kept constant through feedback between the deflection of the bead (d) and the optical table (30). We found that at a force (F) of 8 pN, the average (\pm standard error) velocity (v) was as follows: $v(8 \text{ pN}) = 1,187 \text{ nm/s} \pm 13 \text{ nm/s}$ (Fig. 1b). The distribution

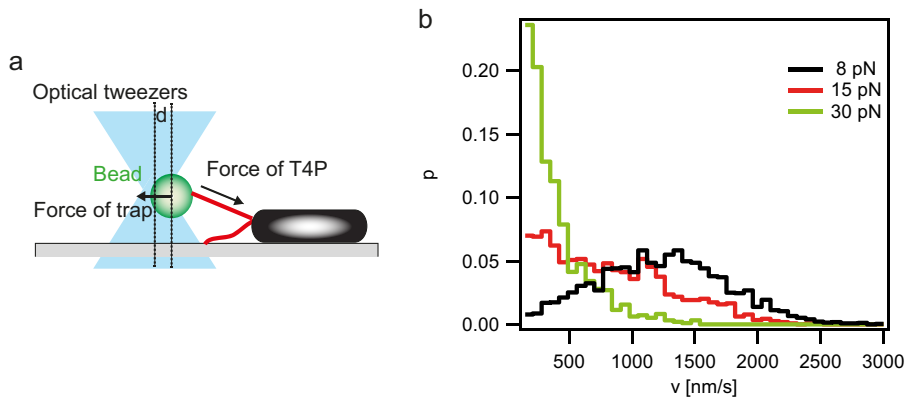


FIG 1 Velocity of *P. aeruginosa* PA14 T4P retraction. (a) Sketch of the experimental setup. (b) Distribution of T4P retraction speeds at clamped forces (F) of 8 pN, 15 pN, and 30 pN (n , >950 retraction intervals of 100 ms for each condition). In these experiments, the *P. aeruginosa* PA14 Δ flgK mutant was used to eliminate any contribution of the flagellum to the adhesion or movement of the cell.

of velocities shifted lower at F of 15 pN, and T4P retraction stalled at F of approximately 30 pN (Fig. 1b).

The position clamp mode of the laser trap was used for characterizing the stalling forces. In this mode, the force acting on the T4P increases linearly with the deflection of the bead (d). Some T4P retraction events showed a single stalling event and subsequent rupture between T4P and the bead (Fig. 2a). In contrast to earlier experiments that characterized T4P retraction in *N. gonorrhoeae* (11), detachment of T4P from

