Type IV Pilin Proteins: Versatile Molecular Modules

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INTRODUCTION

Type IV pilins are small (~7 to 20 kDa) structural proteins with a conserved, hydrophobic α-helical N terminus that is both a transmembrane (TM) domain and a protein-protein interaction domain. In general, the proteins function through their reversible polymerization into helical fibers by dedicated assembly/disassembly systems. In current models, system-specific hexameric ATPases are predicted to undergo conformational changes upon ATP hydrolysis, converting chemical energy into mechanical energy (285, 348). In ways that are not yet clear, the force generated by resulting domain movements in the ATPases is thought to move subunits from the cytoplasmic membrane into the fiber during polymerization and, for type IV pili (T4P), from the fiber back into the membrane during depolymerization. Fibers are predicted to grow by the addition of subunits at the base, with an estimated 12 protomers forming the short type II secretion (T2S) system pseudopilus—a length sufficient to span the periplasm of Gram-negative bacteria—and 500 to 1,000 (or more) subunits forming the quaternary structure of a T4P (349). This dynamic assembly and disassembly of type IV pilin-like proteins is impor-

FIG 1 Alignment of N-terminal sequences of type IV pilin proteins. The leader peptide and first 40 N-terminal residues of representative mature type IV pilin protein sequences were aligned based on the prepilin peptidase cleavage site (marked by an arrow). Pilin-like proteins share the type III signal sequence, which is cleaved before the hydrophobic stretch between the Gly (−1) and Phe (+1) residues, although the +1 residue can vary. The consensus signal sequence used by the PilFind algorithm (195) to identify putative type IV pilin proteins is shown below the alignment. The hydrophobic N terminus of mature pilin proteins is situated in the inner membrane and contains the highly conserved Glu5 (+5) residue (shown in bold); in GspK orthologs, there is a hydrophobic residue at that position. The transmembrane segments, as predicted by Geneious Pro v5.0.3 (Biomatters Ltd.) using TMHMM, are highlighted in blue. Pa, *P. aeruginosa*; Vc, *V. cholerae*; Aa, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*; Mm, *Methanococcus maripaludis*; Nm, *N. meningitidis*; ETEC, enterotoxigenic *E. coli*; Bs, *Bacillis* subtilis; Mv, *Methanococcus voltae*.

tant for the functioning of T4P and T2S systems, as well as for DNA transfer or uptake by a number of naturally competent species (30, 112, 295, 309).

The Signature Type III Signal Sequence

Although type IV pilin proteins are quite divergent in sequence, their defining characteristic is a distinctive N-terminal signal sequence (Fig. 1), denoted "type III" (379) to distinguish it from the type I (recognized by signal peptidase I) and type II (characteristic of lipoproteins) signal sequences. Canonical type I and type II signal sequences are cleaved at the exterior of the cytoplasmic membrane, C-terminal to a stretch of hydrophobic residues. In contrast, proteins with the unique type III signal sequence are called prepilins and are not competent for assembly until the signal is cleaved at the cytoplasmic face of the membrane (369). Prepilins are inserted into the cytoplasmic membrane by the Sec system (22, 144), and the signal sequence is removed by dedicated aspartyl proteases called prepilin peptidases (301). The positively charged signal sequence is likely important for the correct orientation of pilin proteins, with their C-terminal domains outside the cytoplasmic membrane. However, its polar nature may inhibit subsequent extraction of the subunits from the membrane and/or protein-protein interactions between subunits required for fiber assembly. The type III signal sequence motif (Fig. 1) has been used to develop an algorithm called PilFind (http://signalfind.org /pilfind.html) that can accurately identify putative pilins (195).

Both T4P and T2S systems include multiple type III signal sequence-carrying proteins (183). The fibers are composed predominantly of a single subunit, referred to as the major pilin in T4P and the major pseudopilin in T2S. Additional pilin-like proteins are present at lower abundances, and for that reason, they are called the minor pilins/pseudopilins. Some are common to most systems (referred to here as "core" minor pilins/pseudopilins), and the genes encoding them are typically clustered, while other minor subunits are unique to particular species or genera and may or may not be encoded with the core minor subunits (Table 1). Although the minor subunits are present at low levels relative to

the major subunits, they can have profound effects on function (see below).

Proteins with a type III signal sequence have been identified as components of DNA uptake (Com) systems in a wide variety of Gram-negative genera, as well as Gram-positive *Bacillus*, *Clostridium*, and *Streptococcus* species (30); in plasmid-encoded DNA transfer systems (221); as subunits of archaeal flagella (for recent reviews, see references 9 and 151); and even as electrically conductive nanowires in *Geobacter* (327). This broad distribution of type IV pilin proteins among eubacteria and archaea suggests an ancient and versatile modular architecture that has been adapted for many functions.

