Published in final edited form as:

J Mol Biol. 2012 April 20; 418(1-2): 47–64. doi:10.1016/j.jmb.2012.02.017.

STRUCTURE OF THE *VIBRIO CHOLERAE* TYPE IVB PILUS AND STABILITY COMPARISON WITH THE *NEISSERIA GONORRHOEAE* TYPE IVA PILUS

Juliana Li¹, Edward H. Egelman², and Lisa Craig^{1,*}

¹Molecular Biology and Biochemistry Department, Simon Fraser University, Burnaby, BC, Canada V5A 1S6

²Department of Biochemistry and Molecular Genetics, University of Virginia Medical Center, Charlottesville, VA, USA 22908

Abstract

Type IV pili are multifunctional filaments displayed on many bacterial pathogens. Members of the Type IVa pilus subclass are found on a diverse group of human pathogens whereas Type IVb pili are almost exclusively on enteric bacteria. The Type IVa and IVb subclasses are distinguished by differences in the pilin subunits, including the fold of the globular domain. To understand the implications of the distinct pilin folds we compared the stabilities of pilin subunits and pilus filaments for the Type IVa GC pilus from *Neisseria gonorrhoeae* and the Type IVb toxin-coregulated pilus (TCP) from *Vibrio cholerae*. We show that while recombinant TCP pilin is more stable than GC pilin, the GC pili are more resistant than TCP to proteolysis, heat and chemical denaturation, remaining intact in 8 M urea. To understand these differences we determined the TCP structure by electron microscopy and 3D image reconstruction. TCP have a similar architecture to GC pili, with subunits arranged in a right-handed one-start helix and related by an 8.4 Å axial rise and a 96.8° azimuthal rotation. However, the TCP subunits are not as tightly packed as GC pilins and the distinct Type IVb pilin fold exposes a segment of the α-helical core of TCP. Hydrophobic interactions dominate for both pilus subtypes, but base-stacking by aromatic residues conserved among the Type IVa pilins may contribute to GC pilus stability. The extraordinary stability of GC pili may represent an adaptation of the Type IVa pili to harsh environments and the need to retract against external forces.

Keywords

Type IV pili; *Vibrio cholerae*; *Neisseria gonorrhoeae*; toxin coregulated pili; autoagglutination; electron microscopy; cryoEM; 3D image reconstruction; bacterial pathogenesis; colonization; virulence factor; microcolony; helical symmetry; circular dichroism; pilus assembly

© 2012 Elsevier Ltd. All rights reserved.

*Corresponding author: Lisa Craig, Molecular Biology and Biochemistry Department, Simon Fraser University, 8888 University Dr., Burnaby, BC Canada V5A 1S6, licraig@sfu.ca, 778-782-7140 (phone) 778-782-5583 (fax).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Accession numbers

The TCP TEM reconstructions have been deposited in the Electron Microscopy Data Bank under accession numbers EMDB 1954 and EMDB 1955.

INTRODUCTION

Type IV pili (T4P) are filamentous appendages found on many Gram-negative and at least one Gram-positive bacterial pathogen¹. T4P have a broad range of functions that vary from one organism to another and are critical for bacterial virulence. In *Vibrio cholerae*, the toxin coregulated pili (TCP) self-associate to hold bacteria in microcolonies, representing a key step in bacterial colonization of the small intestine to cause the gastrointestinal disease cholera^{2; 3; 4}. TCP are also high affinity receptors for the cholera toxin phage CTX ϕ ⁵. The gonococcal or GC pili play a central role in *Neisseria gonorrhoeae* colonization of the urogenital tract to cause the sexually transmitted disease gonorrhea. GC pili mediate microcolony formation and also bind directly to host cell receptors^{6; 7; 8; 9}. GC pili are retractile and thus possess additional capabilities of twitching motility and DNA uptake^{10; 11; 12}. Many other bacterial pathogens, including *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Salmonella* Typhi, enterotoxigenic and enteropathogenic *Escherichia coli* (ETEC and EPEC, respectively), use T4P for colonization and invasion of human hosts.

T4P are polymers of the pilin subunit, a 15–20 kDa protein that is conserved in its N-terminal segment (~25 residues). Type IV pilins have an unusual N-methylated N-terminal amino acid and a glutamate at position 5. Their C-terminal regions are less well-conserved, but all possess a cysteine pair. The pilin subunits are synthesized with an N-terminal signal sequence that is removed by an inner membrane prepilin peptidase, a bifunctional enzyme that also catalyzes the N-methylation step^{13; 14; 15}. Cleavage of the signal peptide requires a glycine at the –1 position of the prepilin, whereas N-methylation is dependent on Glu5^{16; 17; 18}. Methylation is not essential for pilus assembly as a *P. aeruginosa* prepilin peptidase mutant that can cleave but not methylate pilin is nonetheless capable of synthesizing functional pili¹⁹. In contrast, Glu5 is required for pilus assembly as substitutions at this site resulted in non-piliated strains^{14; 16; 18; 20}. T4P assembly requires 8 or more proteins, many of which are conserved in different bacterial species as well as in the related Type II secretion system^{1; 21}.

Type IV pilins are classified into two subgroups, Type IVa and Type IVb, based on amino acid sequence and length^{22; 23}. The signal peptide, the mature protein and the D-region, delimited by the conserved cysteines, are all longer in Type IVb pilins. The first residue of the mature protein is a phenylalanine in the Type IVa pilins, but this residue varies in the Type IVb pilins. The Type IVa pilins are more highly conserved throughout their amino acid sequence, and share minimal sequence similarity to the Type IVb pilins beyond the N-terminal 25 residues. Genes encoding the Type IVa pilus assembly components are scattered throughout the genome whereas these genes are generally clustered in a single operon for the Type IVb pili²⁴. The Type IVa pilins are found on a diverse group of bacterial pathogens, including *Neisseria meningitidis* and *N. gonorrhoeae*, which infect the brain and urogenital tract, respectively, and *P. aeruginosa*, which colonizes the lungs. In contrast, Type IVb pilins are found almost exclusively on enteric pathogens including *V. cholerae*, ETEC, EPEC and *S. Typhi*.

Structural analysis revealed distinct folds for the Type IVa and IVb pilin subtypes. Crystal structures for three full length Type IVa pilins, PilE from *N. gonorrhoeae*^{25; 26}, PAK pilin from *P. aeruginosa* strain K²⁷ and FimA from *Dichelobacter nodosus*²⁸ reveal similar architectures: a curved extended 53 residue N-terminal α -helix, α 1, the second half of which is embedded in the C-terminal globular domain containing a central anti-parallel 4-stranded β -sheet. Structural differences among these Type IVa pilins occur in the $\alpha\beta$ -loop, which lies between α 1 and the β -sheet, and in the D-region. However, in all three structures, the C-terminal loop, which follows the β -sheet and includes part of the D-region, lies at the

periphery of the globular domain. This loop lies like a thumb at the edge of a closed fist, stabilized by non-covalent interactions as well as a disulfide bond to the β -sheet in the case of GC and PAK pilin. This topology has also been observed in multiple structures of recombinantly expressed N-terminally truncated Type IVa pilins^{29; 30; 31} as well as the minor pilin PilX from *N. meningitidis*³². While no full length structures are available for Type IVb pilins several N-terminally truncated structures reveal an architecture similar to that of the Type IVa pilins, with an N-terminal α -helix, α 1C, embedded in a globular domain containing a central anti-parallel β -sheet and a stabilizing disulfide bond^{3; 27; 33; 34}. However the topology of the β -sheet of the Type IVb pilins is distinctly different, having non-nearest neighbor connectivity, with the C-terminal segment forming the central β -strand of the β -sheet, like a thumb tucked into a fist. As with the Type IVa pilins a disulfide bond stabilizes a peripheral loop but the buried C-terminal segment suggests a more stable protein fold than that of the Type IVa pilins. The significance of these distinct protein folds is not clear but may reflect adaptations to the demands and functions of the pili (retraction, autoagglutination etc.) and/or to the ecological niches occupied by the bacteria.

A pseudo-atomic resolution structure of the Type IVa GC pilus from *N. gonorrhoeae*, derived by fitting the PilE pilin crystal structure into a 12.5 Å resolution electron cryo-microscopy (cryoEM) pilus reconstruction shows pilin subunits arranged in a helical array, held together mainly by hydrophobic associations among the N-terminal α -helices²⁵. Each subunit is related to the next by a 10.5 Å axial rise and a 100.8° azimuthal rotation, following the path of a right-handed 1-start helix. This arrangement places each conserved Glu5 within salt-bridging distance of the positively-charged N-terminal amine of its neighboring subunit to neutralize these two charges in the otherwise hydrophobic environment of the filament core. The GC pilus also has a dominant left-handed 3-start helix, and can be envisioned as being comprised of three separate pilin strands twisting around a central axis. To date, no Type IVb pilus structure has been reported and it is not clear whether or not the topological differences in the globular domain impart different filament architectures. A computational model for *V. cholerae* TCP suggests a similar subunit packing arrangement, with the N-terminal α -helices forming a helical array in the core of the filament and the globular domains exposed on its surface^{3; 27; 35}. However the helical symmetry of these computational TCP models differs markedly from that of the GC pilus structure.

GC pili have been studied extensively as prototypes for understanding pilus assembly and retraction dynamics. T4P polymerization requires an assembly ATPase located on the cytoplasmic side of the inner membrane, but only a subset of the T4P, including GC pili, possess a second ATPase that catalyzes depolymerization or retraction^{10; 11; 12}. Retraction of GC pili is required for twitching motility, natural transformation, intimate adhesion of *N. gonorrhoeae* to host cells, and cortical plaque formation and NF- κ B activation in host cells^{10; 11; 12; 36; 37; 38}. Individual GC pilus filaments can withstand forces in excess of 100 pN, measured using laser tweezers^{12; 39}, and pilus bundles retract with even higher forces⁴⁰. The Type IVa pili from *P. aeruginosa* and *Myxococcus xanthus* possess comparable tensile strength, measured during retraction^{41; 42}. Retraction has not been demonstrated directly for the Type IVb pili but the EPEC bundle forming pilus (BFP) system possesses a putative retraction ATPase, BfpF, and disruption of this protein leads to EPEC hyperpiliation, increased aggregation and adherence, and reduced infectivity^{43; 44; 45}.

