A Nice Return on the "Stalk" Exchange

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Type IV Pili (T4P) are protein filaments widely used by bacterial pathogens for adherence and motility. In this issue of *Structure*, Li and colleagues made clever use of hydrogen/deuterium exchange mass spectrometry techniques to reveal surprising insights into the ultrastructure of T4P.

Type IV Pili (T4P) are 1–4 μm long, <10 nm in diameter, highly flexible and retractable protein fibers expressed by a wide variety of bacteria, including the human pathogens *Neisseria gonorrhoeae, Pseudomonas aeruginosa, Legionella pneumophila, Haemophilus influenzae*, and *Vibrio cholerae*. Bacteria use T4P for adherence to chemically diverse surfaces (both nonliving and living; Figure 1), for uptake of extracellular DNA, and for a form of flagellum-independent, surface-associated motility called "twitching," wherein cycles of pilus extension, tethering, and retraction result in rapid translocation of the cell body. T4P belong to the larger, structurally related type II secretion (T2S) system family, in which a short "pseudopilus" is used to secrete folded proteins and toxins from the periplasm of Gram-negative bacteria to the external milieu.

T4P are composed of small (15–20 kDa) protein subunits called pilins (or pseudopilins in the case of T2S), whose three-dimensional structures are now emerging from a number of systems (Craig et al., 2003; Hazes et al., 2000; Kohler et al., 2004; Parge et al., 1995; Yanez et al., 2007). There are two related subclasses of T4P, type IVa and type IVb, which differ in their overall size and length of their signal sequences (Craig et al., 2004). Based on the structures solved to date, a typical type IV pilin of either subclass has an extended, hydrophobic N-terminal α helix (the stalk) and a compact C-terminal domain containing an antiparallel β sheet terminating in a disulfide-bonded loop (the DSL or D-region) that is crucial for pilus-related functions. At the amino acid sequence level, the first 28 residues of the N termini of pilins and pseudopilins are highly conserved, in part due to the requirement for processing of the subunits prior to assembly

by a single conserved prepilin peptidase, while the C termini are more variable. Because of its hydrophobicity, the first half of the conserved N terminus (denoted α1-N) is often deleted for structural studies; this truncation does not affect the structure of the remaining protein, since full-length and truncated pilin structures have an rmsd of less than 1 Å (Craig et al., 2003; Hazes et al., 2000).

The small size of type IV pilins and T2S pseudopilins has made determination of their structures at high resolution feasible, with the first example, PilE from *N. gonorrhoeae,* solved in 1995 (Parge et al., 1995). However, it has proven more challenging to elucidate the structures of pilus fibers because they are thin, of heterogeneous length, and very flexible. Recently, the first structure of a type IVa pilus from *N. gonorrhoeae* was obtained by computational docking of high resolution structures of individual subunits into a lower resolution (12 Å) structure of an assembled fiber derived from cryo-electron microscopy (Craig et al., 2006). That study generated a pseu-

doatomic model from which hypotheses about the mechanisms of pilus assembly could be formulated. Based on those data, earlier predictions that the conserved hydrophobic N-terminal α helices of the pilins formed the core of the pilus fiber, with the more variable C termini making up its exterior, were confirmed. However, the limits of resolution of cryo-electron microscopy meant that some aspects of pilus ultrastructure remained uncertain, and that the current models were not entirely consistent with the observed physical properties of T4P. In particular, models suggested that a small hole could be present in the center of the pilus fiber, although it was predicted to be occluded by side chains and therefore unavailable for potential transport of DNA or other molecules. The predicted organization of assembled pilin subunits suggested that the hydrophobic N termini of the subunits would be completely buried in the core of the fiber, with the closely packed C-terminal domains surrounding the N termini and exposed to the solvent. However, such a tight-knit configuration
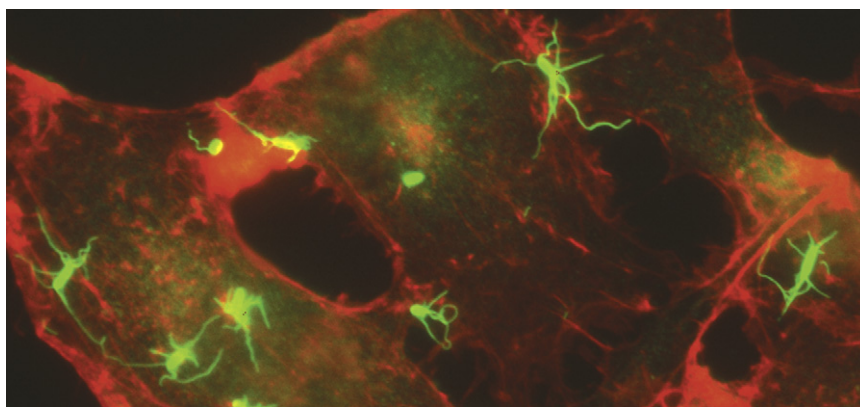

