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The Structure of an Archaeal Pilus

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Abstract

Bacterial pili are involved in a host of activities including motility, adhesion, transformation, and immune escape. Structural studies of these pili have shown that several distinctly different classes exist, with no common origin. Remarkably, it is now known that the archaeal flagellar filament appears to have a common origin with the bacterial Type IV pilus, and assembly in both systems involve hydrophobic N-terminal α -helices that form three-stranded coils in the center of these filaments. Recent work has identified further genes in archaea as being similar to bacterial Type IV pilins, but the function or structures formed by such gene products has been unknown. Using electron cryo-microscopy, we show that an archaeal pilus from *Methanococcus maripaludis* has an entirely different structure from any of the known bacterial pili. Two subunit packing arrangements were identified: one has rings of four subunits spaced by ~ 44 Å and the other has a one-start helical symmetry with ~ 2.6 subunits per turn of a ~ 30 Å pitch helix. Remarkably, both schemes appear to coexist within the same filaments. For the segments composed of rings, the twist between adjacent rings is quite variable, while for the segments having a one-start helix there is a large variability in both the axial rise and the twist per subunit. Since this pilus appears to be assembled from a Type IV pilin-like protein with a hydrophobic N-terminal helix, it provides yet another example of how different quaternary structures can be formed from similar building blocks. This result has many implications for understanding the evolutionary divergence of bacteria and archaea.

Keywords

helical polymers; polymorphisms; cryo-EM; quaternary structure; scanning transmission EM

Introduction

Archaea possess various kinds of cell surface organelles which differ substantially from those of bacteria¹. Archaeal flagella have only recently been characterized^{2–7}, and all evidence supports the notion that the archaeal flagellar filament is related to the bacterial Type IV pilus^{8–10}. However, no structural or genetic information is yet available on archaeal pili, which are observed on the surfaces of many archaeal species^{11–13}. The structure and cellular functions of archaeal pili remain unknown as does the possibility of whether there are different types of pili found in Archaea, as is well known for bacteria. Recently, a genomic analysis identified a diverse group of archaeal proteins that contain class III signal peptides, similar to

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those found in bacterial Type IV pilins and archaeal flagellins, and suggested that these might assemble into cell surface structures, such as pili¹⁴.

Results

Helical symmetry estimates

We have examined pili from the archaeon *Methanococcus maripaludis* with both negative stain and cryo-EM of frozen-hydrated samples. The pili were isolated following detergent extraction of cells and polyethylene glycol precipitation. The filament appears by both negative stain (Fig. 1a) and cryo-EM (Fig. 1b) as a thin and featureless rod. Filaments appeared thinner by negative stain than they did in the frozen-hydrated state. Since we show (below) using cryo-EM that there is a significant hollow lumen in these filaments, the lack of significant stain penetration into the central lumen in images of negatively-stained filaments is probably due to the flattening of these filaments, so that the lumen is crushed.

Despite the radial shrinkage and likely flattening in negative stain, the negatively-stained filaments were useful in estimating the symmetry of the pili. We extracted 1,907 non-overlapping segments (each 300 pixels or 1,260 Å in length) from images of the negatively stained filaments (Fig. 1a), and then added together the power spectrum from each segment (Fig. 1c). This method is independent of any alignment needed to average together images, and is thus unbiased¹⁵. The resulting power spectrum shows three layer lines. Due to the distance of the peaks from the meridian, and the diameter of the filaments, the layer line at $\sim 1/(17 \text{ Å})$ (cyan line) must arise from a one-start helix. The layer line at $\sim 1/(44 \text{ Å})$ (upper red line) is a meridional layer line corresponding to the reciprocal of the axial rise per asymmetric unit. There is intensity on this layer line on the meridian, but the strongest peaks are off the meridian and must correspond to the second maximum of a Bessel function of order 0. The layer line at $\sim 1/(58 \text{ Å})$ (lower red line) arises from either a three-start helix or a four-start helix, based upon the distance of the peak from the meridian. The apparent molecular mass, determined by SDS-PAGE, of the major pilus subunit is $\sim 17 \text{ kDa}$ (Ng *et al.*, manuscript in preparation). Given the relatively small size of the subunit, it is inconceivable that one could have ~ 0.4 subunits per turn of the 17 Å helix (resulting from the observed 44 Å axial rise per asymmetric unit). On the other hand, if there is a point group symmetry (so that the axial rise per asymmetric unit corresponds to a ring of subunits), one would not observe a layer line at $1/(17 \text{ Å})$ arising from a one-start helix. The most likely explanation of this averaged power spectrum is therefore that the pili are a mixture of point group and one-start helical symmetries, and this averaged power spectrum is showing a superposition of these two symmetries.