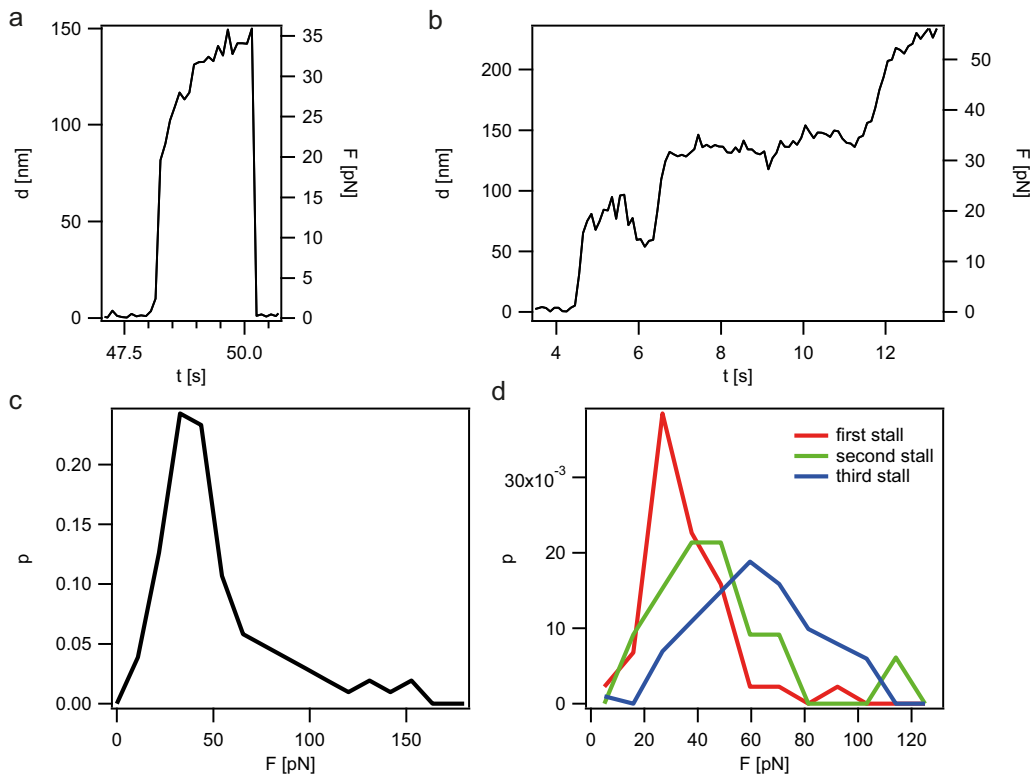


FIG 2 Stalling forces of *P. aeruginosa* PA14 T4P retraction. (a and b) Typical examples for T4P retraction events in position clamp mode. Deflections (d) of the bead from the center of the laser trap (compare with Fig. 1a) and the corresponding forces (F) are shown. (c) Distribution of stalling forces (force at which the velocity was less than 250 nm/s for at least 500 ms) ($n = 103$). (d) Distribution of stalling forces. First, second, and third stalling events of single pilus retractions are plotted ($n > 21$ for each condition). In these experiments, the *P. aeruginosa* PA14 Δ flgK mutant was used to eliminate any contribution of the flagellum to the adhesion or movement of the cell.

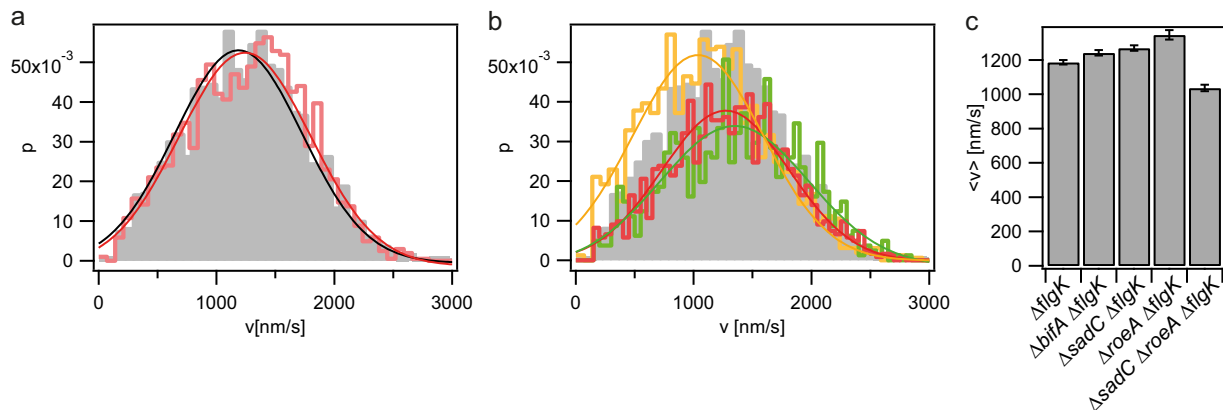


FIG 3 Speed of T4P retraction for diguanylate cyclase and c-di-GMP phosphodiesterase deletion strains. (a) Distribution of T4P retraction speeds for the $\Delta flgK$ parent strain (gray) and the $\Delta bifA \Delta flgK$ mutant (red). (b) Distribution of T4P retraction speeds for the $\Delta flgK$ parent strain (gray) and the $\Delta sadC \Delta flgK$ (red), $\Delta roeA \Delta flgK$ (green), and $\Delta sadC \Delta roeA \Delta flgK$ (orange) mutants. (c) Average T4P retraction speeds of the indicated strains. Error bars, standard errors of the mean of a Gaussian fit (full lines in panels a and b) ($n > 850$ for each condition).

