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MEETING PRESENTATION

Motor Properties of PilT-Independent Type 4 Pilus Retraction in Gonococci

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ABSTRACT Bacterial type 4 pili (T4P) belong to the strongest molecular machines. The gonococcal T4P retraction ATPase PilT supports forces exceeding 100 pN during T4P retraction. Here, we address the question of whether gonococcal T4P retract in the absence of PilT. We show that *pilT* deletion strains indeed retract their T4P, but the maximum force is reduced to 5 pN. Similarly, the speed of T4P retraction is lower by orders of magnitude compared to that of T4P retraction driven by PilT. Deleting the *pilT* paralogue *pilT2* further reduces the speed of T4P retraction, yet T4P retraction is detectable in the absence of all three *pilT* paralogues. Furthermore, we show that depletion of proton motive force (PMF) slows but does not inhibit *pilT*-independent T4P retraction. We conclude that the retraction ATPase is not essential for gonococcal T4P retraction. However, the force generated in the absence of PilT is too low to support important functions of T4P, including twitching motility, fluidization of colonies, and induction of host cell response.

IMPORTANCE Bacterial type 4 pili (T4P) have been termed the "Swiss Army knives" of bacteria because they perform numerous functions, including host cell interaction, twitching motility, colony formation, DNA uptake, protein secretion, and surface sensing. The pilus fiber continuously elongates or retracts, and these dynamics are functionally important. Curiously, only a subset of T4P systems employ T4P retraction ATPases to power T4P retraction. Here, we show that one of the strongest T4P machines, the gonococcal T4P, retracts without a retraction ATPase. Biophysical characterization reveals strongly reduced force and speed compared to retraction with ATPase. We propose that bacteria encode retraction ATPases when T4P have to generate high-force-supporting functions like twitching motility, triggering host cell response, or fluidizing colonies.

KEYWORDS *Neisseria gonorrhoeae*, molecular motor, pilus, twitching motility

Bacterial type 4 pili (T4P) are among the strongest molecular machines known to date. In some species, they generate forces exceeding 100 pN (1–3), i.e., 20-fold higher than the force generated by muscle myosin. Force generation has been linked to diverse functions, including twitching motility (4–7), host cell interaction (8–11), and regulation of biofilm structure and dynamics (12–17). For all of these functions, the retraction ATPase PilT is required. Interestingly, some T4P systems involved in protein secretion, DNA uptake during transformation, or surface sensing bear no *pilT*-like gene. Very recently, it has been shown that T4P can retract in the absence of a retraction ATPase (18–20). The forces generated by these pili, however, are an order of magnitude lower than the force observed for *Neisseria gonorrhoeae* T4P retraction. It remains unclear whether gonococci can retract T4P in the absence of PilT.

The T4P filament is a helical structure built from thousands of major pilin subunits and various minor pilins (21, 22). Recent advances in cryo-electron microscopy together with high-resolution structures of the individual components have given insight into the structure of the complex machinery that shuttles pilin subunits from the cytoplas-

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mic membrane into the growing pilus (23–27) (see Fig. S1 in the supplemental material). Two ATPases power elongation (PilF) (28) and retraction (PilT) (29) of the pilus, respectively. Biophysical, electron microscopy, and crystallographic studies suggest that these motors form oblong hexameric rings (30, 31). It was proposed that sequential ATP binding leads to functionally relevant deformations that propagate around the ring in opposite directions for the elongation and retraction ATPase (24). These sequential deformations of the ATPases would couple to the pilus fiber through a platform complex. (23, 24). Since the T4P fiber is helical, opposite rotations driven by PilF and PilT would then power elongation and retraction of the T4P fiber, respectively. The exact coupling mechanism remains unclear.