In order to establish the helical symmetries for each state, mass per unit length was measured using Scanning Transmission Electron Microscopy (STEM)¹⁶. The STEM results yield a mass-per-unit-length of $1,618.4 \pm 8.8 \text{ (SEM) Da/Å}$ (Fig. 1d). This shows that the averaged axial rise per subunit is $\sim 10.5 \text{ Å}$. The distribution is not obviously bimodal, showing that the two different helical symmetries present must have comparable mass per unit length values. In the case of the one-start helical symmetry (whose pitch is $\sim 17 \text{ Å}$ as suggested by the power spectrum of negatively-stained filaments), the STEM measurements suggest ~ 1.6 subunits per turn. In the case of the point group symmetry, the axial rise per asymmetric unit would be $\sim 32 \text{ Å}$ for C_3 symmetry or $\sim 42 \text{ Å}$ for C_4 symmetry. Since the axial rise per asymmetric unit as suggested by the averaged power spectrum of negatively-stained filaments is $\sim 44 \text{ Å}$ (Fig. 1c), the point group symmetry must be C_4 .

Sorting the filaments and 3D reconstruction

We used the iterative helical real space reconstruction (IHRSR)^{17,18} method to reconstruct the pili from cryo-EM images. The IHRSR approach has been shown to be a powerful method

for reconstructing filaments that are flexible¹⁹, heterogeneous²⁰, suffer from Bessel overlap²¹, or are weakly diffracting²². An interesting question is what happens if one ignores the structural heterogeneity and attempts to use IHRSR on the heterogeneous population of filament segments. We started the procedure with 28,577 overlapping segments (each 480 Å in length) extracted from cryo-EM images of the pili. The IHRSR approach failed to converge to the same solution from different starting points using either of the two symmetries described. This is consistent with our finding that the population must be heterogeneous, and that the failure to achieve convergence in IHRSR is due to heterogeneity^{15,22}. An iterative approach was then used to sort the segments into two groups: C₄ symmetry segments and one-start helical symmetry segments. First, a featureless stacked disk structure with spacings of 44 Å between disks and a continuous one-start helix with a pitch of 17 Å were used as references for an initial sorting. Two groups were generated based on the different subunit packing schemes. Reconstructions were independently generated from the two groups of segments. These two reconstructions were then used as references for a new sorting. With these new references, 54% of the segments were characterized as having C₄ symmetry while the remaining 46% were characterized as having one-start helical symmetry. The validity of the sorting was confirmed by the differences between the averaged power spectra from each group (Fig. 2a,b). The averaged power spectrum for the group sorted as having point group symmetry (Fig. 2a) has a meridional layer line (red arrow) at 1/(44 Å). This layer line is strong and thin, suggesting the axial rise is relatively fixed in this group. The n=4 layer line is not visible, suggesting that the twist between adjacent rings is variable. The averaged power spectrum for the group sorted as having one-start helical symmetry (Fig. 2b) has a layer line at ~ 1/(56 Å) which is weak and broad. The n=1 layer line, observed in negative stain at ~ 1/(17 Å), is not visible in the cryo-EM images which have a much lower signal-to-noise ratio than those in negative stain. We can show (see below) that the failure to see a layer line at ~ 1/(17 Å) is because of additional heterogeneity in symmetry within this group. Further heterogeneity was suggested by the fact that the IHRSR approach failed to converge to the same solution from different starting points in each group (C₄ and one-start symmetries). We therefore attempted to sort each of the two groups into more homogeneous subgroups.

Four-fold rotational symmetry—As suggested by the averaged power spectrum of the first group (Fig. 2a), we expected that most variation is in the twist between adjacent rings. We therefore attempted to use variants of a global reconstruction with different twist imposed as references to sort this group. The variability in twist observed was quite large, with a range on the order of 56° to 88° twist per subunit (data not shown). Although segments can be sorted into these subgroups that differ in twist, that does not necessarily mean that the sorting is valid. Several tests can be done to show that the sorting accurately reflects an intrinsic variability in the data. If we take the segments classified as having a twist closest to 64°, and start the IHRSR algorithm with an initial twist near the population mean, 72°, does the symmetry converge back to a twist of ~ 64°? Similarly, if we take the segments classified as having a twist closest to 80°, and again start the iterations with a twist of 72°, do the cycles converge to a value near 80°? This was indeed the case, arguing that the sorting based upon a variability in twist was valid.

