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The Three-Dimensional Structure of CFA/I Adhesion Pili:

Traveler's Diarrhea Bacteria Hang on by a Spring

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Summary

To survive the harsh environment of a churning intestinal tract, bacteria attach to the host epithelium via thin fibers called pili (or fimbriae). Enterotoxigenic *Escherichia coli* expressing CFA/I pili and related pili are the most common known bacterial cause of diarrheal disease, including traveler's diarrhea. CFA/I pili, assembled via the alternate chaperone pathway, are essential for binding and colonization of the small bowel by these pathogenic bacteria. We elucidate unique structural features of CFA/I pili that appear to optimize their function as bacterial tethers in the intestinal tract. Using transmission electron microscopy of negatively stained sample in combination with iterative three-dimensional helical reconstruction methods for image processing, we have determined the structure of the CFA/I pilus filament. Our results indicate that strong end-to-end protein interactions and weak interactions between the coils of a sturdy spring-like helix provide the combination of strength, stability, and flexibility required to sustain bacterial adhesion and incite intestinal disease. We propose that CFA/I pili behave like a spring to survive the harsh environment of a churning intestinal tract, thereby persisting long enough for these bacteria to colonize the host epithelium and cause enteric disease.

Keywords

Adhesion Pili; Fimbriae; ETEC; Helical Reconstruction; Electron Microscopy and Image Processing

Enteric bacterial infection is a significant worldwide health risk, particularly for infants and small children. Enterotoxigenic *Escherichia coli* (ETEC) are the most frequently isolated cause of community-acquired childhood diarrhea in the developing world, accounting for over 200 million episodes of illness ¹; ² and 380,000 deaths ² per year, and the most common cause of travelers' diarrhea ³. Bacterial adhesion is a key initiating step in the infection process, frequently correlated with the ability of bacteria to colonize host tissue. Adhesion is mediated by colonization factor antigens, with Colonization Factor Antigen I (CFA/I) a commonly isolated virulence factor (see, *e.g.* review by Qadri *et al.* ⁴, and earlier references ⁵; ⁶). The long, thin surface filaments of CFA/I pili (also called "fimbriae") mediate attachment of ETEC to the small intestine during the earliest pathogenic stages in the host, and are essential for binding and colonization of the small bowel ⁷, yet few details of their fine structure are known.

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CFA/I is the archetype of eight genetically related ETEC colonization factors, and is recognized as both a virulence factor⁷ and protective antigen^{8; 9}. As such, CFA/I pili have served as a target antigen for ETEC vaccine development (e.g. 8; 10; 11). In the past three decades, several vaccine candidates have been tested in humans. Nearly all included a heat-labile enterotoxinoid (LT) and one or more CF pili, but none has performed well enough for general licensure and usage.

Pili from the “alternate chaperone pathway” share nominal genetic similarity to those from the “chaperone-usheer” pathway, yet the similarity of their assembly and structure via donor strand exchange is striking^{12; 13; 14}. In both pathways, pilins are secreted from the cell via the General Secretory Pathway, cross the periplasm complexed to a chaperone protein, and are assembled at the outer membrane by exchanging a beta-strand from the chaperone for the N-terminal extension beta-strand of the subsequent pilin subunit. This exchange occurs at or near an outer membrane protein that acts as an usher. Type 1 pili and P-pili (from uropathogenic *E. coli* that cause urinary tract infections involving the bladder and kidneys, respectively), as well as Hib pili (from *Haemophilus influenzae type B* bacteria that cause ear infections, pneumonia, and meningitis in infants and the elderly) are all members of the chaperone-usheer pathway. Each requires a chaperone, usher, major pilin, and 3, 5, or 2 minor pilins, respectively. All are helical structures 7-8 nm in diameter, approximately 1 µm in length^{15; 16; 17}, with 3.0-3.3 subunits per turn of the helix. CFA/I pili, from the alternate chaperone pathway, comprise a chaperone, CfaA, an usher, CfaC, an adhesin, CfaE, and the major subunit, CfaB; no minor pilins are required. Despite the marked genetic sequence divergence between these CFA/I pilins and those from the chaperone-usheer pathway¹⁸, we show here that their overall architecture is similar: CFA/I pili are long helical filaments, ~7.4 nm in diameter, on average 1 µm in length, with 3.17 subunits per turn of the helix (data below).