While all bacteria generating T4P encode elongation ATPases, not all encode retraction ATPases. For example, DNA uptake during transformation has been reported to require a retraction ATPase in *N. gonorrhoeae* and *Vibrio cholerae* (29, 32). However, *Bacillus subtilis* and *Streptococcus pneumoniae* do not carry a clear *pilT* homologue, but they still employ T4P for DNA uptake (33). The first T4P system shown to retract T4P in the absence of a retraction ATPase was the toxin-coregulated pilus of *V. cholerae* (18). The maximum force generated by these T4P was in the range of 4 pN. Furthermore, the Tad pilus of *Caulobacter crescentus* generates somewhat higher force, in the range of 12 pN (19). We note that it is unknown how force generation depends on experimental conditions. For two T4P systems that naturally encode a retraction ATPase, force generation was observed when *pilT* was deleted. First, in *Myxococcus xanthus,* deletion of *pilT* leads to strong reduction of T4P retraction frequency and almost complete loss of twitching motility (2). The force was reduced 2-fold. *M. xanthus* carries four *pilT* paralogues, and it is therefore unclear whether any of them encodes a functional retraction ATPase. Second, the competence pilus of *V. cholerae* showed T4P retraction when *pilT* was deleted (20), while the transformation rate was severely reduced. Forces generated by the competence pilus were on the lower side (8 pN), even in the presence of functional PilT, and dropped by 2-fold in a *pilT* deletion strain. Therefore, it was interesting to find out how severely deletion of *pilT* and its paralogues affected force generation in *N. gonorrhoeae*, one of the strongest T4P machines.

Here, we set out to address this question by characterizing velocity and force generation in gonococcal *pilT* deletion strains. We show that gonococcal T4P indeed retract in the absence of *pilT* and its paralogues. Interestingly, both force and speed of PilT-independent T4P retraction are lower by orders of magnitude compared to retraction in wild-type (wt) T4P, explaining why T4P retraction has been overlooked so far. We investigate putative energy sources of PilT-independent T4P retraction by characterizing the effects of proton motive force (PMF).

RESULTS

T4P retract at low speed in a *pilT* deletion mutant. Deleting the gene encoding the T4P retraction ATPase PilT was long believed to be in accord with generating strains that are incapable of T4P retraction (4). However, recent experiments reported T4P retraction in the absence of *pilT* or homologues (20). We set out to investigate whether T4P retraction occurred in gonococcal *pilT* deletion strains. We used a laser tweezers assay to probe for T4P retraction (Fig. 1a). A bacterium was immobilized with a glass cover slide. Using a laser trap, a polystyrene bead was placed adjacent to the bacterium. When a T4P bound to the bead and retracted, the bead was deflected by distance d from the center of the laser trap. The force acting on the bead is proportional to the optical restoring force (F). In force clamp mode, d was kept constant by moving the microscope stage by distance δ with respect to the center of the laser trap. Thus, by measuring δ, we can determine the velocity of T4P retraction at constant force.

To start with, we clamped the force at $F = 8$ pN. This is the lowest force that we typically used for characterizing T4P retraction in the wt strain with functional PilT. We found that the Δ*pilT* strain indeed deflected the bead from the center of the laser trap, indicating T4P retraction (Fig. 1b). The distribution of velocities showed a maximum