A second, and independent, test for the proposed variability in twist comes from the power spectra. If there is no change in axial rise from ~ 44 Å, and the variability is almost entirely in the twist between rings, then one can predict the change in layer line spacings that should be seen as the twist changes. Since the structure has C₄ symmetry, the pitch of the four-start helices should be $(\Delta z \cdot 360^\circ) / \Delta \phi$, where Δz is the axial rise per subunit and $\Delta \phi$ is the twist per subunit along the four-start helix. The position of the n=4 layer line should therefore be $(4 \cdot \Delta \phi) / (\Delta z \cdot 360)$. For the subgroup with $\Delta \phi = 64^\circ$, the expected n=4 layer line should be at 1/(61 Å); when $\Delta \phi = 72^\circ$, the n=4 layer line should be at 1/(55 Å); and when $\Delta \phi = 80^\circ$, the n=4 layer line should be at 1/(49 Å). Such a large shift should be readily visible. Generating separate

power spectra from the segments initially classified as having a twist closest to either 64° , 72° or 80° (Fig. 3a–c, left halves), the layer lines shift exactly as predicted. Since there is no change in the distance of the peaks from the meridian of the transform as the layer line spacings change, we can eliminate the trivial possibility that the changes we are seeing are simply due to changes in magnification. This variation explains why the $n=4$ layer line is not visible in the global power spectrum of this group (Fig. 2a), and why smaller subsets after sorting generate better power spectra than can be done globally.

We used the IHRSR method to reconstruct the three most populated subgroups. The reconstruction from the segments classified as having a twist of $\sim 64^\circ$ (containing 14% of this group) (Fig. 3d) has C_4 symmetry with an axial rise of 43.9 Å and twist of 65.0° (or -25°) per subunit between adjacent rings. The reconstruction from the segments classified as having a twist of $\sim 72^\circ$ (containing 21% of this group) (Fig. 3e) has C_4 symmetry with an axial rise of 43.8 Å and a twist of 72.0° (or -18°) per subunit between adjacent rings. The reconstruction from the segments classified as having a twist of $\sim 80^\circ$ (containing 12% of this group) (Fig. 3f) has C_4 symmetry with an axial rise of 43.5 Å and a twist of 80.1° (or -9.9°) per subunit between adjacent rings. We do not know the absolute hand of these reconstructions, so the 65° (or -25°) set might actually be -65° (or $+25^\circ$). We will use the convention that the hand is as shown, until such time as the absolute hand can be determined, such as by having an atomic structure of the subunit or by surface shadowing. The power spectra generated from the projections of each reconstruction (Fig. 3a–c, right halves) match the power spectra of the corresponding subgroups (Fig. 3a–c, left halves) very well, which supports the validity of the reconstructions and the sorting. While the different reconstructions differ in the relative twist between adjacent rings, they all show a very similar subunit, similar “fenestrations”, and a very similar modulation of the central lumen. In all three reconstructions the connectivity between asymmetric subunits is along a left-handed four-start helix (Fig. 3e, red line). The lumen at some axial levels is quite constricted (Fig. 3h) while at other axial levels it is quite broad (Fig. 3i). The outer diameter of all the reconstructions is ~ 85 Å, so the large variability in twist does not introduce any variability in diameter. The variable twist also does not introduce any variability in the mass per unit length, which is expected to be ~ 1.56 kDa/Å in this state. This value is quite consistent with the STEM data (Fig. 1d).

Using the 0.5 Fourier Shell Correlation criterion, we find that the resolution of the reconstructions is ~ 20 Å. This estimate is conservative because it compares two independent data sets, in contrast to the standard FSC method that typically compares two halves of a single data set processed by identical methods and aligned to the same reference²³. We think that the main limitation on resolution is the continuous variability of the twist, so that while each of the three sets is more homogeneous than the original population, they are still rather heterogeneous.

One-start helical symmetry—What happens in the group that was sorted to have one-start helical symmetry when we try to further decompose variability? The poor power spectrum of this group (Fig. 2b) suggests strong heterogeneity in the symmetry. We therefore attempted to use variants of a global reconstruction with different symmetries (angular rotation and axial rise per subunit) as references. We used nine arbitrarily chosen symmetries, with three different angles (211° , 216° , and 221°) and three different axial rises (10.7, 11.7, and 12.7 Å). A multi-reference sorting against projections of these reference volumes was used to classify image segments into nine different subgroups. Separate power spectra of segments sorted into three representative subgroups (Fig. 4a–c, left halves) show significant shifts of the layer lines. The layer lines corresponding to one-start helices are not visible, probably because of further variability of pitch within the subgroups and the form of the averaged CTF (see below).