Pili assembled by the chaperone-usheer or alternate chaperone pathways share structural morphology across strains and species, yet each system is uniquely designed to facilitate bacterial survival in its preferred host microenvironment. A comparison of P-pili and Hib pili showed such specialization of pilus structures¹⁹. The major subunit-subunit interactions in Hib pili are essentially parallel to the helical axis¹⁹, producing a strong ropelike structure of three strands wrapped around each other, that cannot unwind under the propulsive forces generated by coughing and sneezing. P-pili have strong interactions that wrap around the helical axis, allowing these pili to unwind into thin fibrillar structures^{17; 20}, maintaining adhesion during urination. Our current studies demonstrate that the architectural organization of CFA/I pili, like that of P-pili and Hib pili, is designed to enhance bacterial pathogenicity in its target tissue, the small intestine. Presenting CFA/I pili on their surface, ETEC are able to stick to the gut lining in the face of rhythmic muscle contractions that variously stimulate vortex mixing and downward flow of the intestinal contents.

Images of isolated, negatively stained CFA/I pili

Most CFA/I pili are long, fairly straight, helical filaments (Figure 1, black arrows), 7.4 nm in diameter and, on average, 1 µm in length. Additional morphologies observed by transmission electron microscopy include gentle curvature of the filament (Figure 1, black arrowheads), sharp bends in the filament (Figure 1, white arrowheads), and regions of the filaments that are unwound to a thin fibril approximately 2-3 nm in diameter (Figure 1, white arrows). Similar structural polymorphism has also been shown for P-pili^{17; 21}, although P-pili have a higher frequency of sharp bends.

Information about the morphological diversity of these pili helps elucidate the correlation between their structure and function. For example, the presence of extended fibrillar structures demonstrates that subunit-subunit interactions are stronger between adjacent subunits (n to n

+1) than between subunits connected along the long axis (n to n+3 or n+4). This behavior is also observed in uropathogenic P-pili, but not in oronasopharyngeal Hib pili (described more fully below).

3-D reconstruction of CFA/I pili

A modified version²⁰ of the Iterative Helical Real Space Reconstruction method (IHRSR; 22) was used for computing the 3-D reconstruction, using SPIDER and WEB software²³. As required for IHRSR, images of intact, relatively straight filaments were selected and boxed as overlapping segments with 90% overlap. It has been shown previously²⁴ that the adhesin, CfaE, is located at the tip of the pilus, and that the major pilin, CfaB, comprises the rest of the helical filament. We have not included the end region of any pili in our data analysis, and therefore report here the macromolecular structure of CfaB assembled into an helical filament.

Shown in the montage (Figure 2a) are 20 of the 49,264 overlapping areas (“particles”) selected from 486 CFA/I pilus filaments on 17 electron microscope images. After rotational alignment and discarding the “bad” images manually, 35,281 were used in refinement of the three-dimensional reconstruction of CFA/I pili.

A prerequisite of the IHRSR method is an approximate helical structure to be used as the initial reference for the iteration procedure. The structures of the CFA/I filament and the CfaB monomer were not available to provide this initial model. Based on the vulnerability of IHRSR to dissimilarity of the initial model with the final true structure, we used the approximate filament diameter measured from our electron micrographs and the known molecular weight (~15kDa) of the CfaB monomer²⁵ to test a series of hypothetical helical models in order to find a suitable one to be used as the initial model for our reconstruction. These hypothetical models are composed of structureless spheres, with radius compatible with the molecular weight of CfaB, positioned into helices with variable parameters for the helical rise per subunit and the rotation of subunits about the helical axis. Tests to obtain the initial reference model included only the best 15% of the data: the 5,529 particles with the highest cross-correlation to the starting model. Test models were built from spheres arranged with the following range of parameters:

Rise per subunit (δz) : 5.6Å - 11.2Å, step size of 0.8Å

Rotation angle between subunits (ϕ): 82° - 152°, step size of 10°

Criteria for a “successful” model are that both δz and ϕ converge in twenty cycles, and the electron density discrepancy between symmetry-related regions is minimized.

The best model (smallest discrepancy between symmetry-related regions; Figure 2b,c, lowest value curves) converged to a solution for the three-dimensional structure of CFA/I pili (Figure 2e). The second-best, and other models, have higher electron density discrepancies between symmetry-related regions (Figure 2b,c) and/or do not converge to a solution (Figure 2d).

This best preliminary model was used as the new starting reference model and 100 iterative cycles were run to refine the 3-D reconstruction. For these iterations, 35,281 particles (of the initial 49,264) were used in each cycle, and the final reconstruction includes the top 70% of these data (24,700 particles). The second best starting model was also refined, but did not converge (Figure 2d), and had significantly higher electron density differences (Figure 2c).

