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Characterization of the Biomechanical Properties of T4 Pili Expressed by *Streptococcus pneumoniae*—A Comparison between Helix-like and Open Coil-like Pili

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Bacterial adhesion organelles, known as fimbria or pili, are expressed by Gram-positive as well as Gram-negative bacteria families. These appendages play a key role in the first steps of the invasion and infection processes, and they therefore provide bacteria with pathogenic abilities. To improve the knowledge of pili-mediated bacterial adhesion to host cells and how these pili behave under the presence of an external force, we first characterize, using force measuring optical tweezers, open coil-like T4 pili expressed by Gram-positive *Streptococcus pneumoniae* with respect to their biomechanical properties. It is shown that their elongation behavior can be well described by the worm-like chain model and that they possess a large degree of flexibility. Their properties are then compared with those of helix-like pili expressed by Gram-negative uropathogenic *Escherichia coli* (UPEC), which have different pili architecture. The differences suggest that these two types of pili have distinctly dissimilar mechanisms to adhere and sustain external forces. Helix-like pili expressed by UPEC bacteria adhere to host cells by single adhesins located at the distal end of the pili while their helix-like structures act as shock absorbers to dampen the irregularly shear forces induced by urine flow and to increase the cooperativity of the pili ensemble, whereas open coil-like pili expressed by *S. pneumoniae* adhere to cells by a multitude of adhesins distributed along the pili. It is hypothesized that these two types of pili represent different strategies of adhering to host cells in the presence of external forces. When exposed to significant forces, bacteria expressing helix-like pili remain attached by distributing the external force among a multitude of pili, whereas bacteria expressing open coil-like pili sustain large forces primarily by their multitude of binding adhesins which presumably detach sequentially.

Introduction

Adhesion of bacteria to host cells is a prerequisite for colonization. For some bacteria, the initial adhesion to host cells is mediated via non-flagellar polymeric cell-surface organelles, pili or fimbria, that bind specifically to host receptors by adhesins.[1–3] Recent studies have revealed that these pili have a direct role in pathogenesis and they are therefore considered as possible vaccine candidates, which makes it important to characterize their structure and role in the adhesion process in some detail, in particular their ability to sustain external forces from various types of flows.[4] Much work has recently been done, using force spectroscopic techniques, in particular force measuring optical tweezers (FMOT), to assess the biomechanical properties of pili expressed by Gram-negative uropathogenic *Escherichia coli* (UPEC).[5] However, other types of pili have not yet been addressed equally rigorously. To improve on the knowledge of bacterial adhesion to host cells mediated by pili, and in particular how pili behave under the presence of an external force, we investigate in this work pili expressed by the Gram-positive *Streptococcus pneumoniae*, which have an architecture that is different from those of the pili expressed by UPEC bacteria.[5,6] Since their force response differs significantly from those of Gram-negative UPEC bacteria, a comparison of the biomechanical properties of the two types of pili is also presented.

E. coli is a member of the Gram-negative family with an ability to colonize and sustain in numerous niches. Some of the strains are broadly categorized as either diarrheagenic or extra-intestinal pathogenic *E. coli* (ExPEC).[7,8] Among the various ExPEC strains, UPEC are commonly associated with community-acquired urinary tract infection. Once inside the urinary tract, UPEC bacteria colonize the bladder giving rise to cystitis, although they can also ascend through the ureters into the kidneys, causing pyelonephritis. In order to manage this, the bacteria have developed various types of pili that can sustain the strong forces that are caused by the rinsing urine flow. For UPEC bacteria, these pili consist of a number of repeated protein subunits that are assembled in a helix-like structure with an adhesin on the tip that binds to the host receptors.[9]

It has recently been shown that these pili, because of their helix-like structure, can be extensively elongated under exposure to force, primarily by a consecutive opening of the layer-

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to-layer (LL) bonds of the rod, and thereby an unfolding of their quaternary structure, which gives rise to a linearized polymer with an open helix-like structure.[10] It is believed that this action helps a complex of pili to distribute the shear forces caused by flows in its natural environment among various pili in such a way that the lifetime for adhesion of a bacterium is prolonged.[11,12]

Moreover, UPEC is capable of adapting to its local environment by expressing more than one type of pili, and predominantly the one that exhibits the combination of biomechanical properties of the rod and adhesive properties of the adhesin that is most efficient for its local surrounding, for example, urine flow and nature of the epithelial cells. This indicates that the differences between the various types of pili are of importance for their colonization ability. Pili expressed by UPEC have therefore been widely scrutinized in previous works in terms of genetics, structure, biomechanical properties, in particular those when exposed to an external force,[10,13–20] as well as their specific adhesion properties.[21]

In contrast to these well-studied types of pili, little is known about the properties and biomechanical function of pili expressed by Gram-positive bacteria, and in particular *S. pneumoniae*. Such bacteria colonize the upper respiratory tract and provoke morbidity and mortality worldwide.[22] This colonization leads most often to infections such as otitis and sinusitis. When the bacteria invade other parts of the human body, general infection induces bacterial pneumonia, other lower respiratory tract diseases, and meningitis.[6,22] Despite their ubiquity, their adhesion mechanisms are not yet well understood.[23] It was only recently discovered that the *S. pneumoniae* bacterium, often referred to as pneumococcus, expresses pili,[1] which is confirmed by the atomic force microscopy (AFM) image presented in Figure 1 A.