We used the IHRSR method to reconstruct three subgroups, classified as having a twist of $\sim 221.0^\circ$, that generated reasonable power spectra. The reconstruction (Fig. 4d) from the segments classified as having an axial rise of $\sim 10.7 \text{ \AA}$ and a twist of $\sim 221.0^\circ$ (containing 11% of this group) converges to a one-start helical symmetry with an axial rise of $10.8 \text{ \AA}$ and a twist of 220.9° per subunit. This twist is defined along a right-handed one-start helix that would have a pitch of $17.6 \text{ \AA}$ ($10.8 \text{ \AA} \times 360^\circ / 220.9^\circ$). There would also be left-handed one-start helix in this symmetry that has a pitch of $28.0 \text{ \AA}$ ($10.8 \text{ \AA} \times 360^\circ / 139.1^\circ$). The reconstruction (Fig. 4e) from the segments classified as having an axial rise of $\sim 11.7 \text{ \AA}$ and a twist of $\sim 221.0^\circ$ (containing 11% of this group) converges to a one-start helical symmetry with an axial rise of $11.7 \text{ \AA}$ and a twist of 221.1° per subunit (yielding a right-handed one-start helix with a pitch of $19.1 \text{ \AA}$ and a left-handed one-start helix with a pitch of $30.1 \text{ \AA}$). The reconstruction (Fig. 4f) from the segments classified as having an axial rise of $\sim 12.7 \text{ \AA}$ and a twist of $\sim 221.0^\circ$ (containing 12% of this group) converges to a one-start helical symmetry with an axial rise of $13.0 \text{ \AA}$ and a twist of 221.1° per subunit (yielding a right-handed one-start helix with a pitch of $21.2 \text{ \AA}$ and a left-handed one-start helix with a pitch of $33.7 \text{ \AA}$). Each of these three values shown for the axial rise (10.8 , 11.7 and $13.0 \text{ \AA}$) is consistent with the mass per unit length histogram (Fig. 1d).

The agreement between the sorting and the IHRSR reconstructions supports the validity of the sorting, as do the good matches between the power spectra of projections of each reconstruction (Fig. 4a–c, right halves) and the power spectrum of each subgroup (Fig. 4a–c, left halves). Cross-sectional contour plots of the second reconstruction (Fig. 4h,i) show a rather constant lumen, in contrast to the large modulation of the lumen with the C_4 symmetry. The outer diameter is $\sim 85 \text{ \AA}$. The surface views of the three reconstructions (Fig. 4d–f) are similar, except for the differences in axial rise (Fig. 4g). In each of the three reconstructions the connectivity between subunits is along a right-handed two-start helix (Fig. 4e, cyan line) and a left-handed three-start helix (Fig. 4e, red line). Using the 0.5 Fourier Shell Correlation criterion, we find that the resolution of the reconstructions is $\sim 20 \text{ \AA}$. We think that the main limitation on resolution is the continuous variability of the axial rise, in contrast to the C_4 symmetry segments where the main variability is in the twist. This variability also explains why the layer line arising from the left-handed one-start helix is not visible at $\sim 1/(18 \text{ \AA})$ to $1/(21 \text{ \AA})$ in the power spectra from the cryo-EM images (Fig. 4a–c), although a layer line is seen at $\sim 1/(17 \text{ \AA})$ in the global power spectrum generated from negatively-stained filaments that have a much higher signal-to-noise ratio. The change in spacing from the observed $1/(17 \text{ \AA})$ in negative stain to the predicted $1/(18 \text{ \AA})$ to $1/(21 \text{ \AA})$ in ice is consistent with the shrinkage that we see radially in negative stain when compared to cryo-EM. Similarly, we do not see a layer line arising from the right-handed one-start helix at $\sim 1/(28 \text{ \AA})$ to $1/(34 \text{ \AA})$ due to both variability as well as the fact that this would be at a spacing that is near a minimum in the averaged CTF (Fig. 4d–f).

Comparison of the two symmetries—A superposition of the reconstructions with C_4 symmetry and one-start helical symmetry (Fig. 5a) in which one subunit in each has been aligned (black arrow) shows the similarity of the structure in terms of the appearance of single subunits, but huge differences in helical symmetry. The difference in helical symmetry can be understood by looking at the helical nets (Fig. 5b,c), which are schematic diagrams showing the helical lattice on the surface of a cylinder. For the filaments with C_4 symmetry, the main connectivity between subunits is along a left-handed four-start helix. For the filaments with one-start helical symmetry, the connectivity switches to the left-handed three-start helices and right-handed two-start helices. The switch in connectivity may be a way of creating an elastic filament out of basic elements that are themselves relatively inextensible.