The hand of the helix was determined by first coating the top surface of the pili with platinum, deposited by rotary shadowing. Computed Fourier transforms of pili imaged by electron microscopy were one-sided, since the bottom surface of the helix was not metal-coated and

therefore provided no signal. Analysis of these Fourier transforms showed that CFA/I pili have a right-handed genetic helix, as do P-pili and Hib pili (data not shown).

Results from our 3-D reconstruction of CFA/I pili (Figure 3) establish that these are helical filaments ~ 7.4 nm in diameter, with a repeat of 19 subunits in 6 turns of the helix (3.17 subunits per turn). There is an $8.3 \text{ \AA}$ ($=0.83$ nm) rise per subunit along the helical axis, and a rotation between subunits of 113° around the helical axis. The resolution of the reconstruction is $12.5 \text{ \AA}$, as determined by the 0.5 cutoff of the Fourier shell correlation produced by a comparison of the data randomly split into two half-data sets. Data were collected at defocus values that did not require correction for the contrast transfer function, and yield about the best resolution attainable for negatively stained samples. The reconstruction reported here is now being used as an initial model for a higher resolution electron cryomicroscopy study of these pili.

A surface view of the reconstruction (Figure 3a) shows a left-handed long pitch helix, with subunits oriented approximately horizontally (perpendicular to the helical axis). A projection map of the CFA/I pilus reconstruction (Figure 3b) mimics the data visible in electron microscope images, as can be seen by comparing this result to the electron microscopy data (Figure 1 and Figure 2a). A cross-section of the CFA/I filament (Figure 3d) is shown taken at the position of the white arrow (Figure 3b).

Each CfaB subunit has a bi-lobed appearance. This is visualized clearly in a surface view of the 3-D reconstruction displayed with a high density threshold cut-off (Figure 3c, white and black outline). This view is also used to show the relationship between subunits, where the adjacent subunit to n is $n+1$, and $n+3$ connects to n via the long-pitch helix.

Adhesion pili are customized for the microenvironment in which they function

To examine the structural specialization required for bacterial adhesion and persistence of attachment in different microenvironments, we have carried out a comparison of our 3D reconstruction of CFA/I pili to those of uropathogenic and respiratory pili (P-pili and Hib pili, respectively). Our results illustrate both the similarities of the basic morphologies and the specialization required for survival in different microenvironments (Figure 4). All three pilus structures are 7-8 nm diameter helices with a central axial hole 2-2.5 nm in diameter. The number of subunits per turn of each helix is similar: 3.17, 3.28, and 3.00 for CFA/I pili, P-pili, and Hib pili, respectively. The major pilin subunits are approximately cylindrical, with diameters of ~ 2 -3 nm and heights of ~ 5 -6 nm. The Hib pilin, HifA is about 20% larger, with the extra mass appearing as a bulge extending out from the cylinder on one side¹⁹.

What varies dramatically are 1) the orientation of the subunit in the filament, and 2) the relative strength of subunit-subunit interactions. While in CFA/I pili and P-pili the subunits are oriented approximately perpendicular to the helical axis, in Hib pili the subunits are almost vertical. This difference in subunit orientation has structural and functional consequences. CFA/I pili and P-pili^{17; 20} have strong n to $n+1$ subunit-subunit interactions, whereas in Hib pili this interaction is either extremely weak or non-existent (Figure 4, yellow arrows). Since in Hib pili the major subunit is tipped almost 90 degrees, the dominant subunit-subunit interactions are along the helical axis (n to $n+3$) rather than around the filament (Figure 4, white arrow). For these pili, deployed by a respiratory disease pathogen, the strength of this connection produces a strong, three-stranded, ropelike structure that is expected to prevent disadvantageous unwinding of the filament during the high velocity shear forces generated by coughing and sneezing¹⁹.

Based on our 3-D reconstruction, CFA/I pili appear to have significantly weaker interactions between subunits on adjacent turns of the helix than do P-pili. That is, the contact between the n and the $n+3$ subunits is weaker in CFA/I pili (Figure 4, white arrows), presumably permitting more facile dissociation. The breakage of the link between adjacent turns of the CFA/I helix could lead to unwinding or to more complex undulations in response to movements in the surrounding fluid (Figure 1). For instance, if the contacts from n to $n+3$ are broken, but the (symmetrically equivalent) contacts from $n+3$ to $n+6$, and from $n+6$ to $n+9$, *etcetera*, are unperturbed, the changes in shape and length caused by dissociation of the n to $n+3$ subunits would lead to a limited local unstacking that could accommodate motion of small sections of the filament without filament breakage and without unwinding of the filamentous structure. Such mobility might be akin to bouncing a toy *Slinky*®, with regions temporarily separating and rejoining. This type of motion could support the ability of CFA/I pili to withstand the peristaltic or churning motions in the gastrointestinal tract, thereby enabling sustained binding of ETEC to the intestinal lining.