To investigate structural differences between the Type IVa and IVb pilins and their impact on pilus architecture and stability, we compared biophysical characteristics of both pilin subunits and pilus filaments from *N. gonorrhoeae* and *V. cholerae* using circular dichroism (CD) spectroscopy and electron microscopy (EM). We further determined negative stain and cryoEM reconstructions of TCP for comparison with the GC pilus structure. Our findings

are discussed in the context of specific T4P functions and the ecological niches occupied by the bacteria on which they reside.

RESULTS

Comparison of stabilities of pilin globular domains

To test whether the Type IVb pilin fold imparted greater structural stability to the globular domain due to its C-terminus being buried in the β -sheet, we compared the thermal stability of soluble *N. gonorrhoeae* PilE and *V. cholerae* TcpA as representatives of the Type IVa and IVb pilin folds, respectively. Both proteins were expressed recombinantly as N-terminal truncations lacking α 1N: Δ N-PilE, residues 27–158,⁴⁶ and Δ N-TcpA, residues 28–199²⁷. Initially a far UV CD scan from 190–260 nm was collected at room temperature to determine the CD profile for each protein and identify an appropriate wavelength at which to follow unfolding. Δ N-TcpA has a CD spectrum characteristic of a predominantly α -helical protein with negative peaks at 208 and 222 nm (Fig. 1A). The Δ N-PilE CD spectrum has an intense negative peak at 208 nm and a shoulder at 220–230 nm (Fig. 1B). Both proteins were heated from 20 to 80 °C, the upper limit of our instrument, and protein denaturation was monitored by CD spectroscopy at peak wavelength, 222 nm for Δ N-TcpA and 208 nm for Δ N-PilE. Δ N-TcpA unfolding occurs between ~47 and 60 °C with a melting temperature (T_m) of 53.5°C (Fig. 1C; see also Supplementary Fig. S2A, lower panel). Δ N-PilE unfolding begins immediately upon heating, indicating instability for this recombinant pilin, and continues through 80 °C with no discrete T_m (Fig. 1D; see also Supplementary Fig. S2B, lower panel). Thus, Δ N-TcpA is considerably more stable than Δ N-PilE, a property that is likely related to Type IVb pilin fold.

Comparison of the thermal stability of Type IVa and IVb pili

As GC pili have been shown to have impressive tensile strength³⁹, we compared their stability to that of TCP using thermal denaturation and far UV CD spectroscopy. GC pili were sheared from *N. gonorrhoeae* strain MS11 cell surfaces and purified by two rounds of precipitation in neutral pH buffer²⁵. TCP were isolated from culture supernatants of *V. cholerae* RT4225, a derivative of classical strain O395 with a mutation in the *tcpA* gene resulting in a His181Ala (H181A) substitution². This alteration causes TCP to grow very long and be shed into the culture media where they can be purified in milligram quantities by ammonium sulfate precipitation³⁵. Far UV CD spectra were first collected for purified TCP and GC pili and these were compared to the CD scans for the soluble pilin monomers. The TCP spectrum is similar to that of the pilin monomer Δ N-TcpA (Fig. 1A), though the intensity of the troughs at 208 and 222 nm are greater. This is likely because the N-terminal α -helix of the pilin subunits is extended by 28 amino acids (α 1N) compared to Δ N-TcpA. Surprisingly, the GC pilus spectrum is markedly different from that of the Δ N-PilE monomer, with a much less intense negative peak at 208 nm and a second negative peak at 228 nm (Fig. 1B), suggesting that the secondary structure of GC pilin differs in the soluble vs. polymerized forms.

Thermal denaturation of the two pilus subtypes was monitored at peak wavelengths, 222 nm for TCP and 228 nm for GC pili. TCP denaturation is highly cooperative, occurring over a narrow temperature range between 55 and 64 °C with a T_m of 59.4 °C, suggesting that the TCP filaments are more stable than their corresponding Δ N-TcpA monomers (Fig. 1C). In contrast, no change was observed in the CD spectrum of GC pili between 22 and 80 °C at its peak wavelength, 228 nm (Fig. 1D), or at other wavelengths tested (data not shown), suggesting that these pili are stably folded over this temperature range. GC pilus stability was investigated further by collecting CD spectra at temperatures between 22 and 80 °C for 5 min and 40 min incubation times. These results show that the characteristic GC pilus CD

spectrum is maintained at 22, 40 and 60 °C but the 228 nm intensity is reduced at 80 °C for both incubation times (Supplementary Fig. S1), indicating that partial unfolding occurs at temperatures above 60 °C.

We examined both pilus types by transmission electron microscopy (TEM) to determine the effects of heating on their gross morphology. Consistent with the CD results, TCP are abundant at 22 °C and 40 °C, although they appear fragmented at 40 °C (Fig. 1E). No TCP were seen upon heating to 60 °C or 80 °C. In contrast, GC pili appear unaffected by heating to 60 °C, and a few filaments are even observed after treatment at 80 °C. Thus, although the Δ N-TcpA monomer is more stable than the Δ N-PilE monomer, the assembled GC filaments are substantially more stable than TCP.

To understand the striking difference between the CD spectra of Δ N-PilE and GC pili we treated both pilus types with the non-ionic detergent octyl- β -D-glucopyranoside (β OG), which disrupts subunit:subunit interactions to dissociate the filaments into pilin monomers. Importantly, this procedure does not cause the pilin subunits to unfold, and has been used to prepare protein for crystal structure determination of full length pilins^{25; 27; 28}. FarUV CD spectra were collected for pili immediately after addition of β OG (0 min) and after 24 hrs incubation at 4 °C. β OG-treatment had an immediate effect on the CD spectra for both pili and the spectra did not change significantly after 24 hrs. Dissociation of the pili was confirmed by EM, which showed few pili in the 0 min samples and no pili after 24 hrs in β OG, and by filtration through a 300 kDa molecular weight cut-off membrane, which retains untreated pili (data not shown). The CD spectra are shown in Supplementary Fig. S2 overlaid upon the spectra for untreated and heat-denatured pili (top panels) and N-terminally truncated pilins (bottom panels). The negative intensity peak at 222 nm seen for untreated TCP and for the Δ N-TcpA subunits is also present in the β OG-treated TCP spectrum, suggesting similar secondary structures for all three samples. However, the spectra for β OG-treated GC pili more closely resembles that of the Δ N-PilE subunits than of untreated pili, further supporting a secondary structure change for the GC pilin subunits upon polymerization into pilus filaments.

Comparison of the chemical stability of Type IVa and IVb pilus filaments

To further explore the relative stabilities of the Type IVa vs. IVb pili, GC and TCP filaments were treated with the chemical denaturant urea. Pili were treated for 1 hr with 0, 2 M, 4 M, 6 M and 8 M urea then analyzed by CD spectroscopy and TEM. The CD spectra for urea denaturation are shown in Fig. 2 from 200 to 260 nm, although the noise levels from the urea are high for data below 212 nm. The 222 nm intensity of the TCP CD spectrum is reduced in the presence of 2 M urea relative to the untreated sample, and the characteristic α -helical spectrum is absent at 4 M urea and higher concentrations, indicating unfolding (Fig. 2A). Consistent with these data, TCP are visible by TEM after treatment with 2 M urea but not with higher concentrations (Fig. 2C). In contrast, the CD spectrum of GC pili appears unaffected by urea concentrations as high as 6 M, and the negative peak at 228 nm is present at ~50% of its original intensity even at 8 M urea (Fig. 2B). GC pili were abundant and morphologically indistinguishable from untreated pili for all urea concentrations tested (Fig. 2C), demonstrating the extraordinary robustness of these filaments. We were however able to completely denature GC pili using 6 M guanidinium hydrochloride, as assessed by CD and TEM analysis (data not shown).

To ensure that the instability of the TCP filaments relative to GC pili was not due to the H181A substitution in TcpA, their susceptibility to urea was compared to that of TCP from wild type *V. cholerae*. Wild type TCP were purified from *V. cholerae* O395 by shearing and precipitation. While the yield and purity of the wild type TCP are lower than for H181A TCP, sufficient amounts were obtained for TEM analysis. Wild type TCP were treated with

varying concentrations of urea and analyzed by TEM. Wild type TCP are stable in 2 M urea but are denatured in 4 M urea, similar to the results obtained for H181A TCP (Supplementary Fig. S3). H181A TCP are also comparable to wild type TCP with respect to resistance to chymotrypsin proteolysis³⁵ and they are functional in that they mediate phage uptake², suggesting that they are a suitable replacement for wild type TCP for our structural analyses.

Comparison of protease susceptibility for Type IVa and IVb pili

Next, we used protease susceptibility to test the relative stabilities of GC pili and TCP and their pilin counterparts. Both pilins were degraded within one hour of treatment at 37°C by all proteases tested using a high protease:pilin mass ratio of 1 (Fig. 3). The pilus filaments required longer treatment durations, with little or no proteolysis evident for four of the six proteases tested after a 1 hour incubation, demonstrating that the quaternary structure of the pilus protects the subunits from degradation. TCP were more susceptible than GC pili to all but one of the proteases tested. In no case were TCP more protease resistant than GC pili. Together, the proteolysis, thermal and chemical denaturation studies demonstrate that GC pili are substantially more stable than TCP.