Several obvious questions are raised by our results. One is whether individual pili are in one symmetry state (either C_4 or one-start helix) and the switching in symmetry is between filaments, and not within filaments. We have tried to answer this by looking at the four longest pili in our data, and classifying segments from these filaments using multiple references. Each

of these filaments suggested that both states are present within individual filaments. Another question is whether the variability in both helical symmetries is due to two or three discrete states, or arises from a continuum (noting that we would be unable to distinguish between many discrete states and a continuum). By looking at different bins generated by sorting against multiple references, all indications are that the variability represents a continuum. This is reflected by the fact that the layer lines in the power spectra from different bins appear to shift continuously in position, with no indication of a few discrete states.

Discussion

The putative proteins involved in pilus assembly in *Methanococcus maripaludis* have been identified as being similar to bacterial Type IV pili in terms of having an N-terminal signal peptide and a predicted N-terminal hydrophobic α -helix¹⁴. Subsequent genetic and biochemical analysis (Ng et al., manuscript in preparation) has confirmed the essential nature of *MMP0236* (*epdB*) and *MMP0237* (*epdC*), two pilin-like genes from *M. maripaludis*, for pilus formation. The symmetry of bacterial Type IV pili has only been determined unambiguously for the *Neisseria gonorrhoeae* pilus²⁴, in which subunits are related by a 10.5 Å rise and a 100.8° azimuthal rotation along a right-handed one-start helix. The N-terminal hydrophobic α -helices in this bacterial Type IV pilin pack together to form a rather solid core, just as a similar packing is observed in archaeal flagellar filaments composed of subunits that appear to be homologs of bacterial Type IV pilin^{3,10}.

The two *M. maripaludis* pilin-like genes have the short atypical signal peptide ending in a conserved glycine followed by a hydrophobic segment which is typical of type IV pilins. In addition, the signal peptide is removed from *EpdC* by a type IV prepilin peptidase homologue, *EppA*¹⁴. Despite being assembled from a subunit that has putative structural homology with bacterial Type IV pilin, we show here that the quaternary structure of the *M. maripaludis* pilus is entirely different from both the bacterial Type IV pilus and the archaeal flagellar filament. In addition, we show that dramatic differences in subunit packing arrangements can exist, corresponding to two different helical symmetries, and these appear to coexist within the same archaeal pili filaments. An interesting question, one that we cannot answer at this time, is whether the two different symmetries that we observe within these pili are due to the presence of the two different pilin-like proteins, *MMP0236* and *MMP0237*. This coexistence of two very different symmetries is very similar to what has recently been reported for an archaeal *Sulfolobus shibatae* flagellar filament⁴, with two distinct subunit packing arrangements: one has helically arranged stacked disks with C_3 symmetry, while the other has a pure helical symmetry with ~ 3.3 subunits per turn of a one-start helix. While for the *M. maripaludis* pili and the *S. shibatae* flagellar filament the two symmetries coexist, it has been known that within families of closely related filamentous phage two distinctly different helical symmetries exist for different members of the family^{25,26}. The symmetry of the class I particles is a 5-fold rotation axis combined with an a helical rotation of ~ 36° between adjacent rings of subunits, while the symmetry of the class II particles involves ~ 5.4 subunits per turn along a one-start helix. It was suggested that despite this large difference in helical symmetry, the protein packing might be conserved in the two classes of filamentous bacteriophage²⁷. However, our results suggest that protein packing is not conserved between the two forms of the *M. maripaludis* pilus and different contacts must occur between proteins within the same filament.

In contrast to the bacterial Type IV pili that have a rather solid core, in the *Salmonella* flagellar filament the N- and C-terminal α -helical D0 domains from adjacent subunits are tightly packed together²⁸ to form a hollow lumen that has a diameter of ~ 20 Å. The bacterial Type III Secretion System (T3SS) has been known to have homology with the bacterial flagellar system²⁹, and models for various components of the T3SS also involve packing of these α -helices to form a hollow lumen^{15,30}. Here we show that archaeal pili filaments with either

C₄ symmetry or one-start helical symmetry have a central lumen. In the case of one-start helical symmetry, there is a rather uniform central lumen that is slightly smaller (at the available resolution) than what is seen in bacterial flagellar system and T3SS EspA filament. For the C₄ symmetry segments, the central lumen is much more modulated. The naive expectation would be that the archaeal pilus would be more similar to the bacterial Type IV pilus and the archaeal flagellar filament, neither of which have a hollow lumen. The possible role of such a lumen in secretion or assembly of the pilus at the distal end is intriguing. Perhaps archaea assemble flagella by incorporation of subunits at the base like bacterial type IV pili while archaeal pili are assembled by incorporation of subunits at the distal end like bacterial flagella.