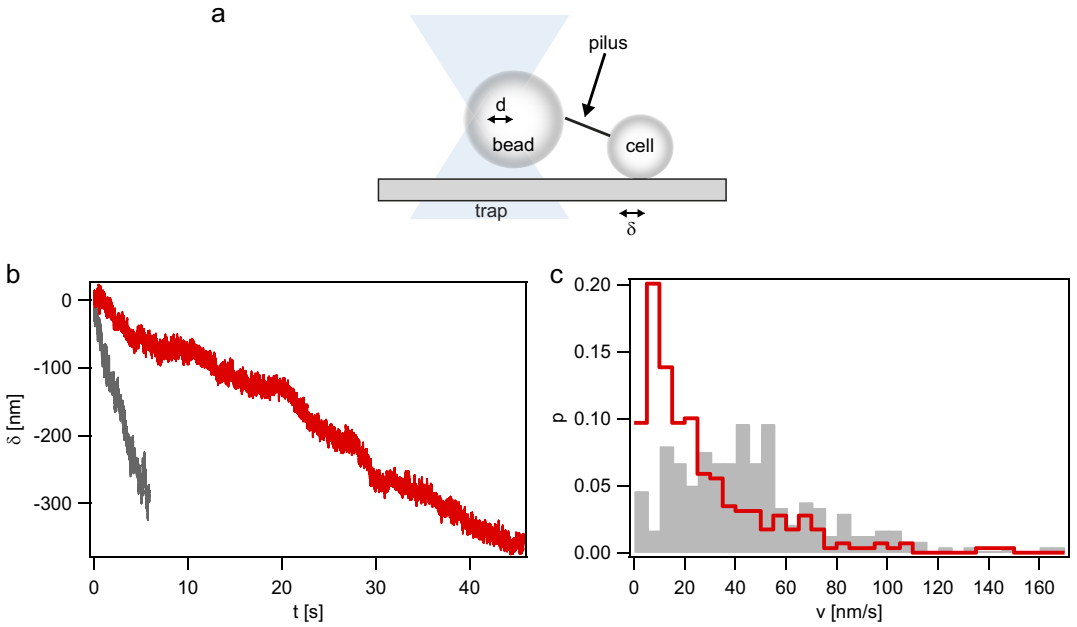

FIG 1 *pilT*-independent T4P retraction in a Δ*pilT* strain (Ng178). (a) Sketch of the experimental setup. A gonococcal cell is attached to a glass surface, and a polystyrene bead trapped in a laser trap is placed in close proximity. When a T4P binds to the bead, retraction deflects the bead from the center of the trap by distance *d*. *d* is proportional to the optical restoring force (*F*). In force clamp mode, *F* is held constant by moving the surface-bound cell by distance δ. Thus, δ is a measure of the T4P length change. (b and c) Typical examples of T4P length change δ as a function of time (b) and velocity distribution at forces clamped at $F = 4$ pN (gray, $n = 239$) and $F = 8$ pN (red, $n = 298$), respectively (c).

around a velocity (v) of 5 nm · s^{-1} and a pronounced tail toward higher velocities (Fig. 1c). We assessed whether similar retractile behavior occurred in a different trapping geometry. A dual trap (17) was used to trap a single spherical bacterium in each trap in position clamp mode. This setup was not influenced by microscope drift. Again, T4P retraction was observed (see Fig. S2a in the supplemental material). Furthermore, we used a configuration where we trapped a spherical bacterium in one trap and a bead in the second trap. Likewise, T4P retraction was observed (Fig. S2b). We conclude that gonococcal T4P retract in the absence of the retraction ATPase PilT.

Next, the effect of force on the speed of PilT-independent T4P retraction was investigated. We measured the velocity when the force was clamped to $F = 4$ pN (Fig. 1b). As expected, the distribution of velocities shifted to higher values compared to those at $F = 8$ pN. The distribution showed a maximum around $v = 40$ nm · s^{-1} and, again, a tail at higher velocities (Fig. 1c). No clear T4P elongation events were observed.

In summary, gonococcal T4P retract in the absence of the retraction ATPase PilT, but the speed is two orders of magnitude lower than the speed measured in the PilT-producing strain.

PilT-independent T4P retraction generates lower force than PilT-driven retraction. Gonococcal T4P are among the strongest molecular machines described so far, generating 150 pN during retraction (5, 34). We addressed force generation in the absence of the T4P retraction ATPase PilT. To this end, T4P retraction was probed using the laser trap in the position clamp mode. As the T4P pulled on the bead, the deflection increased and, concomitantly, the force increased. Eventually, the speed leveled off (Fig. 2a). A stalling event was defined as an event where the force did not change for at least one second, i.e., $dF/dt = 0$ for 1 s or longer. The stalling forces were distributed around a mean (±standard deviation [SD]) of $F = 4.7 \pm 0.7$ pN (Fig. 2b). We note that there were outliers at considerably higher forces. They were disregarded when calculating the average stalling force. Most likely, these stalling events were caused by multiple T4P pulling on the bead simultaneously.

We conclude that PilT-independent T4P retraction generates force in the range of 5 pN, i.e., 20-to 30-fold lower than that generated by PilT-powered T4P retraction.