Pilus stability in the presence of reducing agent

We and others showed previously that the Cys120–Cys186 disulfide bond was necessary for stable pilin expression and hence, pilus assembly^{2,3}. To examine the contribution of the conserved disulfide bond to filament stability, TCP and GC pili were treated with the reducing agent tris(2-carboxyethyl)phosphine (TCEP, 100 mM). For both pili, treatment with 100 mM TCEP at 22 °C for 15 min completely destroyed the filaments, as assessed by TEM (not shown). Thus, the disulfide bond between the conserved cysteines is critical for both subunit and filament stability. Presumably, disruption of this bond destabilizes the pilin subunits, which leads to disintegration of the pili.

Negative stain reconstruction of TCP

To understand the marked differences in thermal, chemical and protease stability between TCP and GC pili and to further characterize the TCP filaments we determined a negative stain reconstruction of TCP from *V. cholerae* strain RT4225 using the Iterative Helical Real Space Reconstruction method (IHRSR,⁴⁷). Negatively stained TCP were imaged by EM in low dose mode (Fig. 4A), micrographs were digitized and straight segments of filaments were selected for analysis. An averaged power spectrum was determined for ~1000 non-overlapping TCP particles (200 pixels or 498 Å in length). A dominant layer line was observed at ~1/43 Å corresponding to a 3-start helix and a weaker layer line at ~1/110 Å corresponds to a 4-start or 5-start helix (Fig. 4A). The approximate helical symmetry (axial rise, azimuthal rotation per subunit) was determined for each combination and IHRSR was performed using these starting symmetries and 8,034 overlapping particles, resulting in a single reconstruction solution with a rise of 8.4 Å and a rotation of 96.8° for the 1-start helix. The TCP reconstruction has 3.7 subunits per helical turn, giving a mass per unit length of 2.4 kDa/Å, based on the 20.3 kDa TcpA subunit. This value was also determined independently by scanning transmission electron microscopy (STEM, Fig. 4B). The reconstruction having a right-handed 1-start helix (+1) as well as a left-handed 3-start helix (−3) and a right-handed 4-start helix (+4) is shown in Fig. 4C, although the mirror image (−1, +3, −4) was equally plausible from the IHRSR results. The resolution of the TCP reconstruction is estimated to be 21 Å based on Fourier shell correlation (FSC) analysis.

CryoEM reconstruction of TCP

We sought to improve the resolution of the pilus structure by determining a cryoEM reconstruction of TCP. For cryoEM, TCP filaments were flash-frozen in vitreous ice in physiological buffer without heavy atom stains to more accurately mimic the native state of the protein (Fig. 5A). TCP cryoEM images were digitized and pilus segments were selected. An averaged power spectrum from 2243 overlapping particles (200 pixels $\times$ 200 pixels) selected from the segments showed a single layer line at $1/43 \text{ \AA}$ corresponding to a 3-start helix with a pitch of $129 \text{ \AA}$ (Fig. 5A). IHRSR⁴⁷ was applied to this relatively small data set using a starting symmetry from the negative stain reconstruction ($8.4 \text{ \AA}$ rise, 96.8° rotation) but the symmetry was unstable over 100 IHRSR cycles, suggesting that the TCP filaments may be heterogeneous in structure, as we have seen for other pili^{48; 49; 50}. Thus, the particles were sorted based on the pitch of the 3-start helix and a smaller data set (1000 particles) with 3-start pitches of $126 \text{ \AA}$ to $132 \text{ \AA}$ was used for IHRSR, with a starting symmetry of $8.2 \text{ \AA} / 96.2^\circ$. The reconstruction converged to symmetry values of $8.5 \text{ \AA}$ rise, 96.7° rotation, which matches that of the negative stain reconstruction (Fig. 5B and Supplementary Fig. S4). However this cryoEM reconstruction showed no improvement over the negative stain reconstruction in terms of providing molecular detail, and in fact had a lower resolution of $27 \text{ \AA}$ as determined by FSC at 0.5. To improve the quality of the cryoEM reconstruction, more TCP images were collected, digitized and processed. IHRSR was performed on 24,816 particles using various starting symmetries close to that of the negative stain reconstruction but these runs all failed to converge. Thus, particles were sorted according to the pitch of the 3-start helix as described above, and several new reconstructions were generated using various bins of particles (3-start helical pitch of $126 \text{ \AA}$ to $138 \text{ \AA}$ and $126 \text{ \AA}$ to $132 \text{ \AA}$). Although some of these reconstructions used a 10-fold increase in number of particles, the reconstructions were no better than the original cryoEM reconstruction using 1000 particles. Presumably, heterogeneity in the TCP filaments evident in the frozen-hydrated filaments but not in the negative-stained filaments limits the resolution of the cryoEM reconstruction.

Pseudo-atomic resolution structure of TCP

Both the negative stain and the cryoEM TCP reconstructions have an undulating surface with a solid core, with density for the pilin globular domains clearly defined (Fig 4C, 5B and Supplementary Fig. S4). The crystal structure of ΔN -TcpA, which lacks the N-terminal 28 residues ($\alpha 1N$)²⁷, was manually docked into the higher-resolution negative stain reconstruction. Although the resolution of this reconstruction is not sufficient to reveal secondary structure elements that would facilitate the fitting process, fitting was guided by the asymmetry of the globular domain. The TcpA globular domain fit well into the right-handed 1-start reconstruction but poorly into its mirror image, thereby defining the reconstruction as a right-handed 1-start helix with subunits related by an $8.4 \text{ \AA}$ axial rise and a 96.8° azimuthal rotation. Importantly, this helical symmetry approaches that of the *N. gonorrhoeae* GC pilus, which has a right-handed 1-start helix with a $10.5 \text{ \AA}$ rise and a 100.8° rotation.

To obtain a pseudo-atomic resolution structure of the TCP filament, a full length model of the TcpA subunit was generated by superimposing $\alpha 1N$ from the full length PAK pilin crystal structure onto ΔN -TcpA²⁷. TcpA and PAK pilin amino acid sequences are 75% similar in $\alpha 1N$ (32% identity). Full length TcpA was super imposed on the docked ΔN -TcpA (Fig. 6A) and the TCP filament model was generated by applying the symmetry parameters for the reconstruction to the single TcpA subunit (Fig. 6B, C). The TCP model fits well into the reconstruction volume, and the N-terminal α -helices of TcpA fill the core of the filament with no significant steric clashes. In this model, $\alpha 1$ of TcpA has the same S-shaped curve seen for PAK and GC pilin and *D. nodosus* FimA, which have kinks at Pro22

and Gly42^{25; 26; 27; 28}. Although TcpA does not possess helix-disrupting residues at these positions, it has glycines at positions 11, 14 and 19 and is thus expected to be curved or at least flexible. We also generated a number of TCP models using an idealized straight α -helix for α 1N but these models produced major steric clashes and were discarded. Thus, the curvature in α 1 appears to facilitate packing in the pilus filament. To compare the TCP model with the TEM reconstructions, the model was filtered to 21 Å resolution. The model closely resembles both the negative stain and the cryoEM reconstructions (Supplementary Fig. S4A), though its accuracy is limited by their moderate resolution. Cross-sections of contour plots of the filtered model were compared with those of the negative stain and the cryoEM reconstructions (Supplementary Fig. S4B). These plots show good correspondence of the subunit positions and connectivity between the model and the reconstructions. Furthermore, the power spectrum of the model (Supplementary Fig. S4C) matches the averaged power spectrum for the negatively stained TCP particles (Fig. 4A), both having equivalent $1/(43 \text{ Å})$ and $1/(110 \text{ Å})$ layer lines, providing further validation for the model.

In the new TCP structure subunits are arranged in a helical array with the N-terminal α -helices (α 1) twisting around the filament axis to form a solid hydrophobic filament core (Fig. 6). The interactions among the N-terminal α -helices hold the subunits together and the globular domains are loosely packed on the filament surface. Although the helical symmetry of the TCP reconstruction is markedly different from our previous computational models for TCP, derived from DXMS analysis and sparse helical symmetry information^{27; 35}, the architecture and subunit:subunit interactions are very similar. The TcpA packing and orientation in the new TCP structure is consistent with the DXMS results, which mapped exposed and buried regions of the pilin subunits within the filament. In both models the N-terminal α -helices form the filament core and the globular domains form the outer surface of the filament. The D-regions (colored magenta in Fig. 6C), which house residues involved in TCP-mediated microcolony formation^{2; 3} protrude from the filament surface and the $\alpha\beta$ -loops (colored green in Fig. 6C) are directed inward and interact with α 1N of a neighboring subunit. The side chain of Glu83 in the $\alpha\beta$ -loop is positioned to form an electrostatic interaction with Arg26 of α 1N in the neighboring subunit in the 3-start helix, as proposed for the DXMS-refined TCP model and supported by mutational analysis³⁵ (Fig. 6C, D). Gaps between the globular domains expose a segment of α 1N, between residues 14 and 29, which was well labeled in the DXMS study (Fig. 6E). Finally, as with the DXMS-based TCP model, the TEM-based TCP structure positions the conserved Glu5 of the N-terminal α -helix within salt-bridging distance of the positively-charged N-terminal amine of Met1 in the neighboring subunit in the 1-start helix (Fig. 6F).

Glu5 is necessary for efficient TCP assembly

To test the importance of Glu5 in TCP filament assembly, we mutated the *tcpA* gene in *V. cholerae* to produce a Glu5Ala substitution in the TcpA subunit (TcpA^{E5A}). TcpA^{E5A} expression was assessed by SDS-PAGE and immunoblotting of whole cell cultures and pilus assembly was assessed by shearing the cells to release pili into the culture supernatant. Pilus assembly was also assessed by autoagglutination and phage transduction assays, which are sensitive to pilus expression, and by TEM of whole cell cultures. The total pilin in overnight cultures was comparable for *V. cholerae* - *tcpA*^{E5A} and the wild type parental strain O395 in terms of expression level and molecular weight, suggesting that TcpA^{E5A} is synthesized and correctly processed by the signal peptidase TcpJ. TcpA^{E5A} has a slightly higher electrophoretic mobility than TcpA^{WT}, which may be a combined effect of the Glu5Ala substitution and loss of N-methylation at Met1, as was shown for a Glu5Val change for *P. aeruginosa* PAK pilin¹⁴. TcpA^{E5A} levels in the sheared cell supernatant were greatly reduced relative to TcpA^{WT} (Fig. 7A). No TCP^{E5A} were observed in whole cell culture by TEM and *V. cholerae*-*tcpA*^{E5A} autoagglutinated poorly relative to the wild type strain (Fig.