While it is generally accepted that sequences diverge more rapidly than structure over the course of evolution^{31,32}, it has been suggested that quaternary structure may be more sensitive to small changes in sequence³³. For example, the overall fold of eukaryotic actin and bacterial ParM has remained relatively similar over huge evolutionary distances³⁴, but the filaments formed by these proteins have diverged greatly³⁵. Dramatic changes in quaternary structure, with only small changes in tertiary structure, may play an important role in evolutionary divergence. Despite being built from a subunit expected to be similar to a bacterial Type IV pilin, we show here that the quaternary structure of an archaeal pilus is very different from both the bacterial type IV pilus and the archaeal flagellum (composed of subunits with homology to bacterial Type IV pilin). What's more, distinctly different quaternary structures exist within the same archaeal pilus. This shows that subunits must have the ability to switch between very different contacts with their neighbors. Polymorphic switching within bacterial flagellar filaments has been extensively studied³⁶ and involves quite small changes in the local packing of subunits that change the macroscopic properties of the flagellar filaments. While we do not yet understand the functions of these archaeal pili, understanding what regulates the polymorphic switching that they can undergo will doubtless be important to understanding functional mechanisms.

Materials and Methods

Purification of pili filaments from *M. maripaludis* ΔflaK

The purification of pili filaments was performed on *M. maripaludis* ΔflaK, which is a non-flagellated but piliated strain. Cells (8 L) were grown for 3 days in Balch III medium at 37°C under an atmosphere of CO₂/H₂ (20:80) with shaking (110 rpm). The cells were harvested by centrifugation (5000×g, 10 mins) and resuspended in buffer (10 mM Tris-HCl, 2% NaCl, pH 7.0). Cell lysis was obtained through the addition of the nonionic detergent OP-10 (Nikko Chemicals Co. Ltd., Tokyo, Japan) to a final concentration of 1% (vol/vol), with DNase-RNase added to reduce viscosity. The reaction was incubated at room temperature with occasional inverting for 90 minutes, after which the sample was centrifuged at 2000×g for 10 min. The resulting supernatant was subjected to polyethylene glycol precipitation with the addition of 1 M NaCl, 20% [wt/vol] polyethylene glycol (M. W. 8000) to a final concentration of 10% (vol/vol) and allowed to incubate on ice, while shaking, for 2 hours. Pellets obtained by centrifugation at 2000×g for 10 min were resuspended and subjected to banding in a KBr gradient for further purification as previously described for flagella isolation³⁷. The diffuse white band containing the pili was removed, diluted with distilled water and pelleted by centrifugation to obtain the final pili sample.

Imaging and Analysis

Two microscopes were used for imaging: a Tecnai 12 transmission electron microscope (TEM) for the negatively stained samples and a Tecnai F20 TEM with a field emission gun source for the unstained frozen-hydrated samples. The negatively stained samples were applied to a carbon film and stained with 2% (w/v) uranyl acetate. The samples for cryo-EM were applied

to either holey-carbon (Quantifoil) or to a continuous carbon film on the grids. The micrographs were taken on film at 30,000 $\times$ magnification (80 keV) for the negatively stained samples and 50,000 $\times$ magnification (200 keV) for the frozen-hydrated samples. Negatives were scanned with a Nikon Coolscan 8000 as 16-bit images using a raster of 4.2 Å/pixel for the negatively stained samples and 2.4 Å/pixel for the frozen-hydrated samples.

The contrast transfer function (CTF) was determined from each cryo micrograph (n=66) using either the carbon film surrounding the hole or the continuous carbon film support. The defocus values spanned the range from 1.7–3.5 μ m. All cryo-EM images were multiplied by the theoretical contrast transfer function (CTF) to correct for phase reversals and to optimize the signal-to-noise ratio. Final reconstructions were then divided by the weighted sum of the squared CTF functions (as the images had been multiplied by a CTF twice: once by the EM, and once by us). For negatively stained samples, no CTF correction was necessary due to the fact that the first zero of the CTF was beyond the resolution of the reconstruction.