Moreover, the pili on the surface of these Gram-positive bacteria do not have a helix-like constitution; they instead exhibit an open coil-like structure composed of at least two protofilaments (Figure 2). In addition, each pilus has a number of adhesins, distributed along the pilus (Figure 1 B).[6,24] It can be hypothesized whether this gives this type of pili elongation properties that differ from those of helix-like pili of Gram-negative bacteria when exposed to force and, in such a case, what the differences are. In this paper, we use FMOT to compare the biomechanical properties of pili expressed by UPEC, represented by P pili, and *S. pneumoniae,* where the former are a typical and well-studied representative of pili of ExPEC bacteria with a helix-like structure.[5,10,14–16,25,26] Since these types of pili have dissimilar structure, a closed helix-like and an open coil-like structure respectively, we use two different models to describe their force–extension behavior before we discuss their difference in response.

Terminology

Since there is not a uniquely defined and fully established terminology regarding pili structure, it is worthwhile to clarify the nomenclature. The term *rod* will in this work refer to the helix-like sequence of repeated subunits for the pili expressed by

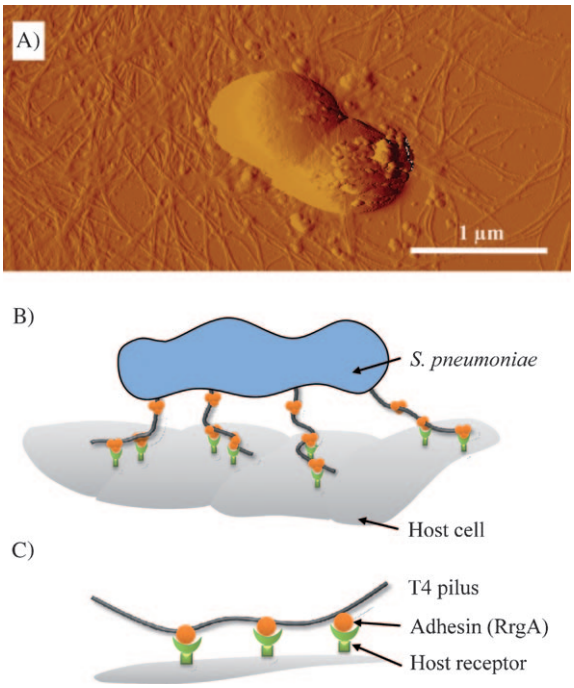

Figure 1. A) AFM image of a pneumococcal bacterium expressing T4 pili. B) and C) Model of adhering *S. pneumoniae*. In contrast to *E. coli* pili, the adhesins are distributed along the organelles and confer multiple anchoring points to the host cells.

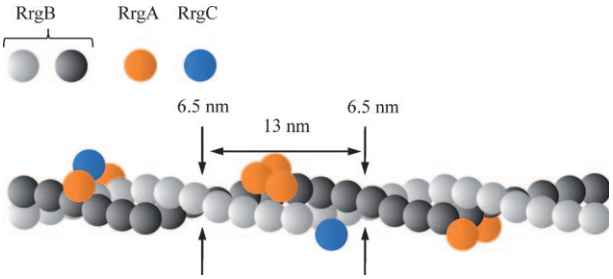

Figure 2. Right-handed schematic illustration of a T4 pilus as a superstructure composed of at least two RrgB protofilaments. Ancillary proteins (RrgA and RrgC) are sited along the coiled-coil backbone. Inspired from Hillerigmann et al.[6]

UPEC bacteria. The quaternary structure of such pili will be referred to as a *closed helix-like structure* when folded, that is, when the consecutive LL subunits bonds are closed, and an *open helix-like structure* when the consecutive layer-to-layer subunits bonds have been opened. In the latter case, when the rod has been fully unfolded, its structure is said to be *linearized*. The structure of the pneumococcal pili will, in this work, be referred to as an *open coil-like structure*, although it is also termed coiled-coil superstructure in the literature.[6] Finally, we present in this work *force–extension* measurements. As depicted below, a force–extension of a pilus consists of a stretching, called an *elongation*, and a contraction (when it is brought back to its original length), referred to as a *retraction*. Thus, elongation and retraction measurements represent two parts of an *extension* measurement.