the bead was an unlikely event at forces less than the stalling force. As a consequence, transient pausing was often observed (Fig. 2b); T4P retraction stalled but resumed after a pause. We assume that the resumption of T4P retraction was caused by binding and retraction of a second or multiple T4P. The stalling force was defined as the force at which the velocity (v) was less than 250 nm/s for at least 500 ms; its distribution showed a maximum at F of 37 pN and a pronounced tail (Fig. 2c). Thus, we plotted the stalling forces of the first, second, and third stalling events independently (Fig. 2d). The force distribution of the first stalling event does not show the tail found when all stalling events were pooled. This observation strongly suggests that we observed the stalling force distribution of single T4P retractions. Thus, the average (\pm standard error) stalling force (F_{stall}) of a single T4P retraction was as follows: $F_{stall} = 31 \text{ pN} \pm 1 \text{ pN}$. We conclude that T4P retraction of *P. aeruginosa* generates mechanical force on the order of ~ 30 pN under the laboratory conditions used in this study.

Impact of oxygen availability on T4P function in *P. aeruginosa* PA14. *N. gonorrhoeae* T4P switch their retraction speed from $2 \mu\text{m/s}$ under aerobic conditions to $1 \mu\text{m/s}$ under anaerobic conditions, while *M. xanthus* is not motile under anaerobic conditions (31, 32). We assessed whether T4P retraction was active in *P. aeruginosa* under conditions that mimic low-oxygen environments. The T4P retraction assay was performed under aerobic conditions and in the presence of an oxygen scavenger and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which disrupts the function of the electron transport chain. While 25% of the sampled bacteria actively retracted their pili under aerobic conditions, no T4P retraction activity was observed when the oxygen scavenger or CCCP was added (see Fig. S1 in the supplemental material), indicating that *P. aeruginosa* shows no T4P retraction in microoxic or anoxic growth conditions and/or when electron transport chain function is impaired. In a control experiment, we found that the addition of CCCP did not alter the viability of *P. aeruginosa* over the experimental period (data not shown).

The force-dependent velocity of T4P retraction is only weakly dependent on the presence of the diguanylate cyclases SadC and RoeA and on the phosphodiesterase BifA. We addressed the question of whether the level of c-di-GMP affects velocity or force generation by T4P. To this end, we measured the velocity of T4P retraction at F of 8 pN in mutants with deletions of diguanylate cyclases and a phosphodiesterase that play important roles in the early stages of biofilm formation by *P. aeruginosa*. The measured force distributions were in good agreement with Gaussian distributions. Neither deletion of *sadC* nor deletion of *bifA* resulted in a significant effect on the distribution of T4P retraction velocities (Fig. 3). Deletion of the *roeA* gene resulted in a very modest but significant increase in velocity from that for the wild type (v_{wt}), as follows:

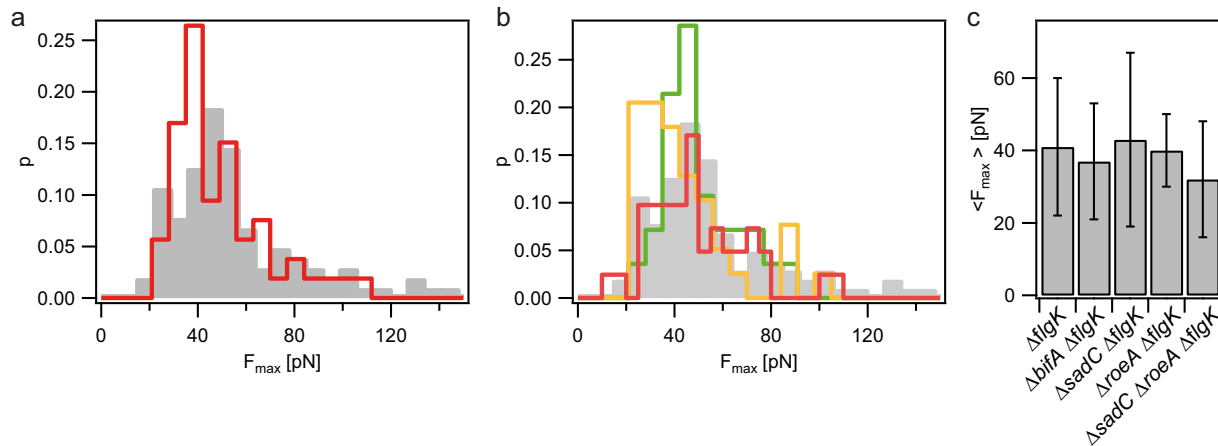


FIG 4 Maximum force of T4P retraction for diguanylate cyclase and c-di-GMP phosphodiesterase deletion strains. (a) Distribution of maximum forces for the $\Delta flgK$ parent strain (gray) and the $\Delta bifA \Delta flgK$ mutant (red). (b) Distribution of maximum force for the $\Delta flgK$ parent strain (gray) and the $\Delta sacC \Delta flgK$ (red), $\Delta roeA \Delta flgK$ (green), and $\Delta sacC \Delta roeA \Delta flgK$ (orange) mutants. (c) Average maximum forces. Error bars, standard deviations. The P value of 0.008, obtained from a Kolmogorov-Smirnov test between the $\Delta flgK$ parent strain and the $\Delta sacC \Delta roeA \Delta flgK$ mutant, indicates a significant difference ($n > 28$ for each condition).

$v_{wt}(8 \text{ pN}) = 1,187 \text{ nm/s} \pm 13 \text{ nm/s}$ to $v_{roeA}(8 \text{ pN}) = 1,346 \text{ nm/s} \pm 28 \text{ nm/s}$, where v_{roeA} is the velocity for the $roeA$ mutant. The double deletion of $sacC$ and $roeA$ reduced the average speed of T4P retraction slightly but significantly: $v_{sacCroeA}(8 \text{ pN}) = 1,037 \text{ nm/s} \pm 19 \text{ nm/s}$, where $v_{sacCroeA}$ is the velocity for the $sacC roeA$ double mutant. Similar to the distribution of stalling forces in the wild-type background, the stalling force distributions of the deletion mutants were non-Gaussian. Deletion of $sacC$, $roeA$, or $bifA$ did not significantly affect the stalling force of T4P retraction, and the $sacC roeA$ strain showed a slightly but significantly reduced stalling force (Fig. 4). In summary, deletion of the diguanylate cyclase genes $sacC$ and $roeA$ or the phosphodiesterase gene $bifA$ showed only minor and nonsystematic effects on the motor properties of T4P, indicating that the motor properties are not systematically dependent on the levels of c-di-GMP.

Coating the surface with the exopolysaccharide PelA enhances T4P-mediated surface interaction. In response to high c-di-GMP levels, *P. aeruginosa* PA14 upregulates production of the Pel exopolysaccharide (EPS) (23, 33, 34). Here, we studied whether the presence of EPS impacts T4P function. Glass coverslips were incubated with a high concentration of *P. aeruginosa* cells for 1 h, allowing these microbes the opportunity to produce surface-associated EPS, as reported (28). Subsequently, most bacteria were rinsed off the surface. From the remaining bacteria, single bacteria were trapped in the laser trap. *P. aeruginosa* assumes an upright position in the laser trap, reminiscent of other rod-shaped bacterial species (35, 36). When T4P retraction is active, the bacteria actively move away from the center of the laser trap (Fig. 5a). While we were unable to determine the force generated by the rod-like bacteria, the probability that the bacteria escape from the trap was determined. In these experiments, increased escape from the trap was associated with increased force generation. An escape event was defined as an event where the distance between the center of the trap and the bacterium was greater than 300 nm.

The probability (P) (\pm standard deviation) of escaping from the trap within 30 s was $47\% \pm 5\%$ for *P. aeruginosa* PA14 after preincubation with the wild-type EPS-producing strain of this microbe (Fig. 5b). The escape probability was not sampled for longer periods of time, because the bacteria tended to lose activity after 30 s of trapping. When the glass coverslide was not preincubated with bacteria (and thus lacked any deposited EPS), the escape probability was significantly reduced to $13\% \pm 5\%$ (Fig. 5b).