We extracted 28,577 overlapping segments, each 200 pixels or 480 Å long, from images of the frozen-hydrated samples. All these segments were down-sampled to 4.8 Å/pixel for data analysis and 3D reconstruction to exclude high-resolution information dominated by noise. We determined that the resolution of the final reconstructions was not limited by the coarser sampling. The shift between adjacent boxes was 10 pixels in all cases. This large overlap improves the signal-to-noise in the reconstruction, as adjacent segments correspond to projections of the structure from different azimuthal angles.

Mass per unit length measurements from STEM

Mass per unit length measurements from Scanning Transmission Electron Microscopy (STEM) were made by similar methods as described previously³⁸. Freeze-dried specimens were prepared according to the standard method of the Brookhaven STEM facility (www.biology.bnl.gov/stem/stem.html). Digital dark-field micrographs of freeze-dried specimens were recorded with 512 $\times$ 512 pixels at raster steps of 1.0 or 2.0 nm per pixel using scanning transmission electron microscope (STEM) from the Brookhaven STEM. The images were analyzed using the PCMass program (available from the Brookhaven STEM resource). The resulting data were normalized to the known mass-per-unit-length of tobacco mosaic virus (131.4 kDa/nm). Histograms were calculated with 1-kDa/nm bins. A Gaussian was then fit to the distribution using the Origin software package (OriginLab company).

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Figure 1. Electron micrographs and helical symmetry estimates

Electron micrograph of negatively stained (a) and frozen-hydrated archaeal pili (b). The scale bar (a) is 250 Å. An averaged power spectrum (c), generated from 1,907 non-overlapping segments (each 300 pixels or 1,260 Å in length) of negatively-stained pili shows three layer lines (black arrows): 1/(17 Å), 1/(44 Å) and 1/(58 Å). The log of the intensities has been taken to reduce the dynamic range and allow all three layer lines to be visible simultaneously. Mass per unit length measurements yielded an average of $1,618.4 \pm 8.8$ (SEM) Da/Å (d).

Figure 2. Sorting based on C_4 symmetry and one-start helical symmetry

The 28,577 segments from the cryo-EM images were sorted into two groups by models with either C_4 symmetry or one-start helical symmetry. 54% of the segments were sorted to have point group symmetry while the remaining 46 % were sorted to have a one-start helix. The averaged power spectrum for the segments sorted as having C_4 symmetry (a) has a meridional layer line (red arrow) at $1/(44 \text{ Å})$. The averaged power spectrum for the segments sorted to have one-start helical symmetry (b) is much poorer, which suggests this group is more heterogeneous than the other group. The broad yet weak layer line at $1/(56 \text{ Å})$ (red arrow) can be interpreted as either $n=2$ or $n=3$, based upon the distance of the first peak from the meridian and the diameter of the filaments..

Figure 3. Sorting of the group with C_4 symmetry

The 15,297 segments of the first class were sorted into five subgroups by differences in the angular rotation between adjacent rings of subunits. The averaged power spectra from the three most populated subgroups (a–c, left halves) are much improved compared to the averaged power spectrum before sorting (Fig. 2a), suggesting reduced heterogeneity in each subgroup. The meridional layer line is fixed while the $n=+4$ and $n=-4$ layer lines shift as expected in the three subgroups (a–c, red lines). The first subgroup was sorted to have a twist of $\sim 64.0^\circ$ and an axial rise of $\sim 43.8 \text{ \AA}$. The averaged power spectrum (a, left half) can be interpreted as having an $n=0$ layer line at $1/(44 \text{ \AA})$, $n=+4$ layer line at $1/(60 \text{ \AA})$ and $n=-4$ layer line at $1/(144 \text{ \AA})$. The reconstruction of this subgroup (d) has a C_4 symmetry with an axial rise of $43.9 \text{ \AA}$ and

a twist of 65.0° . The second subgroup was sorted to have a twist of $\sim 72.0^\circ$ and an axial rise of ~ 43.8 Å. The averaged power spectrum (b, left half) can be interpreted as having an $n=0$ layer line at $1/(44$ Å), $n=+4$ layer line at $1/(53$ Å) and $n=-4$ at $1/(204$ Å). The reconstruction of this subgroup (e) has a C_4 symmetry with an axial rise of 43.8 Å and a twist of 72.0° . The third subgroup was sorted to have a twist of $\sim 80.0^\circ$ and an axial rise of ~ 43.8 Å. The averaged power spectrum (c, left half) can be interpreted as having an $n=0$ layer line at $1/(44$ Å), $n=+4$ layer line at $1/(48$ Å) and $n=+3$ at $1/(350$ Å). The reconstruction of this subgroup (g) has a C_4 symmetry with an axial rise of 43.5 Å and a twist of 80.1° . The power spectra from the projections of each reconstruction (a–c, right halves) match the corresponding averaged power spectrum of each subgroup (a–c, left halves). The reconstructions from the three subgroups are very similar, except for the difference in twist (d–g). The surface thresholds have been chosen to enclose 100% of the expected molecular volume. In all three reconstructions the main connectivity between asymmetric subunits is along a left-handed four-start helix (e, red line). A superposition of the two reconstructions (g) in which one subunit in each has been aligned (black arrow) shows a 15.1° difference in subunit twist at a subunit ~ 44 Å away (red arrow). The yellow reconstruction has a twist of 65.0° and the cyan one has a twist of 80.1° . Two cross-sectional contour plots (h,i) that are spaced 24 Å away from each other have different outer diameters and different size lumens.