Models

T4 Pili Expressed by Gram-positive *S. pneumoniae*

The pneumococcal pili, expressed by the Type 4 strain TIGR4 (T4), have recently been found, by immunoelectron microscopy,[6] to possess an open coil-like quaternary structure composed of at least two protofilaments, as is schematically shown in Figure 2. Individual protofilaments are formed by polymerization of three proteins, RrgA, RrgB, and RrgC, which are covalently linked through catalysis by three sortases, SrtB, SrtC, and SrtD, respectively, assembled into 3.5 nm thick filaments.[4] As is shown in Figure 2, whereas RrgB builds the backbone of the protofilament, the RrgA and RrgC constitute surface-located ancillary proteins.[6,27,28] Moreover, while RrgA is ascribed to mediate adhesion of the T4 pilus, the role of RrgC is not yet fully understood.[24] The RrgA proteins recognize selected extracellular matrix compounds from the host cells, Figure 1C, and thus mediate adhesion during the infection process (confirmed by Nelson et al.[24] who purified recombinant RrgA that bound to epithelial cells).

The protofilaments are tightly intersected at repetitive zones resulting in a pilus diameter of 6.5 nm (Figure 2). Between these zones, the filaments show wider sections with a diameter of about 9.5 nm. They can be up to 3 μm in length and appear as long flexible appendages.[4]

A T4 pilus can be modeled as a continuous semi-flexible polymer that undergoes thermal fluctuations. A common model for describing the force–extension response of linear polymers is the worm-like chain (WLC) model.[29,30] This model treats the polymer as a continuous flexible chain of length L_c with a bending stiffness κ, which is often expressed in terms of a persistence length, L_p, given by κ/kT where k is the Boltzmann's constant and T is the absolute temperature.

Since there is no analytic solution to the WLC model for the entire range of force, approximate solutions have been developed (see for example, Bouchiat et al.[31]). The most common approximation is the interpolated WLC, derived by Bustamante et al.,[30] shown in Equation (1)

$$F = \frac{kT}{L_p}\left[\frac{1}{4}\left(1-\frac{x}{L_c}\right)^{-2} - \frac{1}{4} + \frac{x}{L_c}\right] \quad (1)$$

where x represent the end-to-end distance of the pilus in the direction of the elongation. This relationship is used in this work to analyze the force–extension behavior of T4 pili expressed by Gram-positive *S. pneumoniae*.

Helix-like Pili Expressed by Gram-negative UPEC Bacteria

Helix-like pili expressed by Gram-negative UPEC bacteria consist of a number of repeated protein subunits assembled by a donor-strand exchange. Each subunit donates its amino-terminal extension to complete the immunoglobulin (Ig)-like fold of its neighbor to form a non-covalent immunoglobulin-like polymer, and arranged in a helix-like structure, as is shown in Figure 3A.[32,33] These structures, which are ~7 nm thick and ~1–2 μm long, consist mainly of a rod composed of ~10^3 subunits arranged in a closed helix-like quaternary structure with ~3 layer-to-layer (LL) bonds per turn. UPEC bacteria can express several types of pili, of which P and type 1 pili are the most common. For these two types, the rods are composed of PapA and FimA units, respectively.[18,33] As is shown in Figure 3B, which corresponds to P pili, the force–elongation response of helix-like pili can be divided into three distinct regions, commonly referred to as region I, II, and III, respectively.[10,16,25,34]

For low forces, below a so-called unfolding force, the force-elongation response is linear (region I). This response originates from an elastic elongation of the quaternary structure. For higher forces, the elongation enters region II, where the LL bonds of the quaternary structure of the rod unfold sequentially, at a constant unfolding force. When all LL bonds have been opened, the pilus enters region III, in which the force–elongation response exhibits a soft wave-like shape that originates from a random conformational change of the head-to-tail (HT) bonds. The behavior in this region is governed by properties of both the individual bonds and entropy.