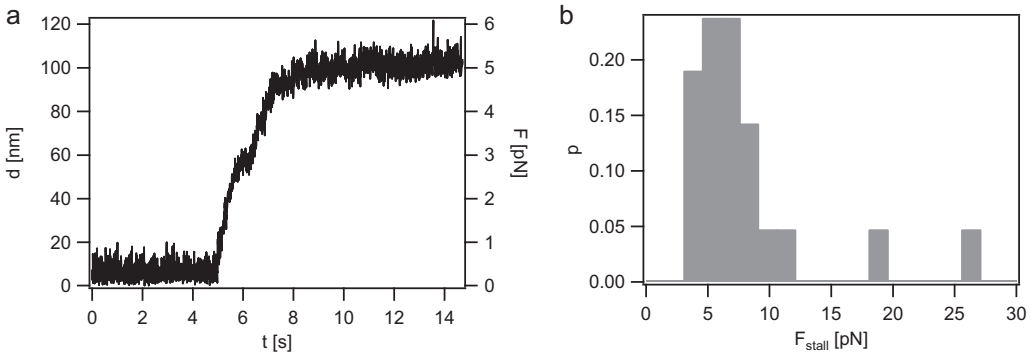

FIG 2 Stalling of *pilT*-independent T4P retraction in the Δ*pilT* (Ng178) strain. (a) Typical stalling event in position clamp mode. Deflection of the bead from the center of the laser trap (*d*) and force (*F*) are plotted as functions of time. (b) Distribution of stalling forces (F_{stall}). $n = 21$.

Deletion of *pilT2* in a Δ*pilT* background reduces the speed of T4P retraction. *N. gonorrhoeae* bears two *pilT* paralogues on its genome, namely, *pilU* and *pilT2*. *pilU* resides in the same operon as *pilT*, and its deletion shows little effect on T4P retraction in wt gonococci (35). Deletion of *pilT2* in the wt background causes a reduction of T4P retraction speed by a factor of ~2 (35). It was conceivable that one of the *pilT* paralogues coded for proteins that powered PilT-independent T4P retraction. To find out whether deletion of the paralogues affected the velocity of T4P retraction, we used the laser tweezers with force clamped to 4 pN. Interestingly, the speed was strongly reduced in the Δ*pilT2* Δ*pilT* strain (Fig. 3). The Δ*pilU* Δ*pilT* strain showed no significant change in T4P retraction compared to the Δ*pilT* strain. As described before, the force clamp assay may be subject to drift of the bacterium with respect to the laser trap. To make sure that the residual bead deflection was not caused by drift, we used the dual-trap assay and probed the following strains for T4P retraction: Δ*pilT2* Δ*pilT*, Δ*pilU* Δ*pilT*, and Δ*pilT2* Δ*pilU* Δ*pilT* strains. All three strains showed T4P retraction (Table S1), indicating that even in a strain that had all three *pilT* paralogues deleted, T4P retract. Examples for retraction of the Δ*pilT2* Δ*pilT* strain are shown in Fig. S3, where the bacteria approached each other by >500 nm within 30 s. We conclude that deletion of *pilT2* slows but does not inhibit T4P retraction.

Depletion of proton motive force slows PilT-independent T4P retraction. Depletion of proton motive force reduces the speed of gonococcal T4P retraction by 2-fold (36, 37). To test whether proton motive force powers *pilT*-independent T4P retraction, Δ*pilT* cells were treated with the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). CCCP shuttles protons across the membrane in the direction of the proton gradient and depletes PMF. Cells were incubated with 50 μM CCCP for 15 min prior to usage in dual laser tweezers. Notably, Δ*pilT* cells

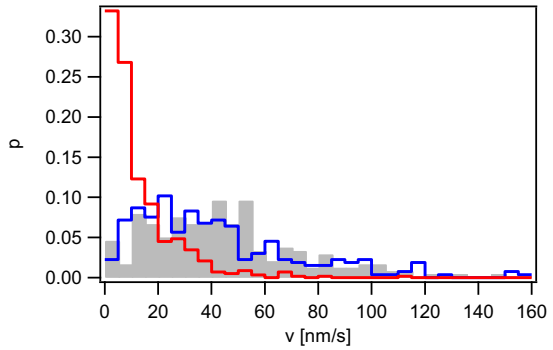

FIG 3 T4P retraction velocity depends on *pilT2*. Velocity distribution of the Δ*pilT* (gray, Ng178, $n = 239$), Δ*pilT* Δ*pilU* (blue, Ng185, $n = 265$), and Δ*pilT* Δ*pilT2* (red, Ng184, $n = 587$) strains at $F = 4$ pN.