7B). Furthermore, *V. cholerae*-tcpA^{E5A} CTX ϕ phage transduction was 50-fold lower than O395, but higher than for a Δ tcpA deletion mutant. Thus, TCP appear to be produced, but at very low levels in *V. cholerae*-tcpA^{E5A}, suggesting that Glu5 is required for efficient TCP assembly, as has been shown for other T4P^{14; 16; 18; 20}.

DISCUSSION

Here we show that two prototypes for the Type IVa and IVb pilus subclasses have pronounced differences in stability. The *V. cholerae* Type IVb pilin subunit Δ N-TcpA is more stable than the *N. gonorrhoeae* Type IVa subunit Δ N-PilE, as we had predicted based on differences in the protein fold. However, the reverse is true for their respective pilus filaments, with GC pili being substantially more resistant than TCP to denaturation and proteolysis. Both pilus types are more resistant to thermal denaturation and protease treatment than their soluble pilin counterparts. This is not surprising, as protein oligomers tend to be more stable than their monomeric equivalents⁵². We expected that the CD spectra for the pilus filaments would be similar to that of their respective pilin subunits, and this was the case for TCP. However, the GC pilus CD spectrum was markedly different from that of Δ N-PilE, and dissociation of GC pili into detergent-solubilized pilin subunits produced a CD spectrum that more closely resembled Δ N-PilE spectrum than that of intact pili. The change in CD spectra for GC pili upon treatment with detergent cannot be attributed to unfolding, as the full-length PilE crystal structure was solved under similar conditions^{25; 27}. Instead, these results point to distinct differences in secondary structure for monomeric vs. polymerized PilE. Interestingly, a secondary structure transition was also reported for the Type III secretion needle protomers from *Shigella flexneri* and *Salmonella typhimurium*, which undergo an α -helical to β -strand change upon polymerization⁵³. Since the PilE crystal structure fits well into the GC pilus cryoEM reconstruction²⁵ the conformational differences between the monomer and polymer forms are likely to be small, such as an extension of the N-terminal α -helix, which would not be apparent at the resolution of the reconstruction. Further investigation and higher resolution filament structures are required to fully understand these intriguing results.

The higher stability observed for GC pili relative to TCP cannot be attributed to the subunit stability and must instead arise from the quaternary arrangement of the PilE subunits in the GC pilus filament. In order to compare the two pilus structures we determined TEM reconstructions of *V. cholerae* TCP and generated a pseudo-atomic resolution filament model. Our new TCP model is similar to our previously-published computational models^{27; 35} with respect to subunit orientation and contacts, but the helical symmetries are markedly different. Earlier models were generated knowing that TCP had a dominant 3-start helix with a pitch of ~ 135 Å, but without knowledge of the positions of the subunits along these 3-start helical strands. Our TCP reconstruction and resulting filament model illustrate the importance of obtaining accurate symmetry values (i.e. the axial rise and the azimuthal rotation of subunits in the primitive 1-start helix and the hand of this helix). Incorrect symmetry values can lead to incorrect filament models that are nonetheless consistent with existing genetic, immunological and biophysical data, as illustrated by our earlier TCP models, and by a TCP model derived from “sparse data”⁵⁴ that relied on our earlier symmetry approximation. Our TCP reconstructions, by precisely defining the helical symmetry of the filament, describe the positions of the TcpA subunits along the 3-start helices, and the TEM density allows their orientations to be determined with some certainty. A higher resolution reconstruction would further improve the precision of the TCP model, but filament heterogeneity makes this a challenging prospect.

The newly-determined TCP symmetry and architecture resembles that of GC pili²⁵. In both TCP and GC pili the interactions among subunits are extensive and occur between globular

domains of neighboring subunits, between globular domains and α 1N segments and between α 1N segments. Each pilin subunit contacts six neighboring subunits. These interactions are extremely complex and have not been determined precisely for either pilus type as the resolution of the reconstructions is not high enough to accurately position the N-terminal α -helices, and the full length TcpA structure is not available. Nonetheless, as both pilins fit well as rigid bodies into their TEM densities, and filament models can be generated that match the reconstructions without major steric clashes, these models provide a strong basis for understanding the subunit interactions and provide clues to explain the relative stabilities of the two pilus types. First, we note that the α 1 segments of TCP are not packed as tightly as GC pili. The larger filament diameter and higher tilt angle of TcpA relative to the filament axis give TCP a larger diameter α -helical core than GC pili, which may explain its lower stability (Fig. 8A). A second factor contributing to pilus stability may be the packing of the subunits on the filament surface. Although the globular domain of TcpA is almost twice the size of PilE (171 vs. 100 residues), its bulky D-region, delineated by Cys120-186, protrudes from the filament surface, increasing the diameter of the filament rather than packing tightly against neighboring subunits (Fig. 8A, B). Furthermore, the C-terminus of TcpA is tucked into the globular domain, leaving a sizable gap between neighboring subunits that exposes a segment of α 1N (Fig. 8C), as demonstrated by DXMS experiments³⁵. In contrast, GC pili have a much narrower gap between subunits due to the peripherally associated C-terminal loop, which partially bridges the space between globular domains (Fig. 8C). Thus, the distinct Type IV pilin folds may in fact define the stability of the pili due to differences in distribution of the D-region bulk and of the C-terminal loop. Finally, the PilEN-terminus has three aromatic residues whose side chains are oriented into the GC pilus core and positioned to stack, with Phe1 of one subunit inserted between Tyr 24 and Tyr27 of an adjacent subunit in the right-handed 4-start helix of the GC pilus (Fig. 8D). Such base stacking may contribute to the higher binding energy for subunit:subunit interactions in GC pili. These aromatic residues are highly conserved in the Type IVa pilins but are notably absent in most Type IVb pilins including *V. cholerae* TcpA (Fig. 8E). Interestingly, we hypothesized that the unique topology of the Type IVb pilins, with their C-terminal segment buried within the globular domain and braced by hydrogen bonding to the β -sheet, might make these pili less reliant on the conserved disulfide bond for stability. However, both TCP and GC pili completely disintegrated in the presence of reducing agent, demonstrating the critical role for the disulfide bond in stability for both pilus types.

The robust stability demonstrated here for GC pili, particularly the thermal stability, is attributed to tight interactions among subunits. Presumably this feature contributes to the remarkable tensile strength documented for GC pili and for *P. aeruginosa* and *M. xanthus* Type IVa pili^{12; 39; 41}. The requirement for mechanical strength most likely depends upon the biological demands of the pili. GC pili must withstand high flow rates in the urogenital tract, and likely encounter high stress forces during retraction for twitching motility and intimate adhesion^{6; 37}. In contrast, *V. cholerae* tend to form TCP-mediated microcolonies in crypts in the lining of the small intestine^{55; 56} where they would be shielded from high flow forces. In general the Type IVb pili are less well characterized than the Type IVa pili and none have been shown definitively to retract. The enteric bacterial pathogens may possess Type IVb pili rather than Type IVa pili because they acquired the genes horizontally from other gut bacteria or phage and found they were sufficient to facilitate colonization, whereas the Type IVa pili further evolved to become more stable as an adaptation to their harsher environments or perhaps to allow pilus retraction. More extensive characterization of TCP and other Type IVb pili with respect to their mechanical stability, retraction capability and range of functions will further clarify the significance of their unique structural features.

TCP and GC pili have comparable helical symmetries: right-handed 1-start helices with a rise per subunit of 8–10 Å and a rotation of ~100°. Furthermore, in both filament models the

conserved Glu5 is positioned to form a salt bridge with the positively-charged N-terminal amine of the neighboring subunit in the 1-start helix. We show here that the conserved Glu5 is necessary for efficient TCP assembly, as it is for GC and other T4P^{16; 17; 18; 20}. These results support a common filament assembly mechanism for all T4P, in which subunits are added sequentially along the right-handed 1-start helix using a conserved inner membrane assembly machinery. We have proposed that Glu5 serves not only to neutralize the positively-charged N-terminus in the otherwise hydrophobic Type IV pilus core, but is also a driving force for filament assembly: incoming pilin subunits, embedded in the inner membrane via α 1N, are attracted by charge complementarity between their Glu5 and the N-terminus on the terminal subunit in the growing pilus filament^{51; 25}. This interaction along with complementarity among the globular domains facilitates the translocation of the pilin subunit from the hydrophobic environment of the inner membrane into that of the pilus filament. While Glu5 is necessary for efficient TCP assembly, some functional pili appear to be expressed in the *V. cholerae*-tcpA^{E5A} mutant, as they are able to auto agglutinate and to transduce phage at low but detectable levels. Both *P. aeruginosa* and *N. gonorrhoeae* Glu5 pilin variants, when expressed heterologously in strains co-expressing wild type pilin, became incorporated into mixed pilus filaments^{18; 57}. However, the morphology of the hybrid *P. aeruginosa* pili was unusual and the *N. gonorrhoeae* strain required that the retraction ATPase, PilT, be disrupted in order for the pilin variants to be assembled into pili¹⁸. These data together indicate that though pilus assembly can proceed in the absence of the Glu5-N1 salt bridge, the process is inefficient and the resulting pili may be aberrant. It is notable that the related Type II secretion pseudopilins also have a glutamate at position 5, and the crystal structure of a ternary complex between the globular domains of the GspI:GspJ:GspK minor pseudopilins from ETEC have quasi-helical symmetry⁵⁸. As in GC pili and TCP, the GspI, GspJ and GspK subunits are arranged in a right-handed helix with a $\sim 100^\circ$ rotation about the filament axis. When the N-terminal α -helices are modeled onto the globular domains in the complex, they are staggered by ~ 10 Å and the Glu5s of GspI and GspJ are within 5–7 Å of the N-terminal amine of their neighboring subunits, GspK and GspI, respectively. These data support a common architecture and assembly mechanism for both Type IV pili and Type II secretion pseudopili.