Type IVa versus Type IVb Pilins

A number of distinctive features divide major pilins into two classes, called T4aP and T4bP. The T4a pilins are a relatively homogeneous class and are found in plant, animal, and human pathogens such as *Pseudomonas*, *Neisseria*, and *Dichelobacter*, as well as in environmental genera such as *Thermus*, *Myxococcus*, *Deinococcus*, *Bdellovibrio*, and *Shewanella* (311). The T4b class is more diverse and is best characterized for enteric bacteria such as enteropathogenic, enterohemorrhagic, and enterotoxigenic *Escherichia coli*, *Salmonella enterica* serovar Typhi, and *Vibrio cholerae*. T4b pilins are further divided into subtypes, including the tight adherence pili (Tad; also called Flp or Fap) that were first identified in *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* (196, 210, 313). Tad pili are distributed among a variety of Gram-positive and Gram-negative species, including well-studied environmental bacteria such as *Caulobacter* (195, 393). Tad pili have smaller subunits (∼7 to 8 kDa) than other T4P and T2S systems (∼15 to 20 kDa). Other T4b subtypes include the plasmid-encoded longus pili of enterotoxigenic *E. coli*, so called because they can reach lengths of 20 μm or more (157). Some species can express multiple kinds of T4P (83, 117, 155, 195), while a subset of archaea coexpress pili and archaeal flagella (200, 296), both of which are formed from subunits of the type IV pilin protein family.

TABLE 1 Type IV pilin proteins in select model systems

Component	Pilin protein(s)			References
Type IVa pili	*Pseudomonas aeruginosa*	*Neisseria* spp.	*Vibrio cholerae* (MSHA[a])	
Major subunit	PilA	PilE	MshA	270, 282, 307
Core minor subunits	FimU, PilV, PilW, PilX	PilH, PilI, PilJ, PilK	MshB, MshC, MshD, MshO	13–15, 270, 416
Noncore minor subunits	PilE	PilX (PilL), PilV, ComP		175, 340, 415
Prepilin peptidase	PilD	PilD	VcpD	146, 271, 298
Type IVb pili	EPEC[b] (bundle-forming pili)	*Vibrio cholerae* (Tcp[c])	R64 thin pili	
Major subunit	BfpA	TcpA	PilS	123, 135, 221
Core minor subunits	BfpI, BfpJ, BfpK	TcpB	PilV	221, 265, 368
Prepilin peptidase	BfpP	TcpJ	PilU	221, 246, 368
Tad pili	*Aggregatibacter actinomycetemcomitans*	*Caulobacter crescentus*	*Pseudomonas aeruginosa*	
Major subunit	Flp	PilA	Flp	117, 210, 359
Core minor subunits	TadE, TadF	NA[d]	TadF	42, 393
Prepilin peptidase	TadV	CpaA	FppA	117, 359, 392
Type II pseudopili	*Klebsiella oxytoca*	*Vibrio cholerae*	*Pseudomonas aeruginosa*	
Major subunit	PulG	EpsG	XcpT	299, 331, 347
Core minor subunits	PulH, PulI, PulJ, PulK	EpsH, EpsI, EpsJ, EpsK	XcpU, XcpV, XcpW, XcpX	299, 331, 347
Prepilin peptidase	PulO	VcpD	XcpA	37, 271, 321

[a] Mannose-sensitive hemagglutinin pili.
[b] Enteropathogenic *E. coli*.
[c] Toxin-coregulated pili.
[d] NA, not applicable.

T4a and T4b major pilins have traditionally been distinguished by differences in the lengths and sequences of their leader peptides (135). The leader peptides of T4a major pilins are usually short (6 or 7 residues), while those of T4bP are longer (15 to 30 residues). The T4a major pilins most often have a methylated Phe at the N terminus following removal of the signal sequence, while T4b pilins can have other, typically hydrophobic, residues at the same position.

Major pseudopilins generally have short leader peptides (6 or 7 residues), although there are exceptions, such as *Erwinia* OutG (22 residues). The archaeal pilins and flagellins have leader sequences ranging from 3 to 20 residues (315). The PilFind algorithm (195) will allow for a more complete bioinformatic analysis of type IV pilin proteins, which in turn will provide better statistics on what is considered a "typical" leader in terms of length and sequence characteristics.