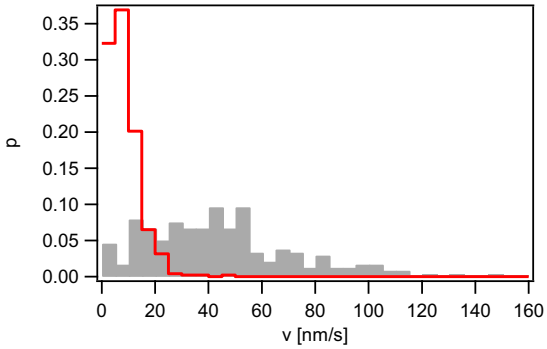

FIG 4 T4P retraction velocity depends on proton motive force. Velocity distribution of the Δ*pilT* strain (Ng178) in the absence (gray, $n = 239$) and in the presence (red, $n = 477$) of 50 μM CCCP at $F = 4$ pN.

were able to generate forces between 15 and 50 min post CCCP treatment (Table S1). However, the speed of T4P retraction was strongly reduced (Fig. 4). We conclude that PilT-independent retraction is slowed but not fully inhibited by depleting PMF.

DISCUSSION

Putative effects of PilT2. When *pilT2* is deleted in a strain generating PilT, the speed of T4P retraction is reduced by ~2-fold through an unknown mechanism (35). Here, we found that in a Δ*pilT* background, deleting *pilT2* again reduces the speed of T4P retraction by ~4-fold. A straightforward interpretation of this result would be that PilT2 can power T4P retraction at a low speed in the absence of PilT. The finding that application of CCCP reduces the speed by a similar factor is in agreement with this result. Depleting the proton motive force reduces the ATP levels considerably within 5 to 10 min (37) and would thus inhibit the ATPase-driven T4P retraction. Other mechanisms explaining the effect of PilT2 are possible. For example, we cannot rule out the possibility that the elongation ATPase PilF is involved in T4P retraction. Interaction of PilT2 with PilF may tune PilF activity.

Comparing T4P retraction in the presence and absence of the T4P retraction ATPase. It is important to note that thus far a gonococcal *pilT* deletion strain was considered to be incapable of T4P retraction (4, 9, 17, 38, 39). In our own studies characterizing single T4P retraction (1, 34) we have overlooked retraction in the absence of *pilT* because the velocity is close to zero in the force range in which we have worked so far. The lowest force was 8 pN, i.e., above the mean stalling force of PilT-independent retraction. Most other studies have probed for T4P retraction by measuring phenotypic consequences of T4P retraction such as twitching motility or DNA transformation. Indeed, deleting *pilT* inhibits gonococcal twitching motility and transformation (4, 29, 40).

T4P retraction in the absence of the retraction ATPase PilT has been recently reported for various bacterial species. First, T4P retraction was reported in T4P systems that naturally lack the gene encoding the T4P retraction ATPase (18, 19). Second, in T4P systems that naturally bear genes encoding the T4P retraction ATPase, retraction ATPases were deleted and yet T4P retraction was still observed (2, 20). Here, we show that deletion of *pilT* in the gonococcal T4P system has a strong effect force generation. While wt T4P generate force in the range of 150 pN (5), the stalling forces measured for the Δ*pilT* strain are in the range of 5 pN. Moreover, the retraction velocity is strongly reduced. It is interesting to compare the forces generated by various T4P systems in the presence and absence of the retraction ATPase. In the absence of the retraction ATPase, the forces generated by T4P retraction are fairly low, in the range of 3 to 12 pN (18, 19). The exception is T4P retraction in an *M. xanthus* Δ*pilT* strain, in which forces in the range of 70 pN are generated (2). However, it is likely that one of the *pilT* paralogues powers retraction for this specific strain. On the other hand, T4P retraction powered by

the retraction ATPase shows a wide range of forces, from 8 pN up to 150 pN (2, 5, 20, 41). Taken together, we conclude that the retraction ATPase PilT consistently increases the force generated by T4P retraction in different bacterial species.