The experiments discussed above are consistent with a model wherein, during preincubation, bacteria deposit EPS onto the coverslide; T4P can exert a force that is higher on the EPS-coated surface than on a plain glass surface. To test whether EPS

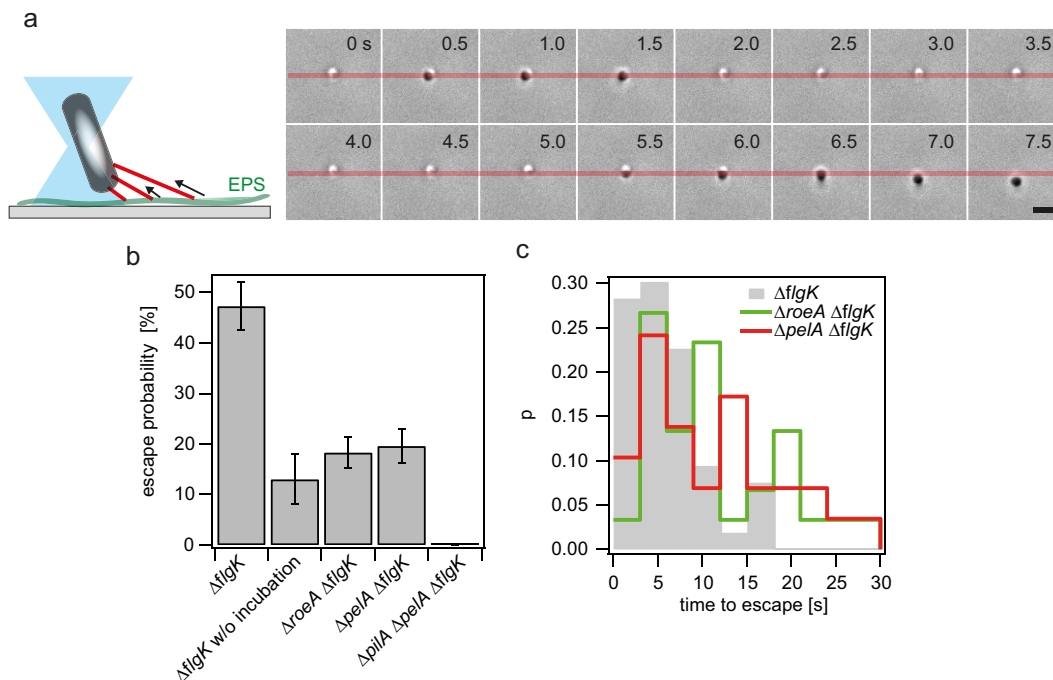


FIG 5 Type IV pili interact with PelA deposited on a surface. (a) Sketch of setup (left) and typical sequence of trapped *P. aeruginosa* (right). Bacteria were incubated on a glass surface for 70 to 80 min. An escape was defined by displacement of the cell body by at least 300 nm from the center of the laser trap (e.g., event starting at 7 s). Bar, 2 μ m. (b) Escape probabilities within 30 s of the $\Delta flgK$, $\Delta roeA \Delta flgK$, $\Delta pelA \Delta flgK$, and $\Delta pelA \Delta pilA \Delta flgK$ mutants. Error bars, standard errors. (c) Distribution of times elapsed before escape for the $\Delta flgK$, $\Delta roeA \Delta flgK$, and $\Delta pelA \Delta flgK$ mutants.

secretion was indeed responsible for the different observed adhesion forces, we repeated the experiment with preincubation of the coverslide using a $\Delta pelA$ mutant strain. PelA is essential for production of the Pel exopolysaccharide (24, 25). We found that the escape probability of the *pelA* mutant was severely reduced to $20\% \pm 3\%$ (Fig. 5b). Furthermore, for the cases where the bacterium escaped, the distribution of escape times was shifted toward considerably higher values (Fig. 5c, red line), indicating that T4P retraction confers a stronger interaction to Pel than to the glass surface.

The diguanylate cyclase RoeA upregulates Pel production (21). Correspondingly, deletion of *roeA* reduces the escape probability to $18\% \pm 3\%$ (Fig. 5b) and shifts the escape time to higher values on par with what was observed for the *pelA* mutant (Fig. 5c). Finally, we confirmed that T4P were responsible for bacterial escape from the laser trap. No escape was observed when the gene encoding the major pilin subunit, *pilA*, was deleted (Fig. 5b). Together, our data indicate that the presence of EPS enhances the adhesion mediated by T4P.