The observed stability of GC pili might appear at odds with their ability to retract. Retraction, like thermal denaturation or mechanical breakage of pili, involves disruption of many non-covalent bonds between subunits. We propose that pilus retraction is a reversal of the pilus assembly process and involves translocation of pilin subunits, one by one, from their hydrophobic environment in the pilus filament to the hydrophobic environment of the inner membrane. Thus, as long as pilus stability is due to hydrophobic interactions among the pilin subunits, pilus retraction should not be hindered. However, the more extensive the hydrophobic interactions, the more resistant the filament will be to denaturation or mechanical breakage, which would disrupt bonds between as well as within the pilin subunits. We show here that although GC pili are highly resistant to thermal and chemical denaturation, they dissociate immediately upon treatment with detergent, leaving the pilin subunits intact and folded. Similar results were obtained for TCP, demonstrating that hydrophobic interactions dominate for both pilus types, providing durable filaments that can, in some cases, rapidly disassemble or retract.

In summary, our results suggest that while the Type IVb subunit fold may be more stable than that of the Type IVa pilins, subunit stability is not synonymous with filament stability. Our TCP TEM reconstructions allow a direct comparison of a Type IVa and IVb pilus structure. Both pilus types have a common architecture and comparable helical symmetries and rely on an invariant Glu5 for efficient pilus assembly, suggesting a common assembly mechanism. Both pilus types are stabilized by extensive hydrophobic interactions, but subtle differences in these interactions and in subunit packing, as determined by their distinct pilin

folds, may explain the superior stability of the GC pili and may be generalizable for all Type IVa pili.

MATERIALS AND METHODS

Bacterial strains and materials

Bacterial strains used in this study are shown in Table 1. The TcpA-6 polyclonal rabbit antibody specific for TcpA residues 174–199⁵⁹ was a gift from Ronald Taylor (Dartmouth Medical School). We are grateful to Katrina Forest (University of Wisconsin-Madison) for providing purified Δ N-PilE. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μ g/ml; kanamycin (Kn), 45 μ g/ml; streptomycin, (Sm), 100 μ g/ml. Isopropyl β -D-1 thiogalactopyranoside (IPTG) was used at 0.4 mM.

Δ N-TcpA expression and purification

Δ N-TcpA was expressed in *E. coli* Origami cells (Novagen) as described²⁷ with minor modifications. Briefly, cells in overnight cultures were sonicated to release the cytoplasmic proteins, which were purified by cation exchange chromatography using a HiTrap SP HP column (GE Healthcare), followed by metal affinity chromatography using a HisTrap HP column (GE Healthcare) and then size exclusion chromatography on a HiPrep 26/60 Sephacryl S-100 HR column (GE Healthcare). The hexahistidine tag was cleaved from purified Δ N-TcpA by treatment with 1 unit of thrombin protease (GE Healthcare) per 500 μ g of substrate at 4°C for 16 hours. The digested sample was loaded onto a HisTrap HP column to remove his-tag. The eluent was dialyzed in phosphate-buffered saline (PBS) with 10 mM ethylene diaminetetraacetic acid. When necessary, protein was concentrated using an Amicon stirred cell concentrator with a YM 10 membrane.

TCP expression and purification

For TCP purification, *V. cholerae* classical strain RT4225 was grown in 800 mls Luria Broth (LB, 1% tryptone, 0.5% yeast extract, 86 mM NaCl, pH 6.5)-Sm/Ap/IPTG for 16 hrs at 30°C, shaking. Strain RT4225 has a mutation in the *tcpA* gene encoding a His181→Ala substitution that causes the pili to fall off the cells into the culture supernatant. To increase pilus expression, the *tcp* transcriptional activator ToxT is overexpressed on plasmid pMT5⁶⁰ under control of the *lac* promoter. Overnight cultures were centrifuged to remove cells and TCP were precipitated from the supernatant using 30% ammonium sulfate, resuspended in PBS and dialyzed exhaustively to remove the ammonium sulfate. All purification steps were performed on ice or at 4°C.

GC pilus expression and purification

GC pili were expressed and purified as described previously²⁵. Briefly, *N. gonorrhoeae* strain MS11 cells were grown on GC medium base plates for 24 hours at 37°C with 5% CO₂. Cells were harvested and pili were sheared from the cells by vortexing. The pili were resuspended in 50 mM CHES buffer, pH 9.5 and purified by dialysis into pilus buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN₃, 1 mM dithiothreitol) followed by centrifugation. Pili were resuspended in PBS.

Far UV circular dichroism

Pilus samples were dialyzed in salt-free phosphate buffer and diluted to 0.3 mg/ml for CD analysis. For urea denaturation, samples were treated with 0 M, 2 M, 4 M, 6 M or 8 M urea for 1 hr at 22 °C prior to CD analysis. CD studies were performed using a 1 mm pathlength quartz cuvette on a Jasco J-810 spectropolarimeter. For thermal denaturation studies, sample temperature was increased from 20°C to 80°C at a rate of 0.5 °C per minute and CD

measurements were collected at the wavelengths specified. For the detergent dissociation, β OG was added to a final concentration of 50 mM and CD spectra were collected immediately and after 24 hrs stirring at 4 °C. Far UV CD scans were collected between 190 and 260 nm at 22°C, 40°C, 60°C and 80°C. All CD data were collected in duplicate. The buffer background readings were subtracted from all spectra and duplicate data were averaged. The Spectra Analysis program within the Spectra Manager package (Jasco) was used to convert ellipticity values in m deg to mean residue molar ellipticity, expressed in units of deg*cm²/dmol.

Proteolysis

TCP and GC pili were incubated with the proteases chymotrypsin, elastase, proteinase K, subtilisin A, thermolysin and trypsin at a protease:protein mass ratio of 1 at 37°C in a shaking incubator. Aliquots were collected at various time points and proteolysis was quenched by boiling in Laemmli sample buffer⁶¹ containing phenylmethylsulfonyl fluoride (10 mM). Proteolysis was visualized by Coomassie-stained SDS-PAGE.

SDS-PAGE and immunoblotting

Aliquots of whole cell culture (20 μ l for Coomassie-stained gel, 5 or 10 μ l for immunoblot) and purified TCP (0.2 μ l, 10 μ g) were boiled in Laemmli sample buffer and analyzed by 15% SDS-PAGE. Gels were either stained with Coomassie Blue or the proteins were transferred from the gels to PVDF membrane and probed using polyclonal antibody TcpA-6⁵⁹. Bound antibody was visualized using goat anti-rabbit antibody conjugated to alkaline phosphatase (Biorad), with 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt / nitro-blue tetrazolium chloride (BCIP/NBT, Biorad) as a substrate.

Transmission electron microscopy

For imaging heat-treated pili, TCP and GC pili at 0.3 mg/ml were heated for 5 min at specified temperatures for 5 min then 5 μ l of the sample was applied to carbon-coated copper grids (Electron Microscopy Sciences) and stained with 1% phosphotungstic acid (PTA). Pili were imaged in a FEI Tecnai F20 SUPERTWIN or Hitachi 8000 STEM at 120 or 200 keV. Chemically-denatured pili were incubated in a final concentration of 0 M, 2 M, 4 M, 6 M or 8 M urea or 6 M guanidinium-HCl for 1 hour at 22 °C then applied to grids, stained and imaged as described above. To test the effect of disulfide bond reduction on pilus stability, TCP and GC pili were treated with 100 mM TCEP for 15 min at 22 °C before being applied to copper grids.

For the negative stain TEM reconstruction, purified TCP were applied to carbon-coated copper grids and stained with 1% PTA. TCP were imaged on an FEI Tecnai F20 electron microscope at 120 keV and images were collected on SO163 film (Kodak) at 51,000X magnification in low dose mode. For cryoEM, 5 μ l of a 1/5 dilution of purified TCP was applied to glow-discharged C-flat holey carbon grids (Protochips Inc.) for five minutes. Grids were blotted for 3.5 seconds using a Vitrobot Mark III Vitrification Robot (FEI) at 4°C, 100% humidity and plunge-frozen in liquid ethane cooled with liquid nitrogen. Grids were stored in liquid nitrogen and transferred to a Gatan 626 cold stage for TEM imaging at 200 keV on a FEI Tecnai F20 TWIN electron microscope at the University of Calgary Microscopy and Imaging Facility. Images were collected on SO163 film (Kodak) at 50,000X magnification in low dose mode.

Iterative Helical Real Space Reconstructions of the TCP filament

Micrographs were digitized at 2.5 Å/pixel using a Nikon Super Coolscan 8000 scanner and the IHRSR algorithm was used to generate the negative stain and cryoEM reconstructions⁴⁷.

TCP segments of varying lengths were selected from the images using the helix boxer program in EMAN⁶². To determine the approximate symmetry of the filaments, 200 pixel × 200 pixel particles were generated from the TCP segments and an averaged power spectrum was determined from 976 non-overlapping particles. The power spectrum (Fig. 4A) shows a dominant layer line at ~1/43 Å corresponding to a 3-start helix and a weaker layer line at ~1/110 Å corresponding to a 4-start or 5-start helix (Fig. 4A). The helical symmetry for the primitive 1-start helix was determined for each combination (−3, +4; −3, −4; −3, +5 and −3, −5), and IHRSR runs were performed with 8,034 overlapping particles (200 pixels length with a 190-pixel overlap, 80 pixels in width, padded to 200 pixels) using these different starting symmetries. Only the starting symmetry corresponding to the −3-start, +4-start combination converged to a reasonable solution, having a rise of 8.4 Å and a rotation of 96.8° for the 1-start helix. To determine the resolution of the reconstruction, the particles were randomly divided into two equal data sets and two volumes were generated. The resulting independently-obtained reconstructions have a FSC 0.5 at 21 Å.