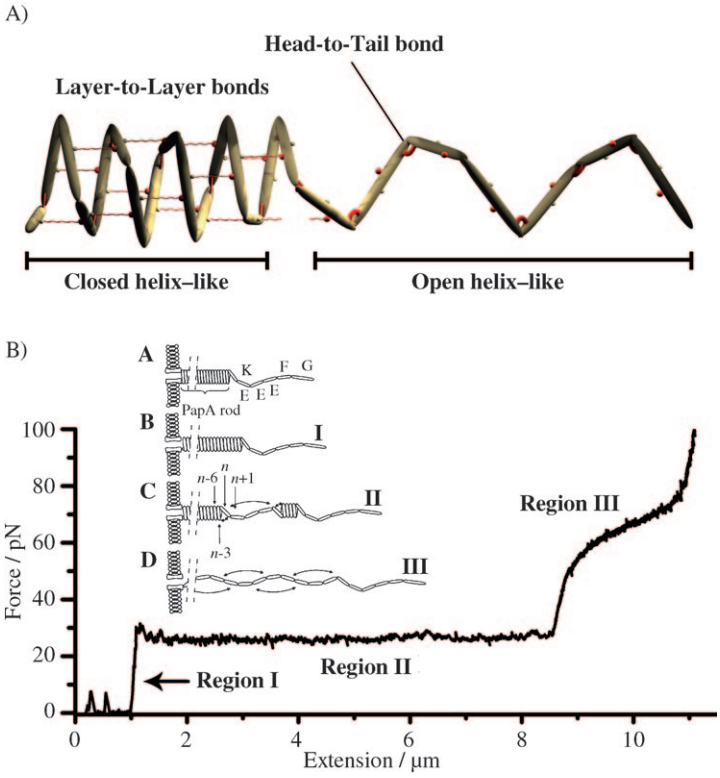

Figure 3. A) Schematic illustration of a helix-like *E. coli* pilus adopted from ref. [5]. B) solid curve: a typical force-elongation curve of a P pilus with its typical elongation regions marked. Inset: modes of elongation of a single pilus: (A) not exposed to force, (B), (C), and (D) elongated into the regions I, II, and III, respectively. The unfolding force of the quaternary structure is ~28 pN.

It has furthermore been shown that this force–elongation response can be well described by the sticky chain model,[35] producing excellent agreement to experimental data.[15,16] This model is based upon a combination of Hooke's law for the elastic elongation of tandem elements and a rate equation for strain-assisted bond-opening according to Bell.[36] The sticky chain model assumes, in this particular case, that the energy landscape of the elongation of the macromolecule along the reaction coordinate consists of three states; one that represents a closed LL bond, a second that represents an open LL bond with the HT bond in its native configuration, and a third state that represents an alternative configuration of the HT bond. The three regions found are a consequence of the interplay between the three possible energy states.

Results

Force–Extension Response of Single *S. pneumoniae* T4 Pili

A typical force–elongation measurement of an individual T4 pilus exposed to stress under a constant elongation speed is shown by the leftmost solid curve (red) in Figure 4A. In contrast to the response from the helix-like pili (Figure 3B), there is no plateau with constant force; instead the force required to elongate the pilus is monotonically increasing in a non-linear manner. This is similar to the elongation behavior of linear macromolecules, for example, single strand DNA, type IV pili, and flagella,[29,37,38] and therefore confirms a linear structure. The significant increase in force, here starting at ~0.7 μm, is due to a combination of an entropic resistance of the pilus to be stretched and the finite contour length of the pilus. In addition, the elongation is significantly shorter for T4 pili than for the helix-like counterparts (a few hundred nm versus a few μm). This demonstrates that the force–extension behavior of T4 pili is significantly different from that of pili expressed by UPEC bacteria, given by Figure 3B.

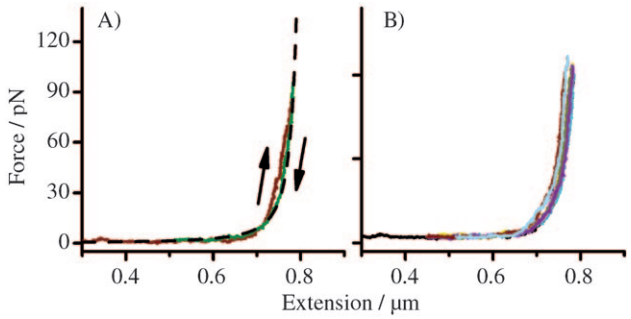

Figure 4. A) A typical force–extension response of T4 pili assessed at constant elongation speed (0.1 $\mu m\,s^{-1}$). Red and green curves represent the elongation and retraction, respectively. The retraction curve has been fitted to Equation (1) for forces up to 100 pN (dashed curve). B) the first four force–extension runs are plotted here.

Figure 4A also shows, by the rightmost solid curve (green), a force–retraction curve. The two curves in panel A show that the T4 pilus can be elongated and retracted in a more-or-less fully reversible manner, that is, with a minimum of hysteresis. This indicates that there are, under the prevailing conditions, no conformational changes or frictional losses in the pilus during extension.

In order to assess the repeatability of the elongation of T4 pili, a given pilus was exposed to a number of repeated elongation and retraction cycles. Figure 4B shows four such consecutive cycles (of a series of 13), taken with ~20 second intervals. As can be seen in the figure, all curves depict a virtually identical behavior, again showing that the elongation and retraction cycle is fully reversible but also indicating that there is no sign of fatigue or alteration of the "bead-bacteria-pilus-bead" system with time or the number of elongations.

Modeling T4 Pili as a Wormlike Chain

Due to the high repeatability of the force–extension response of T4 pili, it is possible to model this behavior. As was alluded to above, since T4 pili are linear macromolecules, their force–elongation response can be described by the well-known WLC model. An example of the interpolated WLC, given by Equation (1), fitted to the force–retraction curve in Figure 4A, is given by the dashed curve in the same figure. As can be seen, the fitted curve matches the curve well.

A fit of Equation (1) to 39 force–elongation and 37 force–retraction curves from a number of pili, similar to the type given in Figure 4A, reveal a persistence length L_p of 2.1 ± 1.7 nm, where those of the elongation curves differ slightly (0.5 nm) from those of the retraction curves.

Force–Elongation Discontinuities

In some series of measurements, "discontinuities" in the force–elongation response were found. Figure 5 shows four typical elongation-and-retraction cycles (from a series of 21 cycles made on a given pilus) in which such discontinuities appeared.