Which biological functions related to T4P retraction require retraction ATPases? T4P systems lacking retraction ATPases are associated with protein secretion (18), surface sensing (19), and DNA in uptake systems in Gram-positive bacteria (33). We propose that high force generation may not be required for these functions. DNA uptake in Gram-negative species is strongly impaired in the absence of the retraction ATPases (32, 40), suggesting a role of high force generation during threading into the periplasm. Twitching motility is another function of T4P (4). T4P elongate, adhere to a surface, and subsequently pull the cell body forward by retraction. The rupture forces of T4P from abiotic surfaces (5, 42) and from other T4P (17) are in the range of 50 pN. During cellular movement, T4P must detach from the surface; otherwise, movement stalls (5, 43). Therefore, the motor force must exceed 50 pN, consistent with PilT-powered retraction. Similarly, T4P retraction regulates the kinetics of T4P-T4P attachment and detachment within bacterial colonies (17). Colonies formed by *N. gonorrhoeae* and *Neisseria meningiditis* generating functional PilT behave like liquids, whereas *pilT* deletion strains behave in a glasslike manner (16, 17). Liquidlike behavior facilitates colonization of blood vessels during meningococcal infection (16) and cell sorting with respect to differential T4P-T4P adhesion forces (14). Another function of T4P retraction that requires high forces is signaling to host cells. When epithelial cells are infected with gonococci or mock infected with T4P-coated beads, cytoskeletal proteins accumulate to the site of infection (8–10, 38). This accumulation depends on PilT and force application for gonococcal and mock infection, respectively (9, 10). We conclude that T4P retraction in the absence of a retraction ATPase is sufficient for some T4P-related functions, but for those functions that call for high forces, retraction ATPases are required.

Implications of retraction ATPase-independent T4P retraction for chemomechanical coupling in the T4P machine. Recently, progress has been made in understanding chemomechanical coupling in the T4P machine (see Fig. S1 in the supplemental material). Most likely, ATP binding and/or hydrolysis induces conformational changes of the retraction ATPase hexamer parallel to the membrane (23, 24, 31). Structural data are consistent with a wave of conformational changes around the ring leading to rotational motion of the platform complex. T4P retraction force, however, is generated perpendicular to the membrane by collapse of pilins from the fiber into the membrane, and it remains to be determined how putative rotation of the platform complex shuffles pilins from the fiber into the cytoplasmic membrane. The fact that T4P retraction occurs in the absence of the retraction ATPase evokes speculations about the energetics of the T4P machine. Deleting the gene encoding the elongation ATPase leads to nonpiliated bacteria (28). So far, to our knowledge, filament assembly has not been reported in the absence of an elongation ATPase. These two facts strongly suggest that the energy provided by ATP hydrolysis in the elongation ATPase is required for T4P polymerization. Part of this energy may be stored in the T4P fiber and power T4P depolymerization when the elongation ATPase has dissociated from the complex.

Interestingly, little T4P elongation was observed in this study. If the retraction and elongation ATPases bound alternatively and stochastically, then we would expect to see occasional switching from slow T4P retraction to fast elongation (indicative of binding of the elongation ATPase). The lack of T4P elongation in our study may suggest that T4P have to retract fully prior to binding of the elongation ATPase. This finding is consistent with processive retraction of toxin-coregulated pili in *V. cholerae*, in which it was proposed that insertion of minor pilins blocked elongation and triggered retraction (18). Previously, we observed that T4P retraction frequently switched to elongation in gonococcal strains that had strongly reduced concentrations of PilT (34, 44). However, the frequency of these elongation events increased strongly with external force, and

elongation events were rarely observed at forces as low as 8 pN (34), in agreement with results of the present study.

Conclusion. We have shown that gonococcal T4P retract in the absence of the retraction ATPase PilT. Both the speed and the maximum force of *pilT*-independent retraction are orders of magnitude lower than those in PilT-powered retraction, explaining why *pilT*-independent T4P retraction has been overlooked so far. Our findings, together with recent results for other species, strongly suggest that T4P retraction without PilT is a general phenomenon. We thus propose that the T4P elongation ATPase is necessary to provide energy for fiber formation, while retraction occurs spontaneously. Figuring out the chemomechanical coupling within the T4P machine, especially the dynamics of the platform complex in the presence and absence of the retraction ATPase, will be a future challenge.