The size of the mature (processed) pilin has also been used as a criterion to distinguish the two subclasses, with T4b pilins being larger, on average, than T4a pilins (~180 to 200 residues versus 150 to 175 residues), except for the Flp pilins, which are significantly smaller (~50 to 80 residues). As more type IV pilin proteins with a range of overlapping sizes are identified through high-throughput sequencing and metagenomic approaches (195), sequence length is likely to become a less useful feature for distinguishing between the two classes. A more informative way to determine whether the pilin-like proteins in a particular bacterial strain are T4a, T4b, or T2S subunits is to consider their corresponding assembly system(s), as each has characteristic components (31). For example, T4a but not T4b assembly systems often include homologs of the PilMNOP inner membrane complex proteins, while T2S systems typically have GspLMC components instead (31, 125, 311, 386). Among the exceptions to this rule of thumb are T4a pili encoded in single large clusters (likely part of former mobile elements), such as the mannose-sensitive hemag-

glutinin (MSHA) pili of *V. cholerae* and other environmental bacteria (57, 149, 270, 350, 408). T4b assembly systems are more diverse with respect to their specific components, but both Tad and Com systems have characteristic elements (195). For comprehensive comparisons of T4a, T4b, and T2S assembly systems and their components—which are beyond the scope of this article—the reader is directed to previously published references (31, 99, 112, 113, 125, 139, 195, 205, 275, 309, 311, 313, 393).

STRUCTURES OF TYPE IV PILIN PROTEINS

Outside their conserved N termini, type IV pilin proteins can have widely divergent sequences. Despite this limited sequence identity, X-ray crystallography and nuclear magnetic resonance (NMR) studies have revealed the remarkable underlying structural similarity in this protein family. For comprehensive reviews on the particulars of type IV pilin structure, the reader is directed to previous references (112, 113, 168, 311). Below, we highlight recent findings regarding the structures of type IV pilin proteins.

Although several structures have been solved, thus far they are limited to Gram-negative major pilins (both T4a and T4b subfamilies) (26, 36, 114, 297, 306, 326, 420), a *Neisseria*-specific minor pilin from *Neisseria meningitidis* (176), and T2S major and minor pseudopilins from a number of species (16, 233, 236, 421, 422). To date, there are no structures available for the core minor pilins (named FimU, PilV, PilW, and PilX in *Pseudomonas aeruginosa* and PilH, PilI, PilJ, and PilK in *Neisseria*) (Table 1) from any T4P system, for the Tad/Flp pilins, for competence-specific pilins, or for any of the archaeal type IV flagellin or pilin subunits. This lack of structural depth is unfortunate, as it precludes a more complete understanding at this time of the flexibility and adaptability of the predicted T4P fold in other members of this family. The poor sequence conservation of the C-terminal domains of type IV pilin proteins usually prevents the generation of high-confidence mod-

els based on existing structures and the use of molecular replacement methods in crystallography studies.

Ironically, even though the extended N-terminal helix of type IV pilin proteins is their most distinctive trait, the majority of structures solved lack most of this region. To date, only three full-length structures are available (114, 169, 306), all of which are for T4a pilins. As techniques for purification and manipulation of membrane proteins improve, it will be important to add additional full-length structures for comparison. The pilins are typically truncated by ~28 residues or more to remove the hydrophobic first half of the N-terminal α-helix, improving their solubility. The minimal impact of this deletion on pilin structure was confirmed by comparison of the N-terminally truncated and full-length versions of the *P. aeruginosa* PAK pilin, which were essentially identical, with a root mean square deviation for all atoms of 0.69 Å (114). Interestingly, it is possible to induce the formation of hollow pilus-like nanotubes from N-terminally truncated pilins by using a hydrophobic small molecule to replace the missing hydrophobic domains (27).

Although the N-terminal segment appears dispensable for folding of the C terminus, it has important functional, and potentially regulatory, roles in T4P biology (see below), and thus minor sequence differences in this region can be quite significant. For example, Aas et al. (4) showed that point mutations at the highly conserved Glu5 residue (Fig. 1) of the *Neisseria gonorrhoeae* major pilin, PilE, precluded assembly unless the mutant protein was co-expressed with wild-type subunits. The mixed fibers had altered T4P phenotypes, including differences in retraction dynamics, which were dependent upon the nature of the substitution. Similarly, a recent study of *Myxococcus xanthus* (424) showed that mutation of the Ala residue at position 20 of the mature pilin (Ala32 in prepilin sequence numbering) to Val resulted in formation of adhesive pili that were deficient in retraction. Alteration to Gly or Ser gave wild-type phenotypes, while other substitutions resulted in unstable pilins or pilins that were unable to assemble. These studies show that very small variations at key residues can have major consequences for function.

