Enterotoxigenic *Escherichia coli* CS1 Pilus: Not One Structure but Several

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The chaperone/usher pathway describes the biogenesis of a diverse group of more than 200 extracellular polymers that mediate bacterial adhesion to host cells (1). Well-studied exemplars include P pili and type I pili of uropathogenic *Escherichia coli* (2), the “alternate” chaperone/usher pathway pili CS1 and CFA/I of enterotoxigenic *E. coli* (3, 4), the common pilus of many pathogenic and commensal *E. coli* bacteria (5), and secreted nonpilus adhesins, such as the F1 antigen of *Yersinia pestis* (6). The hallmarks of these systems are the structures of the subunits and their assembly mechanisms.

Each subunit ultimately adopts a seven-stranded β-sandwich (an immunoglobulin [Ig]) fold. But, seemingly in contradiction to Anfinsen’s hypothesis that all of the requisite information for protein folding is contained within a polypeptide (7), in this fascinating class of proteins the expected C-terminal β-strand (strand G) is lacking (8, 9). Expressed heterologously, these incomplete folds are unstable. The G strand is donated by an N-terminal extension (Nte) of the subsequent subunit to be added at the base of the filament (10). The Nte completes the 3-dimensional (3D) fold of the subunit, affording an assembled polymer with remarkable stability and blurring the lines between tertiary and quaternary structure.

The fundamental mechanism of the chaperone/usher pathway is conserved and fantastical, an important biophysical story for microbiologists. Assembly of subunits relies on a periplasmic chaperone that protects subunits from premature interactions and an outer membrane usher that guides subunits to their proper liaisons. There has been dizzying progress in the past dozen years on the structural and thermodynamic bases for assembly (1, 2, 11–14). In outline, the chaperone “complements” the fissure in the subunit by “donating” the 7th β-strand in a process aptly named donor strand complementation. The usher, which is an outer membrane β-barrel, promotes the displacement of the chaperone’s complementing strand from the bottom-most subunit and the zipper in of the Nte from the next subunit, still stabilized by its own interaction with a chaperone and poised at the usher. This process is called donor strand exchange. In most systems, more than one subunit type is assembled serially into the polymer, and the order of these is precisely controlled by the relative affinities of Ntes for their cognate binding pockets in pilin grooves (15).

In this issue, Galkin, Kolappan, et al., from the collaborating groups of Egelman and Craig, describe the X-ray crystal structure of the CS1 pilus major subunit CooA, as well as the electron microscopy (EM) reconstruction of the native filaments. They combined the two types of data to yield a detailed molecular model for the interactions of CooA subunits within the filament (16). This hybrid approach is a strong suit of the Craig/Egelman collaboration (17, 18). The unique conclusion is that the CS1 filaments populate multiple helical packing arrangements. The molecular models now available for the CooA-CooA interactions within the filaments are detailed enough to predict which residues must change conformation in order to accommodate the distinct pilus structures.

In order to build the highest-quality molecular model of a large biological assembly using results from multiple techniques, each type of data should be collected from the same protein(s). Thus, although a structure was available for CfaB, the 53% sequence-identical subunit of Cfa/I pili, determining the crystal structure of the CooA subunit was a necessary step. In this and previous crystallographic analyses (3, 5), a clever cut-and-paste of the codons for the Nte amino acids onto the 3’ end of the pilin gene allowed expression of soluble subunits in which the donor strand is appended at the C terminus (donor-strand-complemented, or DSC, subunits). The CooADSC crystal structure confirmed that the 3D structures of CooA and CfaB are nearly identical. Indeed, even a homology model of CooA built using the software program Phyre2 (19) using CfaB as a template leads to a predicted structure that mimics the experimentally determined one. Thus, the skillfully executed CooA crystal structure did not on its own lead to novel biological insights but rather was a key piece of a larger story.

EM experiments supplied the remaining crucial data—the envelope of the CS1 pili and the revelation of polymorphism in the filaments—that allowed merging of the CooA X-ray crystallographic coordinates with the lower-resolution helix structure. The average overall architecture of the purified CS1 filaments was a key starting point and was determined by combining results from three EM techniques. First, helical symmetry based on calculated diffraction patterns from transmission EM (TEM) images of negatively stained filaments reports a major spacing at 27.6 Å for the 1-start helix. Pili diameter is also estimated from these TEM images. Second, scanning TEM is the technique of choice for measuring the mass/unit length of filaments. Given the observed value of 1.76 kDa/Å and the known mass of each CooA monomer (15.2 kDa), it is straightforward to calculate that the filament rises 8.6 Å for every subunit. If the one-start helix has a major repeat distance, or pitch, of 27.6 Å and there is 1 subunit every 8.6 Å, then there are 3.2 subunits per turn of the helix. Third, the handedness of the helix must be established. However, the untitled negative-stain TEM images do not preserve any information about hand, because they are essentially projections of each filament onto a plane.

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By shadowing samples from an angle with a spray of heavy metal, only one side of filaments is imaged, and calculated diffraction patterns report on the handedness of the helix (20); in this case, the 1-start helix is right-handed.