Virulence is a complex integration of many factors, where each bacterial pathogen possesses an array of mechanisms designed specifically to adhere, evade the host's immune responses, and promote infection. Simultaneously, the host is trying to recognize and mount an attack against any such invaders. It is possible to limit or eliminate infection by interfering with bacterial binding to the host, providing the impetus for our studies on pili whose sole function is adhesion. Our studies on the architecture of adhesion pili have begun to elucidate strategies by which bacteria remain attached to the host cell under physiologic conditions designed to thwart infection. It is hoped that detailed structural knowledge of CFA/I pili will promote design of new interventions to prevent ETEC disease.

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Figure 1.

Enterotoxigenic *E. coli* (ETEC) express CFA/I pili on their surface. These pili are $\sim 1 \mu\text{m}$ long helical filaments, 7.4 nm in diameter. Isolated pili either retain their helical structure and are visualized as straight filaments (black arrows) gently curved (black arrowheads), or sharply bent filaments (white arrowheads), or appear to be unwinding into extended thin fibrillar structures (white arrows). Isolated CFA/I pili were placed on carbon-coated copper grids, stained with 1% uranyl acetate, and imaged in a Philips CM12 or FEI TF20 electron microscope. Images were taken at 60,000x or 50,000x, respectively. Magnification bar, 250Å.

Figure 2.

(a) Sample CFA/I pili “particles”, including segments that will be discarded: row 2, column 1 due to the bend in the filament, and row 2, column 5 due to the crossing filament. The particle in row 4, column 3 will be discarded if it is not centered properly in subsequent iterative cycles. Images for the reconstruction were all taken on an FEI TF20 electron microscope, and selected using the EMAN boxer software ²⁶, and processed using a modified version of the IHRSR ²² in combination with the SPIDER/WEB software package ²³. Magnification bar, 50 Å. (b) Differences in electron density of symmetry-related regions of five sample starting models. Shown here are starting models with a rotation angle of 112° between subunits, and a rise per subunit of 5.6-11.2 Å in steps of 0.8 Å. The two models with the lowest electron density differences in the final iteration have converged to the same solution from different starting symmetries. The full dataset was used to test the best and second-best starting models. (c) The electron density differences were consistently lower in the best model. (d) The second-best starting model did not converge to a solution, even after 100 iterative cycles, whereas the best starting model (e) converged quickly. In panels (d) and (e) both the rotation angle (lighter line) and the rise per subunit (darker line) are shown in the graphs. In all panels, the abscissa corresponds to the iterative cycle number.

Figure 3.

3-D reconstruction of CFA/I pili showing that the right-handed helical filament has a repeat of 19 CfaB pilin subunits in 6 turns of the helix (3.17 subunits per turn) with a 0.83 nm rise per subunit along the helical axis. Shown here are (a) a surface view of the structure, (b) a projection view which mimics the original EM data, (c) a surface view showing only the high electron density of the structure; this view is helpful for viewing the bi-lobed domain structure of the CfaB subunit (white and black lines) and to see the progression of subunits along and around the helical filament: subunit n is adjacent to $n+1$, subunit $n+2$ is around the back of the filament, and subunit $n+3$ is the subunit which stacks above subunit n in the coiled helical structure. (d) a cross-section of the helix is shown as a grey-scale map; this view is taken at vertical location marked by the arrow in panel (b). Magnification bar in panel (d), 50 Å, is for all panels.

Figure 4.

To compare the structures of CFA/I pili, P-pili, and Hib pili, surface views of enterotoxigenic *E. coli* CFA/I pili (left, green), uropathogenic *E. coli* P-pili (middle, pink) and oronasopharyngeal *H. influenzae* Hib pili (right, blue) are shown at high electron density thresholds. These surface views show only the strongest protein density, and for clarity, the far side of each helix is not shown. The strong connections between subunits around the helical axis of CFA/I pili (left panel, yellow arrow), and the relatively weak connections between subunits that are the stacked coils of the helix (left panel, white arrow), are compared with P-pili (center panel) and Hib pili (right panel). Note that Hib pili have essentially no connections around the helical axis, and therefore the yellow arrow appears to point to a black region of the map. Under stress a morphological transition may confer limited unstacking properties to CFA/I pili, allowing motion without breaking, thereby supporting sustained binding of ETEC in the gastrointestinal tract.