In order to investigate the origin of these discontinuities, the pilus was elongated by exposure to large forces. Although the optical tweezers (OT) can apply forces up to (and above) 150 pN under the pertinent conditions, the detection system does not give rise to a linear response for such high forces. Instead, the response curves tend to "bend over", as is shown in Figure 5. The force threshold for a linear response was found to be in the 80–120 pN range, depending on the stiffness of the trap (i.e. the power of the trapping laser); it was assessed to ~120, ~100, and ~80 pN for trap stiffness of 250, 210, and 160 $pN\,\mu m^{-1}$, respectively.

Since this non-linearity is only caused by the detection system (and not any properties of the pilus), the pili were elongated up to these high forces. However, no quantitative conclusions from this high-force region were drawn, even though it was noticed to which extent the retraction curves retraced the elongation curves, that is, whether any discontinuities or hysteresis appeared.

Figure 5 shows that although the various curves are not fully identical, the discontinuity phenomenon is rather reproducible. In all of the curves displayed, there is a discontinuity at a force

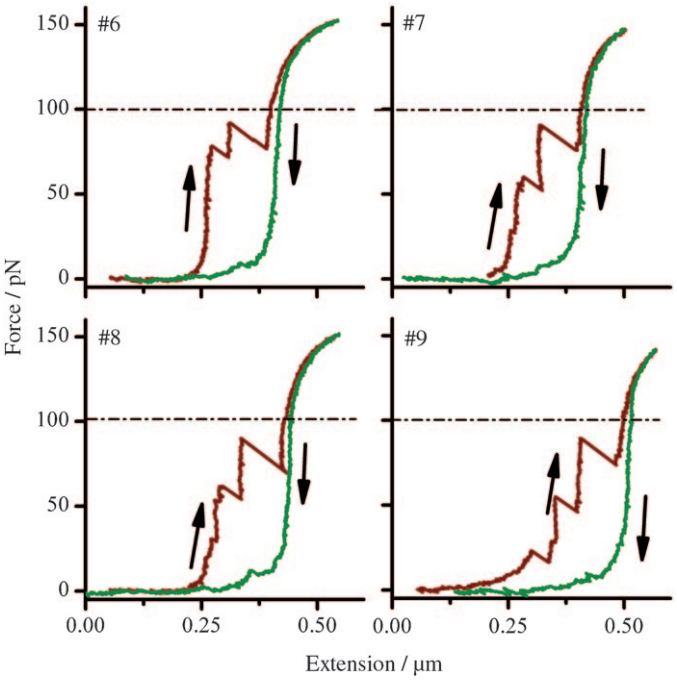

Figure 5. Repetitive elongation–retraction cycles of a single T4 pilus, for consecutive runs #6–9 of 21 cycles. Red curves represent the elongation process, at a constant velocity of 0.1 $\mu m\,s^{-1}$, green curves the retraction process. The dashed solid line represents the threshold for linear response for the pertinent trap stiffness, $\kappa = 213\ pN\,\mu m^{-1}$.

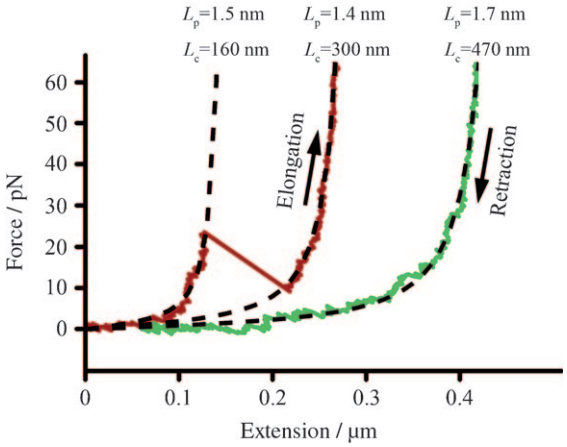

Figure 6. Solid curves: An elongation-retraction curve, with one visible discontinuity during elongation. The pilus was exposed to further discontinuities at forces outside the range of the plot, why the retraction curve appears shifted from the elongation curve. Dashed curves: fits of Equation (1) to the various segments of the elongation-retraction curve. The results indicate that although the discontinuity gives rise to an elongated contour length, the persistence length is not affected.

slightly above 50 pN, corresponding to an elongation of the system of ~40 nm, followed by a second discontinuity at a force slightly below 100 pN, giving rise to a slightly longer elongation, ~80 nm. In one of the panels (run #9) an additional discontinuity at around 20 pN can be seen.

Occasionally, discontinuities in the force–elongation response appear already for low forces (i.e. forces well below the force threshold). One example is shown in Figure 6. Such features allow for a quantitative investigation of the elongation behavior of the pili prior to, and following, a discontinuity. Fits of Equation (1) to the separate parts of the elongation curve are presented in Figure 6 and they show that the contour length of the pilus indeed increases with each discontinuity, in this case from 160, via 300 to 470 nm. The persistence length is, however, not significantly affected.