DISCUSSION

Force generation by *P. aeruginosa* T4P. By direct force measurements, we confirmed that T4P in *P. aeruginosa* are force-generating molecular motors. The speed of T4P retraction was previously determined by direct visualization of T4P retraction to be 0.5 μ m/s at 29°C (12). Here, we measured 2-fold-higher velocities at 37°C (a physiologically relevant temperature) in the presence of low external force. With the different temperatures taken into account, the results are in reasonable agreement. Interestingly, the forces generated by T4P retraction in *P. aeruginosa* are considerably lower than those generated in *N. gonorrhoeae* and *M. xanthus*, where forces exceeding 100 pN were observed (9, 11). Replacing the gene encoding major pilin subunit *pilE* in *N. gonorrhoeae* by the major pilin of *P. aeruginosa*, PAK, supported high force generation in the range of 100 pN (37), suggesting that the difference is unrelated to the structure of the major pilin. We note that our experiments were conducted in standard lysogeny

broth (LB) medium. We cannot exclude that other nutritional conditions support higher force generation in *P. aeruginosa*.

The twitching motility phenotypes of *P. aeruginosa* and *N. gonorrhoeae* show remarkable differences (38). The motility of *P. aeruginosa* has been described as slingshot-like, with periods of low velocity (0.3 $\mu\text{m/s}$) lasting for several seconds followed by short translations at velocities of 1 $\mu\text{m/s}$ (39). In contrast, *N. gonorrhoeae* performs a random walk with directional persistence at a speed comparable to that of single T4P retraction (40). Since the motor forces (100 pN) are considerably higher than the detachment forces (on the order of 10 pN), the movement can be explained by a tug-of-war between multiple pili emanating in random directions (11). When the ratio between motor force and detachment force is decreased, trapping is observed for *N. gonorrhoeae* (41). As shown in this report, for *P. aeruginosa*, the motor forces are considerably lower, and therefore the prerequisite for a tug-of-war-like movement (motor forces much larger than detachment force) is most likely not fulfilled. In fact, detachment of T4P from the beads used for probing the motor properties was an unlikely process, indicating that the detachment forces were higher than the motor forces. Therefore, it was impossible to assess the force-dependent detachment force systematically.

The data presented here lead to a better understanding of the molecular mechanisms generating slingshot-like motility of *P. aeruginosa*. During the periods of slow movement, multiple T4P are likely attached and pulling in different directions, generating a force balance and resulting in only slow movement. Eventually, a T4P ruptures and perturbs the balance. As a result, the dominating T4P retracts at its typical speed of $\sim 1 \mu\text{m/s}$, generating a rapid "twitching" movement.

The role of c-di-GMP in motor properties of T4P. The level of c-di-GMP is an important determinant of the transition from the planktonic lifestyle to the biofilm state. For *Escherichia coli*, a direct connection between c-di-GMP levels and the speed of flagellar rotation has been identified through the role of a molecular brake that binds to the flagellar rotor in a c-di-GMP-dependent way (42). For *P. aeruginosa*, it is known that reversal frequencies of flagellar rotation are affected by the c-di-GMP level, but the molecular mechanism has not been identified (22, 23, 43). In terms of T4P, earlier reports showed that the level of piliation changes during biofilm formation through cAMP-dependent upregulation of the pilus machinery (3), but how the c-di-GMP level impacts T4P function had not been explored. Here, we modulated the levels of c-di-GMP by deleting genes that play roles in early biofilm formation and investigated their effect on T4P motor properties. We found no major changes in velocity or force generation when we decreased the production level of c-di-GMP by deleting diguanylate cyclase SadC or RoeA. The velocity was slightly increased (12%) by deleting *roeA* and decreased (by 14%) when *roeA* and *sadC* were deleted. The stalling force was decreased by 22% in a $\Delta roeA \Delta sadC$ strain. While these effects were statistically significant, we consider them modest and unlikely to have a large impact on the function of T4P. Likewise, when the degradation of c-di-GMP was impaired by mutation of the BifA phosphodiesterase, the motor properties of T4P were unaffected. Mutations in *sadC* and *bifA* caused a defect in twitching motility (44). While the motor properties are largely unchanged, as shown in this study, the density of T4P may be affected, causing the defect in twitching motility. However, approximately 30 other proteins in *P. aeruginosa* are involved in the synthesis and/or degradation of c-di-GMP, including some which impact twitching motility (44). It is formally possible that one or more of these proteins impact motor function in a manner analogous to the molecular brake described above (42).