Figure 1. *Pseudomonas aeruginosa* Adhere to Human Epithelial Cells Using Type IV Pili
A flagellum-minus mutant of *P. aeruginosa* strain PAK (stained green; Alexa488) adheres to human epithelial cells (stained red; rhodamine-phalloidin) using its type IV pili, seen here as flexible fibers extending from the cell poles.
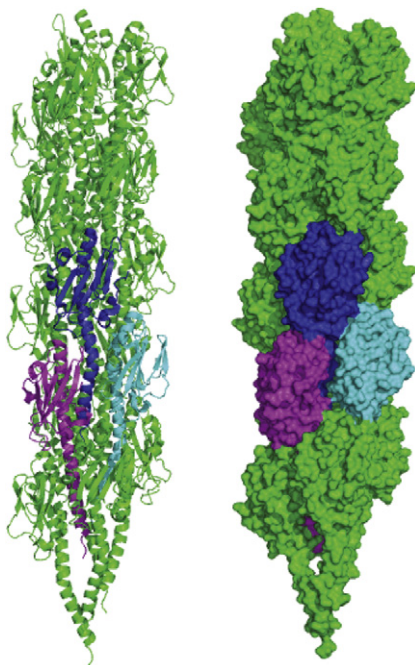
Figure 2. Model of a Type IVa Pilus from *Neisseria gonorrhoeae*

The arrangement of the pilin subunits in a 3-start helical array is shown on the left, with three individual subunits shown in blue, magenta, and cyan. On the right is a surface representation of the same model; the gap visible between the magenta and cyan subunits which exposes the N-terminal helix of the blue subunit may contribute to pilus flexibility. Diagram created using Pymol (Delano Scientific) with PDB code 2HIL (Craig et al., 2006).

would predict a more rigid filament than was apparent through analysis of micrographs that show T4P are quite flexible and capable of bending sharply to form hairpin loops (Figure 1).

Using the type IVb pilin TcpA (for *toxin-coregulated pilus subunit A*) from *V. cholerae* as a model system, Li and colleagues (Li et al., 2008) set out to address these and other uncertainties using a technique called hydrogen/deuterium exchange mass spectroscopy (HDX-MS), a powerful approach not previously applied to this particular problem. HDX-MS involves the incubation of individual monomers of a protein complex, as well as the assembled complex, in an aqueous solution containing D_2O for timed intervals, during which

solvent-exposed hydrogens from peptide backbone amides are exchanged for deuterium atoms. After quenching of the reaction, the paired samples are protease-digested and mass spectrometry is used to identify fragments whose mass is altered between the monomer versus complexed states. This analysis allows for the differentiation of peptides that do not undergo HDX because they are buried in the monomer state from those that are protected from exchange due to protein-protein interactions in the complex.

Among the key findings that arose from the analysis by Li and coworkers (Li et al., 2008) was the absence of HDX on residues that were predicted to line the core of the assembled pilus fiber, confirming that the pili are not hollow. The overall pattern of labeling of the monomer and assembled filaments was more similar than might have been expected, implying that large regions of the monomers continue to be exposed to the solvent after they are assembled into pili. Therefore, packing of the subunits is loose and inter-subunit interfaces are relatively small. Another interesting and unexpected observation was the pronounced labeling of a short segment of the α1-N portion of the N-terminal helix or stalk of the pilins in the assembled filament, previously thought to be occluded from the solvent through the formation of extensive hydrophobic interactions within the core of the fiber and masking of the core by the C termini of the subunits. The labeled segment of the helix (residues 13–23) is amphipathic, with its polar face exposed to the solvent, and was noted by the authors to have several residues with small side chains (Gly, Ala, Ser) that could increase solvent accessibility to the amide nitrogens. The fairly loose packing of the C termini of the pilins observed in this study allows for a pronounced gap to form between subunits, which may help to explain how the pili can exhibit the considerable flexibility associated with these organelles. Interestingly, a similar, though narrower, gap between subunits was predicted in the type IVa pilus model

derived previously through cryo-electron microscopy (Figure 2), though the potentially exposed region of the N-terminal helix in the type IVa subfamily is not predicted to be amphipathic. Instead, type IVa pilins have a Pro residue at position 22 that induces a kink in α1-N that may also contribute to pilus flexibility. Although the exact mechanisms may differ, the allowance for "wiggle room" between subunits so that T4P can bend sharply without breaking may be a widely conserved feature.

The use of HDX-MS to report on solvent accessibility of the pilin subunits in assembled T4P yielded a substantial information return on the effort invested, and is a very useful step forward on the road to understanding how these common protein complexes function. Application of the same techniques to type IVa pili, and to T2S pseudopili, would be of great value to gain a comprehensive picture of the architecture of these important virulence factors.

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