Discussion

In order to understand how bacteria adhere to host cells during the early stage of infection, and how they can sustain significant external forces from various types of rinsing flows, it is of importance to have knowledge about their structure as well as their biomechanical function, in particular their elongation behavior under exposure to stress. Even though pili expressed by Gram-negative and Gram-positive bacteria both mediate adhesion during the early stages of colonization, it has been known for a while that they differ substantially in their assembly mechanism as well as architecture. On the other hand, it has not yet been elucidated to which extent this provides them with dissimilar biomechanical properties.

Until recently, most biomechanical studies of pili have been concerned with pili expressed by Gram-negative bacteria, for example, from *Hemophilus influenzae* (type b),[39] *E. coli* (type 1, P and S pili),[10,13–21,40] and *Yersinia* spp. These pili have in common that they are assembled through a periplasmic chaperone-usher pathway and consist of a non-covalently connected array of subunits that forms a helix-like structure. Of particular importance for this work are the force–elongation studies of the P and type 1 pili that recently have been performed by force–spectroscopic techniques.[25]

Conversely, subunits of pneumococcal pili are connected covalently. Recently, Kang et al.[41] showed that the pili expressed by Gram-positive *Streptococcus pyogenes* are stabilized by isopeptide bonds between consecutive subunits. In addition Hilleringmann et al.[6] identified and characterized, by the use of immuno-electron microscopy on purified pili, the architecture of pili expressed by *S. pneumoniae* as an open coil-like structure consisting of at least two protofilaments, referred to as a coiled-coil superstructure. They also showed that the T4 pilus has a compact but flexible quaternary structure that constitutes a starting point for understanding the T4 pili architecture.

Adhesion and pathogenicity of Gram-negative bacteria can additionally be mediated by various types of non-helix-like phenotypic structures, like type IV and curli, (reviewed by, for example, Fronzes et al.[42] and Craig et al.[43]). Since the latter are assembled by a few parallel strands,[43–45] they have architectures that are analogous to those of *S. pneumoniae*.

The measurements presented above, performed by FMOT on individual T4 pili expressed by living *S. pneumoniae* cells in situ, complement these pictures. In particular, they highlight the differences in the force-extension response of open coil-like and helix-like pili.

Flexibility

As was alluded to above, the persistence length of T4 pili was assessed to 2.1 ± 1.7 nm, which is similar to the length of a RrgB subunit, but slightly shorter than those of P and type 1 pili previously found to be 3.3 ± 0.6 nm[21] and 3.3 ± 1.6 nm,[17] respectively. This suggests that T4 pili have a structure that is significantly more flexible than closed helix-like pili, and similar to, or slightly more flexible than, open helix-like pili, expressed by UPEC. The flexible structure of T4 suggests that the pili are capable of following the topographical variations of the host tissue and thereby adhere with a multitude of anchoring points, which would be beneficial for adhesion under in vivo conditions.

Discontinuities

As was illustrated in the Figures 5 and 6, some measurements show discontinuities in the force–elongation response. Such features can in principle originate from a number of processes, of which the most plausible are: i) force-induced structural changes within the pilus, ii) partial detachment of the bacteria from the mounting bead, or iii) partial detachment of the pilus from the trapped polystyrene bead.

Since the discontinuity phenomenon was intermittent, that is, dissimilar amongst different series of runs, it suggests that the discontinuities do not originate from any structural changes within the pilus, which therefore rules out the process (i).

As illustrated in Figure 8 A, the interaction between the poly-L-lysine-coated bead and the bacterium is mainly electrostatic; in our experiments the bacteria surface was negatively charged whereas the polymer chain of poly-L-lysine was positively charged. This results in a strong attachment, in agreement with what has been assessed in previous work.[46,47] For example, it has been shown by AFM that a single Gram-positive *Staphylococcus epidermis* bacterium can withstand forces of more than 2 nN without detaching from such a surface.[46] In addition, the influence of the PBS solution on the bacterial envelope and its subsequent electrostatic charge was investigated by AFM imaging by Yang et al.[46] and it was concluded that despite an exposure to an extensive force, no changes or alteration in the binding were observed. This suggests that the force applied by the trap in our measurements should not be sufficient to detach the bacteria. This makes the process (ii) improbable.

Finally, adhesion between the pili and the small polystyrene bead is essentially mediated by non-specific bindings. Since the small bead will eventually, in the presence of the force applied by the FMOT, detach from the pilus, it is possible to conclude that the binding strength is in the pN range. This makes the process (iii) plausible.

All this implies that we attribute the discontinuities to detachment of anchoring points of the pilus from the trapped bead.