MATERIALS AND METHODS

Growth conditions. Gonococcal base agar was made from 10 g/liter Bacto agar (BD Biosciences, Bedford, MA), 5 g/liter NaCl (Roth, Darmstadt, Germany), 4 g/liter K_2HPO_4 (Roth), 1 g/liter KH_2PO_4 (Roth), 15 g/liter Bacto proteose peptone no. 3 (BD), and 0.5 g/liter soluble starch (Sigma-Aldrich, St. Louis, MO), and supplemented with 1% IsoVitaleX: 1 g/liter D-glucose (Roth), 0.1 g/liter L-glutamine (Roth), 0.289 g/liter L-cysteine–HCl · $H_2$0 (Roth), 1 mg/liter thiamine pyrophosphate (Sigma-Aldrich), 0.2 mg/liter Fe(NO3)3 (Sigma-Aldrich), 0.03 mg/liter thiamine HCl (Roth), 0.13 mg/liter 4-aminobenzoic acid (Sigma-Aldrich), 2.5 mg/liter β-NAD (Roth), and 0.1 mg/liter vitamin B_{12} (Sigma-Aldrich). GC medium is identical to the base agar composition but lacks agar and starch.

Bacterial strains. All bacterial strains were derived from the gonococcal strain MS11 (VD300). In all strains, we deleted the G4 motif by replacing it with the *aac* gene conferring resistance against apramycin. The G4 motif is essential for antigenic variation of the major pilin subunit (45). Pilin antigenic variation modifies the primary sequence of the pilin gene. To generate the Δ*pilT2* Δ*pilT* strain (Ng184), Ng150 (46) was transformed with genomic DNA from Δ*pilT2* (35) and subsequently with DNA from the Δ*pilT* strain (Ng178). To generate the Δ*pilU* Δ*pilT* strain (Ng185), Ng150 (46) was transformed with genomic DNA from GU2 (47) and subsequently with DNA from the Δ*pilT* strain (Ng178). To generate the Δ*pilT2* Δ*pilU* Δ*pilT* strain (Ng186), Ng150 (46) was transformed with genomic DNA from GU2 (47) and the Δ*pilT2* strain (35), and subsequently with DNA from the Δ*pilT* strain (Ng178).

Transformants were selected on agar plates containing the respective antibiotics (Table S1).

Characterization of T4P retraction. Retraction velocities and stalling forces were measured using an optical tweezers setup assembled on a Zeiss Axiovert 200 microscope (34). In short, all measurements were carried out in retraction assay medium consisting of phenol red-free Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 2 mM L-glutamine (Gibco), 8 mM sodium pyruvate (Gibco) and 30 mM HEPES (Roth). A suspension of bacteria and carboxylated latex beads with a diameter of 2 μm (Polysciences, Warrington, PA) was applied to a microscope slide and sealed. All measurements were performed at 37°C. The trap stiffness was determined by power spectrum analysis of the Brownian motion of a trapped bead to be 0.5 pN/nm ± 10%. The retraction velocities were measured in force clamp mode. During the experiment, a bead was trapped and held close to an immobilized bacterium at the surface. Eventually, a pilus attached to the bead, and its retraction lead to a deflection out of the equilibrium position. As soon as the deflection of the bead reached the threshold deflection corresponding to a force of 4 or 8 pN, a force feedback algorithm held the displacement constant by moving the sample in the *x-y* plane using a piezo stage. Stalling forces were measured in position clamp mode.

Double laser trap. In order to investigate single cell interactions, we followed a previously developed protocol (17). Two optical traps were installed in an inverted microscope (Nikon TE2000 C1). The trapping laser (20I-BL-106C, 1,064 nm, 5.4 W; Spectra Physics) was directed into a water immersion objective (Plan Apochromate VC; 60×; numerical aperture [NA], 1.20; Nikon). Manipulation of the laser was done with a 2-axis acousto-optical deflector (DTD-274HD6 colinear deflector; IntraAction Corp., USA). Bacterial interaction was recorded with a charge-coupled device (CCD) camera (Sensicam qe; PCO, Kelheim, Germany). The optical trap was calibrated via the equipartition and drag force methods. At 100% laser power, the average stiffness is 0.11 ± 0.01 pN/nm. The linear regime extends up to 80 pN.

Depletion of proton motive force. Cells were incubated with 50 μM CCCP for 15 min prior to usage in dual laser tweezers. To check that 15 min is sufficient to affect cells, twitching motility of *pilT*-expressing Δ*G4* cells was checked by bright field microscopy. Consistent with previous results (37), cells showed low-speed twitching motility after 15 min of treatment with 50 μM CCCP and high-speed twitching motility without CCCP.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00778-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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