CryoEM images from a small number of micrographs were digitized. For each micrograph, the contrast transfer function (CTF) was determined for the carbon film surrounding the hole using the B show program in B soft⁶³. Micrographs were corrected for phase reversals by multiplying by the CTF. Individual TCP filaments were selected using helix boxer in EMAN, and 2234 overlapping particles were generated (200 pixels length with a 190-pixel overlap, 80 pixels wide padded to 200 pixels, 2.54 Å/pixel). An average power spectrum for all 2234 particles revealed a single n=3 layer line at 1/43 Å corresponding to a pitch of 129 Å for the 3-start helix. Particles were decimated to 5.08 Å/pixel and IHRSR was run using the 2234 overlapping particles and starting symmetries approximating that of the negative stain reconstruction. None of the IHRSR runs converged after 100 cycles, suggesting heterogeneity in the filaments. To reduce heterogeneity, the particles were sorted using a series of reference volumes in which the pitch for the 3-start helix was varied (108 Å to 156 Å) based on the observed layer line in the averaged power spectrum. Almost half of the particles had a pitch in the range of 126–138 Å. One thousand particles with pitches between 126–132 Å were used for IHRSR with a starting symmetry of 8.2 Å, rise and 96.2° rotation. The IHRSR run converged to a final symmetry of 8.5 Å rise, 96.7° rotation for the cryoEM reconstruction. The final volume was corrected for the CTF using a Weiner filter⁶⁴. The reconstruction has a resolution of 27 Å based on a FSC of 0.5.

Since a relatively small number of particles was used for the TCP cryoEM reconstruction, a larger set of images was collected and processed as described above. In this case a total of 24,816 overlapping TCP particles were selected (200 pixels length with a 190-pixel overlap, 80 pixels in width, padded to 200 pixels) and IHRSR was run using starting symmetries approximating that of the negative stain reconstruction. Not surprisingly, none of the IHRSR runs converged after 100 cycles. The particles were then sorted according to the 3-start helical pitch as described above. Again, almost half of the particles had a 3-start helical pitch in the range of 126 Å to 138 Å. Various combinations of these particles were used for IHRSR: 10,415 particles with a pitch of 126 Å to 138 Å; 7,189 particles with a pitch of 126 Å to 132 Å; and 3780 particles with a pitch of 132 Å. Although each reconstruction converged to a symmetry very close to that of the negative stain reconstruction, no improvement was obtained in terms of resolution or molecular detail.

To generate a pseudo-atomic resolution TCP model, the 1.3 Å crystal structure of N-terminally-truncated TcpA²⁷ was manually docked into the negative stain reconstruction using Chimera (<http://www.cgl.ucsf.edu/chimera>). The N-terminal 28 residues (α1N) were modeled using the coordinates for *P. aeruginosa* PAK pilin²⁷. The TCP pseudo-atomic resolution structure was generated by applying the symmetry parameters for the negative stain reconstruction, 8.4 Å rise and 96.8° rotation, to the docked subunit. Preferred models

were selected having reasonable packing of the N-terminal α -helices and close contacts between the N-terminal amine and Glu5 in neighboring subunits, and between Arg26 and Glu83, which were shown by pair wise mutagenesis to interact³⁵. The final model was selected based on its similarity to the negative stain and cryoEM reconstructions, as determined by examining cross-sections of contour plots of the reconstructions and the model filtered to 21 Å.

Scanning transmission electron microscopy

TCP were freeze-dried at the Brookhaven National Laboratory STEM facility. Digital dark-field images were collected and the data were analyzed using the PCMass program. TCP data were normalized to tobacco mosaic virus using a mass per unit length of 13.1 kDa Å⁻¹.

Auto agglutination assay

After overnight growth, 2-ml *V. cholerae* cultures were allowed to settle for 10 minutes at room temperature then scored visually for auto agglutination.

Generation of *V. cholerae*-tcpA^{E5A}

The TcpA^{E5A} mutant was generated by allelic exchange as described in Lim et al.³.

Phage transduction assay

Overnight cultures of *V. cholerae* cells were incubated for 30 min at 22 °C with equal volumes of filtered supernatant from overnight cultures of *V. cholerae* CL101 cells, which produces CTX-Kn phage^{2; 5}. Cells were serially diluted and plated on LB-Kn agar plates, grown overnight at 37 °C and colony-forming units were counted.

Figure preparation

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081) and the PyMOL Molecular Graphics System, Schrödinger, LLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Edgar Young, Svetla Taneva and Rosemary Cornell for insightful discussions, Joseph Wall and Beth Lin (Brookhaven National Laboratory Scanning Transmission Electron Microscopy Facility) for STEM analysis, Tobias Fürstenhaupt (University of Calgary Microscopy and Imaging Facility), Li Yang (Simon Fraser University Nano-imaging Facility) and Sumaiya Islam for technical support. This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR, RGPIN312152 to LC) and the National Institutes of Health (AI022160 to LC and EB001567 to EHE). LC is supported by salary awards from CIHR and the Michael Smith Foundation for Health Research. JL is the recipient of a Frederick Banting and Charles Best Canada Graduate Scholarship from CIHR.

ABBREVIATIONS

Ap	ampicillin
BFP	bundle-forming pili
βOG	octyl β-D-glucopyranoside

CD	circular dichroism
CTF	contrast transfer function
DXMS	deuterium exchange mass spectrometry
EM	electron microscopy
FSC	Fourier shell correlation
EPEC	enteropathogenic <i>Escherichia coli</i>
ETEC	enterotoxigenic <i>E. coli</i>
GC pili	gonococcal pili
IHRSR	Iterative Helical Real Space Reconstruction
IPTG	isopropyl β -D-1 thiogalactopyranoside
Kn	kanamycin
LB	Luria Broth
Sm	streptomycin
T4P	Type IV pilus/pili
TCEP	tris(2-carboxyethyl)phosphine
TCP	toxin coregulated pilus/pili
TEM	transmission electron microscopy

REFERENCES

1. Ayers M, Howell PL, Burrows LL. Architecture of the type II secretion and type IV pilus machineries. *Future Microbiol.* 2010; 5:1203–1218. [PubMed: 20722599]
2. Kim TJ, Lafferty MJ, Sandoe CM, Taylor RK. Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. *Mol. Microbiol.* 2000; 35:896–910. [PubMed: 10692166]
3. Lim MS, Ng D, Zong Z, Arvai AS, Taylor RK, Tainer JA, Craig L. *Vibrio cholerae* El Tor TcpA crystal structure and mechanism for pilus-mediated microcolony formation. *Mol. Microbiol.* 2010; 77:755–770. [PubMed: 20545841]
4. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc. Natl. Acad. Sci. USA.* 1987; 84:2833–2837. [PubMed: 2883655]
5. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science.* 1996; 272:1910–1914. [PubMed: 8658163]
6. Higashi DL, Lee SW, Snyder A, Weyand NJ, Bakke A, So M. Dynamics of *Neisseria gonorrhoeae* attachment: microcolony development, cortical plaque formation, and cytoprotection. *Infect. Immun.* 2007; 75:4743–4753. [PubMed: 17682045]
7. Park HS, Wolfgang M, van Putten JP, Dorward D, Hayes SF, Koomey M. Structural alterations in a type IV pilus subunit protein result in concurrent defects in multicellular behaviour and adherence to host tissue. *Mol. Microbiol.* 2001; 42:293–307. [PubMed: 11703655]
8. Punsalang AP Jr, Sawyer WD. Role of pili in the virulence of *Neisseria gonorrhoeae*. *Infect. Immun.* 1973; 8:255–263. [PubMed: 4200266]
9. Swanson J. Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *J. Exp. Med.* 1973; 137:571–589. [PubMed: 4631989]