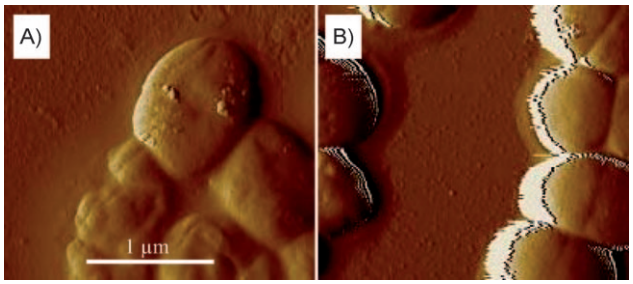

Figure 7. AFM micrographs of BHN33 (A) and BHN134 (B) showing no expression of any pili.

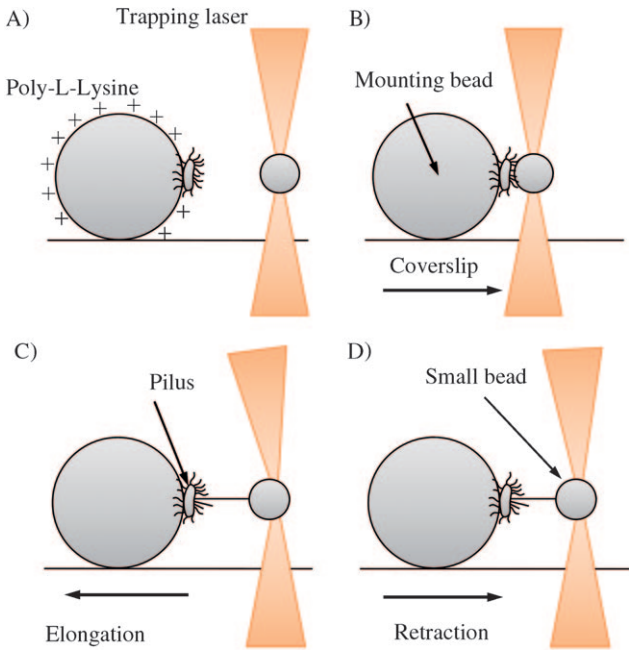

Figure 8. Mounting and measurement procedures. Measurement procedure adopted before.[10] The mounting bead was attached to the coverslip, both functionalized with poly-L-lysine which charges the surfaces positively. The bacterium, charged negatively, was then mounted to a 9.6 µm bead by the use of the OT. A) A small bead was trapped by the OT and served both as a surface onto which the bacterial pili could bind and as a force indicator in the OT system. B) The trapped bead was brought in close proximity to the bacterium forming a non-specific bond between the bacterial pili and the bead. C) A force was then exerted on the pili by translating the coverslip with the large bead. The induced displacement of the trapped bead is thereby a measure of the exerted force. D) When the desired extension of the pilus had been achieved, the coverslip was stopped and translated back. The response was acquired during elongation as well as retraction.

Rebinding

The existence of repeatable elongation-and-retraction cycles, even in the presence of subsequent discontinuities, as is illustrated in Figure 5, indicates that rebinding of the detached anchoring points takes place during the retraction process, and thereby supports the conclusion above. However, since such rebinding takes place predominantly under low forces, it is, in general, not observed in the force–retraction response. Moreover, consecutive elongation-and-retraction cycles show occasionally slightly dissimilar behaviors, which indicate somewhat different configurations of the anchoring points on the small bead.

Bacterial Adhesion Strategies

It is clear that pili expressed by UPEC bacteria and *S. pneumoniae* show completely different force–extension behavior, and it is plausible that they represent two different strategies to stay attached to host cells when exposed to large and fluctuating external forces in an in vivo situation.

Helix-like pili expressed by UPEC bacteria have one single adhesin at the distal end of the pilus. An external force gives rise to an unfolding of the closed helix-like quaternary structure. Although the attachment time of a single pilus is unaffected by the unfolding process, given by the expected attachment time of the adhesin-receptor bond, $\langle t \rangle_1$, such time of a complex of pili is determined by the degree of cooperativity between the various pili, that is, their ability to distribute an external force among various pili by means of unfolding.[12] In contrast, as shown in Figure 2, ancillary RrgA adhesins, which have been ascribed to mediate adherence of the T4 pilus, are distributed along the pilus structure. It is therefore assumed that the adhesins on the T4 pili serve as multi-anchoring points.[28] When such a pilus is exposed to an external force, it is likely that its detachment can be delayed by the consecutive detachment of a large number of adhesins from their host receptors. The expected attachment time of a T4 pilus with N adhesins, $\langle t \rangle_N$ is then equal to $\langle t \rangle_N = N\langle t \rangle_1$. Although the interactions in this work are non-specific, the stepwise detachment process for specific interactions is suggested to be analogous to the partial detachments observed in Figures 5 and 6.

The detachment of a multipili binding system is expected to depend also on the intrinsic biomechanical properties of the attachment organelles. Pili expressed by UPEC bacteria have shown to possess an exceptional ability to elongate by unfolding their closed helix-like structure, which is considered to be crucial for multipili attachment exposed to high forces. UPEC bacteria are assumed to rely on this elongation behavior to redistribute an external force to a multitude of pili, which thus will share the force.[12] The ability to redistribute an external force in a multipili attachment system is also present for T4 pili that elongate by the stretching of the macromolecule as well as the zipper-like detachments of adhesins. Since this elongation is significantly shorter for T4 pili than for helix-like pili, T4 pili possess a reduced ability to share an external force.