Figure 4. Sorting of the group with one-start helical symmetry

The 13,280 segments of the second group were sorted into nine subgroups by differences in the angular rotation and axial rise per subunit. Nine arbitrarily chosen symmetries, with three different angles (211° , 216° , and 221°) and three different axial rises (10.7, 11.7, and 12.7 Å) were used. The averaged power spectra of three representative subgroups (a–c, left halves) are much improved compared to the averaged global power spectrum before sorting (Fig. 2b), suggesting reduced heterogeneity in each subgroup. The layer lines shift in different subgroups (a–c, red lines) as expected. The first subgroup was sorted to have a twist of $\sim 221.0^\circ$ and an axial rise of ~ 10.7 Å. The averaged power spectrum (a, left half) can be interpreted as having an $n=-2$ layer line at $1/(47 \text{ Å})$ and $n=+3$ at $1/(66 \text{ Å})$. The reconstruction of this subgroup (d)

has an axial rise of 10.8 Å and a twist of 220.9°. The second subgroup was sorted to have a twist of ~221.0° and an axial rise of ~11.7 Å. The averaged power spectrum (b, left half) can be interpreted as having an $n=-2$ layer line at $1/(50 \text{ Å})$ and $n=+3$ at $1/(72 \text{ Å})$. The reconstruction of this subgroup (e) has an axial rise of 11.7 Å and a twist of 221.1°. The third subgroup was sorted to have a twist of ~221.0° and an axial rise of ~12.7 Å. The averaged power spectrum (c, left half) can be interpreted as having an $n=-2$ layer line at $1/(56 \text{ Å})$ and $n=+3$ at $1/(82 \text{ Å})$. The reconstruction of this subgroup (g) has an axial rise of 13.0 Å and a twist of 221.1°. The power spectra from projections of each reconstruction (a–c, right halves) match the averaged power spectrum of each class (a–c, left halves). The surface threshold for each of the reconstructions has been chosen to enclose 100% of the expected molecular mass.

The reconstructions from the three subgroups are very similar, except the difference in axial rise (d–g). In all the three reconstructions, the connectivity between subunits is along a right-handed two-start helix (e, cyan line) and a left-handed three-start helix (e, red line). A superposition (g) of two reconstructions (d,f) in which one subunit in each has been aligned (black arrow) shows a 13.2 Å difference in axial rise at a subunit ~70 Å away (red arrow). The yellow reconstruction has an axial rise of 10.8 Å and the cyan one has an axial rise of 13.0 Å. Two cross-sectional contour plots (h,i) spaced 9.6 Å away from each other show similar outer diameters and lumen size.

Figure 5. Comparison between the two subunit packing schemes

A superposition of the reconstructions with C_4 symmetry and one-start helical symmetry (a) in which one subunit in each has been aligned (black arrow) shows the similarity of the structure of each single subunit but huge difference in subunit packing. The yellow surface has a one-start helical symmetry with a twist of 221.0° and an axial rise of $11.7 \text{ \AA}$. (Fig. 3e), while the cyan surface has C_4 symmetry with an axial rise of $43.8 \text{ \AA}$ and a twist of 72.0° (Fig. 4e). The aligned subunit in the cyan structure is shown as mesh for clarity.

A helical net (b,c) shows the lattice of subunits on the surface of a cylinder, using the standard convention that the cylindrical surface has been cut open and we are looking at the inside of the surface. Two helical families are labeled in the helical net for the C_4 helix (b). Within segments having this symmetry, the strongest observed connectivity between subunits is along the left-handed four-start helices. Three helical families are labeled in the helical net for the one-start helix (c). A left-handed one-start helix having ~ 2.6 subunits per $30 \text{ \AA}$ pitch turn is labeled. The strongest observed connectivity between subunits within segments having a one-start symmetry is along the left-handed three-start helices and the right-handed two-start helices.