Putative role of EPS in T4P-mediated adhesion. T4P mediate attachment of bacteria to abiotic surfaces (17, 41, 45, 46) and to host cells (17, 47). Moreover, T4P-T4P-mediated attractive forces control the shape, fusion, and sorting in microcolonies formed by *N. gonorrhoeae* (1, 48–51). Here, we provide evidence that the exopolysaccharide Pel enhances the surface adhesion mediated by T4P. The deposition of exopolysaccharides can influence the interaction between *P. aeruginosa* and surfaces in at least two ways. First, it has been reported that Pel is involved in surface attachment of

TABLE 1 Bacterial strains used in this study

| Strain | Relevant genotype ^a | Source or reference |
|---------|---------------------------------------|---------------------|
| SMC5845 | $\Delta flgK$ | 52 |
| SMC6591 | $\Delta sadC \Delta flgK$ | This study |
| SMC6592 | $\Delta roeA \Delta flgK$ | This study |
| SMC6593 | $\Delta sadC \Delta roeA \Delta flgK$ | This study |
| SMC6594 | $\Delta bifA \Delta flgK$ | This study |
| SMC7296 | $\Delta pelA \Delta flgK$ | This study |
| SMC7297 | $\Delta pelA \Delta pilA \Delta flgK$ | This study |

^aAll strains are mutants of *P. aeruginosa* PA14. Mutants bear a deletion of the coding region of the indicated gene(s), and these strains do not carry an antibiotic marker.

P. aeruginosa PAO1 by generating short-ranged attractive forces (52). Thus, the presence of Pel might directly enhance bacterial interaction with a surface. Second, T4P may bind to EPS, thus enhancing the strength of attachment between the T4P and the surface. Consistent with this idea, for *Myxococcus xanthus*, direct interaction between T4P and its secreted slime material has been observed (53).

MATERIALS AND METHODS

Bacterial strains. All strains used in this study were isogenic with the flagellum-defective mutant *P. aeruginosa* PA14 $\Delta flgK$ (Table 1). This mutant was used to eliminate any contribution of the flagellum to adhesion or the other assays performed here. As such, the $\Delta flgK$ mutant served as the positive control in all studies. All the strains were grown on lysogeny broth (LB) at 37°C. All the mutants used in this study contained a deletion of the coding region of the indicated gene, and they carried no antibiotic marker.

Construction of $\Delta flgK$ mutants. In-frame deletion mutants of *P. aeruginosa* were constructed via allelic exchange as previously described (54). DNA fragments were amplified from *P. aeruginosa* PA14 genomic DNA by PCR to generate upstream and downstream fragments of the *flgK* gene with nucleotide tails complementary to plasmid pMQ30. The primer pairs *flgK* KO P1 (tgtaaacgacgcccagtgccaagcttgca tgctgtTCATGAACAGCCAGACCACC) and *flgK* KO P2 (TCTCTCGGTTAAGGCGCTCACATGGGTCAGTTCCTCC TTG) (upstream fragments) and *flgK* KO P3 (CAAGGAGGAACTGACCCATGTGAGCGCCTTAACCGAGAGA) and *flgK* KO P4 (ccatgattacgaattcgagctcggtaccgggggatccACGGTATTGAGCAAGCTGCG) (downstream fragments) were used to generate the PCR fragments. The lowercase letters mark sequences complementary to the cloning vector pMQ30.