10. Wolfgang M, Lauer P, Park HS, Brossay L, Hebert J, Koomey M. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in pilated *Neisseria gonorrhoeae*. *Mol. Microbiol.* 1998; 29:321–330. [PubMed: 9701824]
11. Wolfgang M, Park HS, Hayes SF, van Putten JP, Koomey M. Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in *pilT*, a twitching motility gene in *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. USA.* 1998; 95:14973–14978. [PubMed: 9844000]
12. Merz AJ, So M, Sheetz MP. Pilus retraction powers bacterial twitching motility. *Nature.* 2000; 407:98–102. [PubMed: 10993081]
13. Kaufman MR, Seyer JM, Taylor RK. Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram-negative bacteria. *Genes Dev.* 1991; 5:1834–1846. [PubMed: 1680773]
14. Strom MS, Lory S. Amino acid substitutions in pilin of *Pseudomonas aeruginosa*. Effect on leader peptide cleavage, amino-terminal methylation, and pilus assembly. *J. Biol. Chem.* 1991; 266:1656–1664. [PubMed: 1671038]
15. Zhang HZ, Lory S, Donnenberg MS. A plasmid-encoded prepilin peptidase gene from enteropathogenic *Escherichia coli*. *J. Bacteriol.* 1994; 176:6885–6891. [PubMed: 7961448]
16. Pasloske BL, Paranchych W. The expression of mutant pilins in *Pseudomonas aeruginosa*: fifth position glutamate affects pilin methylation. *Mol. Microbiol.* 1988; 2:489–495. [PubMed: 2902505]
17. Macdonald DL, Pasloske BL, Paranchych W. Mutations in the fifth-position glutamate in *Pseudomonas aeruginosa* pilin affect the transmethylation of the N-terminal phenylalanine. *Can. J. Microbiol.* 1993; 39:500–505. [PubMed: 8330261]
18. Aas FE, Winther-Larsen HC, Wolfgang M, Frye S, Lovold C, Roos N, van Putten JP, Koomey M. Substitutions in the N-terminal alpha helical spine of *Neisseria gonorrhoeae* pilin affect Type IV pilus assembly, dynamics and associated functions. *Mol. Microbiol.* 2007; 63:69–85. [PubMed: 17140412]
19. Pepe JC, Lory S. Amino acid substitutions in PilD, a bifunctional enzyme of *Pseudomonas aeruginosa*. Effect on leader peptidase and N-methyltransferase activities in vitro and in vivo. *J. Biol. Chem.* 1998; 273:19120–19129. [PubMed: 9668097]
20. Horiuchi T, Komano T. Mutational analysis of plasmid R64 thin pilus prepilin: the entire prepilin sequence is required for processing by type IV prepilin peptidase. *J. Bacteriol.* 1998; 180:4613–4620. [PubMed: 9721303]
21. Sandkvist M. Biology of type II secretion. *Mol. Microbiol.* 2001; 40:271–283. [PubMed: 11309111]
22. Strom MS, Lory S. Structure-function and biogenesis of the type IV pili. *Annu. Rev. Microbiol.* 1993; 47:565–596. [PubMed: 7903032]
23. Craig L, Pique ME, Tainer JA. Type IV pilus structure and bacterial pathogenicity. *Nat. Rev. Microbiol.* 2004; 2:363–378. [PubMed: 15100690]
24. Pelicic V. Type IV pili: *e pluribus unum?* *Mol. Microbiol.* 2008; 68:827–837. [PubMed: 18399938]
25. Craig L, Volkmann N, Arvai AS, Pique ME, Yeager M, Egelman EH, Tainer JA. Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol. Cell.* 2006; 23:651–662. [PubMed: 16949362]
26. Parge HE, Forest KT, Hickey MJ, Christensen DA, Getzoff ED, Tainer JA. Structure of the fibre-forming protein pilin at 2.6 Å resolution. *Nature.* 1995; 378:32–38. [PubMed: 7477282]
27. Craig L, Taylor RK, Pique ME, Adair BD, Arvai AS, Singh M, Lloyd SJ, Shin DS, Getzoff ED, Yeager M, Forest KT, Tainer JA. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol. Cell.* 2003; 11:1139–1150. [PubMed: 12769840]
28. Hartung S, Arvai AS, Wood T, Kolappan S, Shin DS, Craig L, Tainer JA. Ultra-high resolution and full-length pilin structures with insights for filament assembly, pathogenic functions and vaccine potential. *J. Biol. Chem.* 2011; 286:44254–44265. [PubMed: 22027840]

29. Audette GF, Irvin RT, Hazes B. Crystallographic analysis of the *Pseudomonas aeruginosa* strain K122-4 monomeric pilin reveals a conserved receptor-binding architecture. *Biochemistry*. 2004; 43:11427–11435. [PubMed: 15350129]
30. Hazes B, Sastry PA, Hayakawa K, Read RJ, Irvin RT. Crystal structure of *Pseudomonas aeruginosa* PAK pilin suggests a main-chain-dominated mode of receptor binding. *J. Mol. Biol.* 2000; 299:1005–1017. [PubMed: 10843854]
31. Nguyen Y, Jackson SG, Aidoo F, Junop M, Burrows LL. Structural characterization of novel *Pseudomonas aeruginosa* type IV pilins. *J. Mol. Biol.* 2010; 395:491–503. [PubMed: 19895819]
32. Helaine S, Dyer DH, Nassif X, Pelicic V, Forest KT. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. *Proc Natl Acad Sci USA*. 2007; 104:15888–15893. [PubMed: 17893339]
33. Ramboarina S, Fernandes PJ, Daniell S, Islam S, Simpson P, Frankel G, Booy F, Donnenberg MS, Matthews S. Structure of the bundle-forming pilus from enteropathogenic *Escherichia coli*. *J. Biol. Chem.* 2005; 280:40252–40260. [PubMed: 16172128]
34. Xu XF, Tan YW, Lam L, Hackett J, Zhang M, Mok YK. NMR structure of a type IVb pilin from *Salmonella typhi* and its assembly into pilus. *J. Biol. Chem.* 2004; 279:31599–31605. [PubMed: 15159389]
35. Li J, Lim MS, Li S, Brock M, Pique ME, Woods VL Jr, Craig L. *Vibrio cholerae* toxin-coregulated pilus structure analyzed by hydrogen/deuterium exchange mass spectrometry. *Structure*. 2008; 16:137–148. [PubMed: 18184591]
36. Aas FE, Wolfgang M, Frye S, Dunham S, Lovold C, Koomey M. Competence for natural transformation in *Neisseria gonorrhoeae*: components of DNA binding and uptake linked to type IV pilus expression. *Mol. Microbiol.* 2002; 46:749–760. [PubMed: 12410832]
37. Higashi DL, Zhang GH, Biais N, Myers LR, Weyand NJ, Elliott DA, So M. Influence of type IV pilus retraction on the architecture of the *Neisseria gonorrhoeae*-infected cell cortex. *Microbiology*. 2009; 155:4084–4092. [PubMed: 19762436]
38. Dietrich M, Bartfeld S, Munke R, Lange C, Ogilvie LA, Friedrich A, Meyer TF. Activation of NF- κ B by *Neisseria gonorrhoeae* is associated with microcolony formation and type IV pilus retraction. *Cell Microbiol.* 2011
39. Maier B, Potter L, So M, Seifert HS, Sheetz MP. Single pilus motor forces exceed 100 pN. *Proc Natl Acad Sci USA*. 2002; 99:16012–16017. [PubMed: 12446837]
40. Biais N, Ladoux B, Higashi D, So M, Sheetz M. Cooperative retraction of bundled type IV pili enables nanonewton force generation. *PLoS Biol.* 2008; 6:e87. [PubMed: 18416602]
41. Winther-Larsen HC, Wolfgang MC, van Putten JP, Roos N, Aas FE, Egge-Jacobsen WM, Maier B, Koomey M. *Pseudomonas aeruginosa* Type IV pilus expression in *Neisseria gonorrhoeae*: effects of pilin subunit composition on function and organelle dynamics. *J. Bacteriol.* 2007; 189:6676–6685. [PubMed: 17573479]
42. Clausen M, Jakovljevic V, Sogaard-Andersen L, Maier B. High-force generation is a conserved property of type IV pilus systems. *J. Bacteriol.* 2009; 191:4633–4638. [PubMed: 19429611]
43. Anantha RP, Stone KD, Donnenberg MS. Role of BfpF, a member of the PilT family of putative nucleotide-binding proteins, in type IV pilus biogenesis and in interactions between enteropathogenic *Escherichia coli* and host cells. *Infect. Immun.* 1998; 66:122–131. [PubMed: 9423848]
44. Bieber D, Ramer SW, Wu CY, Murray WJ, Tobe T, Fernandez R, Schoolnik GK. Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. *Science*. 1998; 280:2114–2118. [PubMed: 9641917]
45. Zahavi EE, Lieberman JA, Donnenberg MS, Nitzan M, Baruch K, Rosenshine I, Turner JR, Melamed-Book N, Feinstein N, Zlotkin-Rivkin E, Aroeti B. Bundle forming pilus retraction enhances enteropathogenic *Escherichia coli* infectivity. *Mol. Biol. Cell.* 2011; 22:2436–2447. [PubMed: 21613538]
46. Hansen JK, Demick KP, Mansfield JM, Forest KT. Conserved regions from *Neisseria gonorrhoeae* pilin are immunosilent and not immunosuppressive. *Infect. Immun.* 2007; 75:4138–4147. [PubMed: 17562775]

47. Egelman EH. A robust algorithm for the reconstruction of helical filaments using single-particle methods. *Ultramicroscopy*. 2000; 85:225–234. [PubMed: 11125866]
48. Galkin VE, Orlova A, Rivera C, Mullins RD, Egelman EH. Structural polymorphism of the ParM filament and dynamic instability. *Structure*. 2009; 17:1253–1264. [PubMed: 19748346]
49. Trachtenberg S, Galkin VE, Egelman EH. Refining the structure of the *Halobacterium salinarum* flagellar filament using the iterative helical real space reconstruction method: insights into polymorphism. *J. Mol. Biol.* 2005; 346:665–676. [PubMed: 15713454]
50. Wang YA, Yu X, Overman S, Tsuboi M, Thomas GJ Jr, Egelman EH. The structure of a filamentous bacteriophage. *J. Mol. Biol.* 2006; 361:209–215. [PubMed: 16843489]
51. Craig L, Li J. Type IV pili: paradoxes in form and function. *Curr. Opin. Struct. Biol.* 2008; 18:267–277. [PubMed: 18249533]
52. Ali MH, Imperiali B. Protein oligomerization: how and why. *Bioorg. Med. Chem.* 2005; 13:5013–5020. [PubMed: 15993087]
53. Poyraz O, Schmidt H, Seidel K, Delissen F, Ader C, Tenenboim H, Goosmann C, Laube B, Thunemann AF, Zychlinsky A, Baldus M, Lange A, Griesinger C, Kolbe M. Protein refolding is required for assembly of the type three secretion needle. *Nat. Struct. Mol. Biol.* 2010; 17:788–792. [PubMed: 20543831]
54. Campos M, Francetic O, Nilges M. Modeling pilus structures from sparse data. *J. Struct. Biol.* 2011; 173:436–444. [PubMed: 21115127]
55. Olivier V, Salzman NH, Satchell KJ. Prolonged colonization of mice by *Vibrio cholerae* El Tor O1 depends on accessory toxins. *Infect. Immun.* 2007; 75:5043–5051. [PubMed: 17698571]
56. Yamamoto T, Yokota T. Electron microscopic study of *Vibrio cholerae* O1 adherence to the mucus coat and villus surface in the human small intestine. *Infect. Immun.* 1988; 56:2753–2759. [PubMed: 3417355]
57. Pasloske BL, Scraba DG, Paranchych W. Assembly of mutant pilins in *Pseudomonas aeruginosa*: formation of pili composed of heterologous subunits. *J. Bacteriology*. 1989; 171:2142–2147.
58. Korotkov KV, Hol WG. Structure of the GspK-GspI-GspJ complex from the enterotoxigenic *Escherichia coli* type 2 secretion system. *Nat. Struct. Mol. Biol.* 2008; 15:462–468. [PubMed: 18438417]
59. Sun DX, Mekalanos JJ, Taylor RK. Antibodies directed against the toxin-coregulated pilus isolated from *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. *J. Infect. Dis.* 1990; 161:1231–1236. [PubMed: 1971653]
60. DiRita VJ, Neely M, Taylor RK, Bruss PM. Differential expression of the ToxR regulon in classical and El Tor biotypes of *Vibrio cholerae* is due to biotype-specific control over *toxT* expression. *Proc. Natl. Acad. Sci. USA*. 1996; 93:7991–7995. [PubMed: 8755590]
61. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
62. Ludtke SJ, Baldwin PR, Chiu W. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* 1999; 128:82–97. [PubMed: 10600563]
63. Heymann JB. Bsoft: image and molecular processing in electron microscopy. *J. Struct. Biol.* 2001; 133:156–169. [PubMed: 11472087]
64. Frank J, Penczek P. On the corrections of the contrast transfer function in biological electron microscopy. *Optik*. 1995; 98:125–129.
65. Yang Z, Hu W, Chen K, Wang J, Lux R, Zhou ZH, Shi W. Alanine 32 in PilA is important for PilA stability and type IV pili function in *Myxococcus xanthus*. *Microbiology*. 2011; 157:1920–1928. [PubMed: 21493683]
66. Segal E, Billyard E, So M, Storzbach S, Meyer TF. Role of chromosomal rearrangement in *N. gonorrhoeae* pilus phase variation. *Cell*. 1985; 40:293–300. [PubMed: 2857113]
67. De Lorenzo V, Timmis K. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* 1994; 235:386–405. [PubMed: 8057911]