In conclusion, in contrast to helix-like pili, a single T4 pilus can maintain attachment far longer than a single adhesin. They cannot elongate to the same degree as helix-like pili, and they do not take up any significant force unless fully stretched, which implies that the cooperative effect of a multipili attachment complex is considered less important for a T4 system. These structures thereby suggest that the open coil-like pili are designed to withstand forces a longer time on a single pilus level while the high cooperativity of helix-like pilus is advantageous in a multipili scenario.

Conclusions

The molecular structure of T4 pili expressed by *S. pneumoniae* is different from that of pili expressed by UPEC bacteria; while the latter has a helix-like shape with a single adhesin at the tip of the pilus, the former has an open coil-like form composed of at least two protofilaments with adhesins distributed along the pili. This gives rise to dissimilar force–elongation responses. Whereas pili expressed by UPEC bacteria exhibit three characteristic regions in their force–elongation response, of which the longest constitutes a constant force plateau (~a few μm), it has been found in this work that T4 pili have a force–elongation response of a typical WLC shape with a weak response over a wide range of elongation but with a significantly increasing force in a short elongation interval (~0.1 μm). The short persistence length of the T4 pili suggests that their structure is more flexible than that of the helix-like pili from UPEC bacteria. It is hypothesized that these differences can be related to dissimilar invasion strategies in vivo. It is plausible that the ability of UPEC pili to undergo long elongations while exposed to force provides a high degree of cooperativity (i.e. a possibility for a complex of pili to distribute an external force among the various pili), which is beneficial for multiple pili binding. Since the elongation range over which T4 pili take up any substantial force is significantly shorter, it is suggested that they have a lower degree of cooperativity. On the other hand, it is suggested that their multitude of adhesins, which are distributed along the pilus and can detach sequentially, serves as an alternative means to sustain external forces.

Experimental Section

Biological Model System: Experiments were performed on a strain of *Streptococcus pneumoniae* (BHN155) in which a *rlrA* pathogenicity islet (coding for T4 pili) had been introduced.[1] A negative control was performed by AFM imaging of two strains, BHN33 and BHN134, that lack the *rlrA* islet.[1,27] As is shown in Figure 7, these strains do not express any surface organelles. This indicates that the bacteria studied expressed T4 pili. The bacteria were grown on horse blood agar plates with 200 $\mu g\,mL^{-1}$ spectinomycin at 5% CO_2 and 37 °C for 20–24 h.

Mounting and Measurement Procedures: Bacterial pili are thin structures that are not visible in bright field microscopy; however, a trapped bead in an optical trap gives real time information regarding its environment and the forces present. We used FMOT to assess the force–extension behavior of single T4 pili. The system is described in refs. [15,48]. Samples and suspension of *S. pneumoniae* and *E. coli* were manufactured as briefly described here.

The bacteria expressing pili were suspended in a PBS solution (1x, pH 7.4 at room temperature) with 3 μm polystyrene beads (Duke Scientific Corp., Palo Alto, CA). 25 μl of this suspension was then placed between two coverslips. Large 9.6 μm beads (Duke Scientific Corp., Palo Alto, CA) were immobilized to the surface of the lower coverslip through heating at 60 °C for 60 min. Poly-L-Lysine (Sigma–Aldrich, Stockholm, Sweden) was coupled to the large beads in order to provide a positively charged surface for attachment of single negatively charged bacterial cells. This creates strong electrostatic bonds with bacteria, substantially stronger than the bead-pili interaction, which ensures that the bacterium is properly fixed during an experiment (Figure 8A).

A single bacterium was then trapped with the OT and mounted to an immobilized large bead. Moreover, a 3 μm bead was subsequently trapped and brought to close proximity of the mounted

bacterium to interact with pili (see Figure 8B). The coverslip, with the large bead and the mounted bacterium, was retracted from the trapped bead using a piezostage (Figure 8C). The resulting force-extension response of the pilus was probed and recorded until the interaction ruptured or until the retraction was triggered that then brought the coverslip back.

Atomic Force Microscopy: Imaging of bacterial pili using AFM was performed essentially as described earlier with some modifications.[49] Bacterial cells from solid medium were suspended 50 μL in filtered water before 10 μL were placed onto freshly cleaved ruby red mica (Goodfellow Cambridge Ltd, Cambridge). The cells were incubated for 5 min at room temperature and blotted dry before being placed into a dessicator for a minimum of 2 h. Images were collected in a Nanoscope V AFM (using Veeco software) by TappingMode™ with standard silicon cantilevers oscillated at resonant frequency (270–305 kHz) in air at a scan rate of approximately 0.5–1.5 Hz. The final images were flattened and/or plane fitted in both axes using image processing and presented in either height or amplitude (error) mode.

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