PCR products were cloned into pMQ30 by *in vivo* homologous recombination in *Saccharomyces cerevisiae* INVSc1 (Invitrogen) as previously described (54). The pMQ30-*flgK* deletion construct was transformed by electroporation into *E. coli* strain S17 and introduced into *P. aeruginosa* $\Delta sadC$ (strain SMC 4465), $\Delta bifA$ (SMC 3351), $\Delta roeA$ (SMC 3812), $\Delta sadC \Delta roeA$ (SMC 3809), $\Delta pilA$ (SMC 3782), and $\Delta pelA$ (SMC 2893) mutants by conjugation. The $\Delta pilA \Delta flgK \Delta pelA$ mutant was built by introducing the pMQ30-*pelA* knockout construct (SMC 6955) into *P. aeruginosa* $\Delta pilA \Delta flgK$ by conjugation. Integrants were isolated on gentamicin (20 μ g/ml) and nalidixic acid (20 μ g/ml), followed by sucrose counterselection. Resolved integrants were confirmed by PCR and sequencing.

T4P retraction assay. Prior to laser tweezer experiments, bacteria were cultivated on agar plates (BD Bacto Agar; Fisher Scientific) containing LB (lysogeny broth; Roth) at 37°C for at least 8 h and then diluted in LB medium to an optical density (OD) of 0.002. Fifty microliters of the vortexed cell suspension was applied to polystyrene-coated coverslides. The diluted cells were incubated on the coverslides for an additional 30 min at 37°C. Subsequently, the medium was exchanged by a microsphere suspension of 2- μ m carboxylated polystyrene beads (Invitrogen) before the sample was sealed. The observation time was limited to 20 min to avoid potential oxygen depletion.

Single pilus retraction events were measured with optical tweezers in position clamp and force clamp modes (30). In short, in position clamp mode, the T4P pulls the bead out of the center of the laser trap, and the optical restoring force increases linearly with the deflection. This mode is useful for determining the stall force but less convenient for determining speed, since the elastic properties of the T4P are nonlinear. In force clamp mode, software-based feedback clamps the deflection of the bead from the center of the trap at a constant position, thus keeping the force acting on the T4P constant. Pilus retraction events were analyzed in Matlab as previously described (31), but the parameters were adjusted to account for lower retraction velocities and stronger force dependence. In particular, a stalling event was detected when the velocity was lower than 250 nm/s for at least 500 ms. Prior to analysis, the data were down-sampled from 20 kHz to 10 Hz.

Oxygen depletion studies. The effects of depletion of oxygen and proton motive force on T4P dynamics were assessed using the oxygen scavenger system protocatechuic acid (PCA)-protocatechuate-3,4-dioxygenase (PCD) and CCCP (carbonyl cyanide *m*-chlorophenylhydrazine), respectively, as described previously (32). To ensure that CCCP treatment did not affect the survival of *P. aeruginosa*, we treated cells with 50 μ M CCCP for 15 min and subsequently plated them on agar plates. The number of CFU was comparable to the number of untreated cells.

Escape assay. Prior to laser tweezer experiments, bacteria were cultivated overnight on agar plates containing LB at 37°C. Subsequently, bacteria were suspended in LB medium to an OD of 0.2 and

incubated on coverslips for 70 to 80 min to ensure the secretion of exopolysaccharides onto the surface of the cover slides. Subsequently, the cells sitting on the cover slides were washed repeatedly (10 to 20 times) using a pipette and fresh medium to ensure cell densities feasible for the experimental procedure.

The characterization of bacterium-surface interaction followed a previously established protocol for *N. gonorrhoeae* (48, 49). While for *N. gonorrhoeae* it was possible to select for spherical monococci and thus measure the escape force (49), *P. aeruginosa* bacteria are rod shaped and thus align vertically within the laser trap. As their T4P reside at the cell pole, retraction causes tilting of the bacterial body with respect to the major axis of the laser trap; therefore, quantification of escape forces was not possible. Instead, we characterized the frequencies at which *P. aeruginosa* escaped for the laser trap. In short, bacteria moving close to the surface were trapped in the optical trap and placed 2 to 3 μm above the surface. A single bacterium was sampled for 30 s, since photodamage became obvious at longer periods of time. Time-lapse movies were recorded at 0.1 Hz. An escape event was defined as a displacement (d) of >300 nm from the center of the laser trap.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00859-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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