HIGHLIGHTS

- *N. gonorrhoeae* Type IVa pili are much more stable than *V. cholerae* Type IVb pili
- electron microscopy reconstructions of the *V. cholerae* Type IVb pilus are presented
- the Type IVa and IVb pili have similar structures despite distinct pilin folds
- pilus stability is attributed to compact subunit packing and interface aromatics

Figure 1. Thermal stability comparison for *V. cholerae* and *N. gonorrhoeae* Type IV pilins and their respective pilus filaments

Far UV CD spectra for (A) ΔN -TcpA and TCP filaments and (B) ΔN -PilE and GC pili. (C) Thermal denaturation of ΔN -TcpA and TCP measured by CD spectroscopy at the wavelengths indicated. (D) Thermal denaturation of ΔN -PilE and GC pili. CD plots are averages of two independent experiments. (E) TEM images of negatively-stained pili treated at the temperatures indicated for 5 min.

Figure 2. Urea denaturation of TCP and GC pili

Far UVCD spectra for (A) *V. cholerae* TCP and (B) *N. gonorrhoeae* GC pili after treatment with various concentrations of urea. CD plots are averages of two independent experiments. (C) TEM images of negatively-stained pili treated with urea.

Figure 3. Comparison of Type IVa vs. IVb pilin and pilus protease susceptibility

Coomassie-stained SDS-PAGE analysis of TCP, GC pili, Δ N-TcpA pilin and Δ N-PilE pilin digested with a panel of proteases at 37 °C for the indicated times using a mass ratio of 1:1 protein:protease. Calculated molecular weights are 20.3 kDa for full length TcpA (TCP), 17.2 kDa for PilE (GC pili), 18.0 kDa for Δ N-TcpA and 15.0 kDa for Δ N-PilE. Bands seen in Δ N-PilE at 1, 6 and 24 hr time points are chymotrypsin self-degradation products that appear at ~14 kDa.

Figure 4. Negative stain reconstruction and STEM analysis of TCP

(A) Negative stain image of purified classical *V. cholerae* TCP. Inset: averaged power spectrum for 976 non-overlapping TCP segments, 200 pixels in length. (B) STEM histogram for TCP giving an average mass/length of 2.4 kDa/Å. (C) Negative stain reconstruction of TCP obtained from 8,034 overlapping particles showing a right-handed 1-start helix (+1), a left-handed 3-start helix (−3) and a right-handed 4-start helix (+4). The reconstruction has an axial rise of 8.4 Å and an azimuthal rotation of 96.8° for the +1 helix.

Figure 5. CryoEM TCP reconstruction

(A) CryoEM image of frozen-hydrated TCP filaments. Inset: averaged power spectrum for 2234 TCP segments, 200 pixels in length. (B) CryoEM reconstruction of TCP having an axial rise of $8.5 \text{ \AA}$ and an azimuthal rotation of 96.7° .

Figure 6. Pseudo-atomic resolution structure of *V. cholerae* TCP

(A) Side and end views of a single TcpA pilin subunit docked into the negative stain TEM reconstruction. The arrow indicates the path of the +1 helix. (B) Side and end views of the TCP filament model shown as a cartoon representation, with the left-handed 3-start helical strands colored red, yellow and blue. The relationship between subunits in the +1 helix is indicated. (C) Side and end views of TCP to show the surface exposure of the D-region (magenta), the involvement of the $\alpha\beta$ -loop (green) in subunit:subunit interactions, and the solid core of the filament. Met1, Glu5, Arg26 and Glu83 are shown in stick representation in the top panel. (D) Close-up of the stabilizing salt bridge between Arg26 on $\alpha 1$ and Glu83 in the globular domain of a neighboring subunit. (E) A segment of $\alpha 1N$ is exposed between gaps in the globular domains. (F) Close up of the N-termini showing the potential charge neutralization between Glu5 on one subunit and the N-terminal amine on the adjacent subunit in the one-start helix.

Figure 7. Effect of Glu5Ala substitution on TcpA expression and TCP assembly

(A) Immunoblot of TcpA from wild type *V. cholerae* O395+pMT5 and *tcpA* mutants showing total TcpA in whole cell cultures and TcpA from assembled pili in sheared cell supernatant. MWM, molecular weight markers, kDa units. Coomassie-stained OmpU (37 kDa) is shown below as a loading control. (B) Results of TEM analysis, auto agglutination and phage transduction assays to assess the presence of functional pili in *V. cholerae* O395+pMT5 and *tcpA* mutant strains.

Figure 8. Close packing of the pilin subunits and conserved N-terminal aromatic residues may stabilize GC pili

(A) Top view of the α -helical core of TCP and GC pili showing differences in diameter and packing of the α -helices. The globular domain is shown for a single subunit (yellow). The conserved cysteines, Cys120 and Cys186 in TcpA, Cys121 and Cys151 in PilE, are shown in ball-and-stick representation and the C-terminal segment beyond the second cysteine is colored red. (B) Side view of TCP and GC pili with a single subunit colored as in (A) to show the differences in the mass distribution in the globular domain and in the position of the C-terminal segment. The C-terminal residue is indicated. (C) Surface representation of TCP and GC pili oriented to show the gap between globular domains exposing a segment of α 1N (circled). The TCP gap measures ~ 16 Å in width and ~ 25 Å in length and the GC pilus gap is ~ 7 Å by ~ 32 Å. (D) Left. Cross-section of the GC pilus α 1 segments in the filament core showing the close proximity of aromatic residues Phe1 of one subunit (eg. yellow sticks) and Tyr24 and Tyr27 (cyan sticks) of an adjacent subunit in the +4-start helix, which can potentially stack to increase the binding energy of the filament. Each subunit has a different color, with oxygen atoms of the aromatic side chains shown in red. Only Phe1 of the yellow subunit is visible in this view. Right. Side view GC pilus showing only the yellow and cyan subunits from the left panel. These subunits are separated by an axial

distance of 42 Å in the +4 helix. (E) Phe1, Tyr24 and Tyr27 are highly conserved in Type IVa pilins but not Type IVb pilins. Ng, *N. gonorrhoeae* strain MS11 (GenBank Accession number P02974); Nm, *N. meningitidis* (CAA30557); Pa, *P. aeruginosa* (top, strain K, P02973, bottom, strain K122-4, P17838); Dn, *D. nodosus* (X52403); Mb, *Moraxella bovis* (AAA53087); EHEC, enterohemorrhagic *E. coli* O157-H7 (AAG54412); Mx, *M. xanthus*⁶⁵; Ec, *Eikenella corrodens* (P35645); ntHi, non-typeable *Haemophilus influenzae* (AAX87353); Ft, *Francisella tularensis* Schu S4 FTT0889c, CAG45522); Cp, *Clostridium perfringens* (Gram positive, NP_563200). Nm PilX (AAF62322) is a minor pilin subunit. Vc, *V. cholerae* (ABQ19609); ETEC, enterotoxigenic *E. coli* (CofA, BAA07174; LngA, AAC33154); Cr, *Citrobacter rodentium* (AAO17805); EPEC, enteropathogenic *E. coli* (Z68186); ST, *S. Typhi* (AAF81215); R64, Inc11 plasmid R64 (BAA77979).

Table 1**Bacterial strains**

Bacterial strains	Description	Reference/Source
<i>N. gonorrhoeae</i> MS11	variant C30	66
<i>V. cholerae</i> O395	Classical Ogawa, pMT5: <i>toxT</i> , Sm ^R	60
<i>V. cholerae</i> RT4225	O395, <i>tcpA</i> ^{H181A} , pMT5: <i>toxT</i>	2; 60
<i>V. cholerae</i> SI009	O395, <i>tcpA</i> ^{E5A} , pMT5: <i>toxT</i>	This study
<i>V. cholerae</i> SJK70	O395, Δ <i>tcpA</i>	R.K. Taylor
<i>V. cholerae</i> CL101	O395, pCTX-Knφ.	2; 5
<i>E. coli</i> S17λpir	<i>thi pro recA hsdR</i> ⁻ <i>M</i> ⁺ [RP4-2-Tc::Mu::Km ^R Tn7] λ <i>pir</i> Tmp ^R Sp ^R , Sm ^R	67