With this set of average helical parameters in hand, determination of the ∼20-Å resolution map of CS1 pili proceeded by iterative helical real space reconstruction (21) (as opposed to classic Fourier-Bessel treatment [22]). Furthermore, segments of filaments were treated as single particles and could be binned into three self-consistent groups that refined with distinct helical parameters. The accuracy to which these parameters could be distinguished is impressive. Two of the classes are similar; an approximately 25° rotation of the subunits about their own long axes changes the predicted packing interactions between the \( n+1 \) and \( n-2 \) subunits without disrupting the helix. The third class has a rise which differs by 1.3 to 1.4 Å and a twist that differs by 1.2 to 1.3° from the first two. Not only are the vertical interactions between the \( n+1 \) and \( n-2 \) subunits altered, but even the donor strand complementation interactions between subunits \( n \) and \( n+1 \) must change in order for the crystal structure of the subunit to be able to pack into this filament. Galkin, Kolappan, and coauthors suggest a dramatic sliding of the A1 β-strand by five residues so that a loop between A1 and A2 takes the place of the A1 strand, and the A1 residues serve to elongate the Nte so that subunits can be further apart (see Fig. S5 in the supplemental material for reference 16). The recent crystal structure of the common pilus EcpA subunit provides an example of such a loosened A1 strand (5).

The take-home message is that CS1 pili are heterogeneous, with \( n+1:n-2 \) packing interactions of CooA subunits that dictate at least four helical classes: two very similar well-packed structures, a more-steeply rising helix that plausibly occurs when donor-strand-complementing subunits slip apart because of repositioning of \( β \) strand A1, and a very thin, fully unraveled filament which maintains only \( nn+1 \) interactions. Was this polymorphism expected, and what are the implications?

More than 15 years ago, it became clear through negative-stain EM studies of type I pili that there were two incarnations of these filaments, wound and unraveled (23). Polymorphism of the wound structures was suggested (24), but no high-resolution reconstructions were available, nor were crystal structures of the subunits. Type IV pili were also reported to undergo dramatic unraveling when subjected to shear stresses (25). Now, with the advent of the real space helical reconstruction refinement procedure, Vibrio cholerae type IVb pili and archaeal flagella have similarly been found to exhibit polymorphism (18, 26). It seems certain that as this technique becomes a mainstream method for analyzing helical filaments, observations of polymorphism will become the norm.

The second exciting outlook from this study and others that combine X-ray crystallography and EM reconstructions is that we are beginning to understand the localized discrete structural changes in subunits that propagate to cause long-range changes in helical structures or change their antigenic or adhesive properties. The structural plasticity of the type I pilus adhesin FimH increases adhesion under high flow forces (27) via a “finger trap” model for FimH conformational change (28). CfaB uses cis-trans isomerization of a conserved proline to transition from its wound to its unraveled state (3). Still to be worked out is the nature of the structural shift in type IV pilin subunits during unraveling.

In closing, there are a few general guidelines for authors, reviewers, and readers that could make this and other exciting papers that capitalize on hybrid structural methods optimally accessible and therefore of greatest value to Journal of Bacteriology readers.

(i) Structures must be deposited in appropriate databases; atomic coordinates (and structure factors) in the Protein Data Bank (29) and density maps from EM reconstructions in the EM-DataBank (30). Such deposition is a requirement of this and most journals, is universally met, and has completely changed the way the scientific community interacts with structural information. For the current CS1 results reported by Galkin et al., coordinates have pdb code 4HJI and the three distinct helical assemblies have EMDataBank codes 1951, 1952, and 1953 (16).

(ii) Taking this a step further, metadata that accompany coordinates and/or density maps should provide as much user-friendly information as possible. Unlike deposition itself, this is not a requirement for publication, but it is crucial for maximum impact. For example, if an article describes the fitting of atomic coordinates into an EM-derived electron density map, then the fitted coordinates can be included in the EMDataBank deposition to facilitate visualization of biologically important details. Deposits that link EM maps to preoriented pdb files are immediately visualizable in standard software programs such as PyMOL (31). Similarly, if a paper describes the use of an X-ray structure(s) to model a higher-order assembly, authors should at least provide within the pdb file the matrix transformations needed to recreate the biological assembly and at best actually make the assembly coordinates available as a supplemental file. These simple steps would allow more readers to become familiar with the deliverables of structural biology.

(iii) Structures should be made to come alive in articles. The use of pdb files with embedded, manipulatable 3D viewing (32) is already encouraged by some journals (33). Another straightforward option is to include a link to the NSF-supported Java-based application Firstglance (http://bioinformatics.org/firstglance/fgij/) that will allow the reader to call up the pdb file in a simple Web-based viewer that is browser independent.

(iv) Wording must maintain rigor and accuracy without snubbing microbiology readers who are not card-carrying structural biologists. Structural biology papers in a microbiology journal are special because while the broad impact of the results may resonate with the audience, the readership may not be intimately familiar with the techniques. It is incumbent on authors to make it possible for readers to assess their work.

(v) Conversely, readers should take the time to learn to assess structure papers critically, which does not mean learning how to solve a structure. Like any other experimental methods, crystallography, NMR, and EM have strengths, limitations, and statistics! Journal of Bacteriology readers should download an easy-to-use and readily available three-dimensional viewing program such as PyMOL (http://www.pymol.org/) (31), Jmol (http://www.jmol.org/), or Swiss PDB viewer (http://www.expasy.org/spdbv/) (34) and learn its fundamentals. The consumer will reap additional benefits if her reading of an article is accompanied by a hands-on exploration of the structure. As the launching point for developing hypotheses about biological function or as the culmination of years of genetic and biochemical research, structural results provide much more tangible promise for explaining the microbial world than the aesthetic thrill of “seeing” biological macromolecules.
REFERENCES