General Architecture of Type IV Pilin Proteins

Full-length type IV pilin proteins resemble a lollipop or a ladle (Fig. 2). The extended N-terminal α-helix has two subdomains: α1-N (spanning amino acids ~1 to 28) and α1-C (amino acids ~29 to 52). The hydrophobic α1-N region protrudes from the globular C-terminal domain and forms the central core of the assembled pilus fiber (113). The α1-N domain is multifunctional, acting as a transmembrane segment to retain individual pilin subunits in the cytoplasmic membrane prior to assembly, as a protein interaction domain for subunit-subunit interactions in the fiber, and potentially as a regulatory domain (see below). In the case of T4P, where hundreds to thousands of subunits can form a single fiber, mature subunits disassembled from the pilus when it is retracted are thought to reenter the cytoplasmic membrane via the α1-N domain for use in subsequent rounds of assembly. In contrast, subunits of the short T2S pseudopilus may be degraded as a means of pseudopilus retraction (129). The α1-C region embedded in the C-terminal globular domain is amphipathic and packs against the head domain, which generally consists of a 4- to 7-stranded antiparallel β-sheet oriented 45° or more relative to the long axis of the α1 helix. Sequence differences in the loop regions and in the orientation of secondary structure elements

within the C-terminal domain contribute to the structural diversity associated with these proteins.

In the full-length structures of the T4a pilins *P. aeruginosa* PAK PilA and *N. gonorrhoeae* PilE, there is a shallow S-shaped kink in the N-terminal helix (114, 306), generated by Pro22 and Gly/Pro42 (26, 114, 126, 172, 219, 297, 306). The resulting curvature is thought to reduce intersubunit packing, thereby contributing to the flexibility of the fiber (67, 114, 251, 306), but may also contribute to fiber assembly-disassembly dynamics by making otherwise rigid transmembrane domains more flexible (78). Although a full-length structure of a T4b pilin has yet to be solved, N-terminal sequences of that subclass lack the characteristic Pro residues of T4a sequences. Instead, they have Gly residues that could impart flexibility. Li et al. (251) showed that solvent exposure of assembled *V. cholerae* TcpA subunits was similar to that of unassembled monomers, suggesting that limited interactions between T4b subunits in fibers exposed large areas of their surfaces. Portions of the N-terminal α-helices—previously assumed to be buried in the assembled fiber—were also solvent exposed. Those data suggested that despite the lack of N-terminal Pro residues, the packing of T4b pilins in the pilus is looser than that of T4a pilins, contributing to fiber flexibility. The looser packing means that they are less resistant to proteases than T4a pili, which are quite stable and, amazingly, remain intact even in 8 M urea (250).

The *N. meningitidis* noncore minor pilin PilX lacks a kink in the N-terminal helix, despite having a Gly residue at position 42; however, the full-length protein may have a curved N-terminal helix, as it has a Pro residue at position 22 (176). Although there are no other T4P minor pilin structures yet available, analysis of the representative *P. aeruginosa* minor pilins shows that only FimU and PilE have a conserved Pro22 residue, while the *N. meningitidis* minor pilin PilH, equivalent to FimU, also has a Pro residue at position 22. The major pseudopilin GspG—named XcpT and EpsG in *Pseudomonas* and *Vibrio*, respectively—has a conserved Pro22 residue. The minor pseudopilin GspH and its relatives have a Pro residue in the α1-C domain. Korotkov and Hol (236) solved the structure of a heterotrimeric complex of GspIJK, and based on its architecture, they hypothesized that it may form the tip of the T2S pseudopilus. It is possible that the Pro22 residue is important only for controlling the extent of intersubunit packing along the fiber to provide flexibility and is less important for those subunits located at the pilus or pseudopilus tip.

The 4- to 7-stranded β-sheet of type IV pilin proteins is a conserved structural motif that forms the majority of the C-terminal domain of the protein. In all of the T4a pilins, as well as the minor pilin and pseudopilin structures solved to date, this region is composed of a β-meander with 3 to 4 β-strands having nearest-neighbor connectivity (16, 112, 176, 233, 236, 244, 421, 422). The T4b pilins are somewhat different, in that the antiparallel β-sheet is composed of 5 to 7 β-strands in the case of *V. cholerae* TcpA and *S.* Typhi PilS or of a mix of parallel and antiparallel β-strands in the case of BfpA from enteropathogenic *E. coli* (EPEC) (Fig. 2) (36, 114, 326, 420), with non-nearest-neighbor connectivity.

What Makes Type IV Pilin Proteins So Diverse?

Although type IV pilins have generally similar architectures, their amazing functional diversity comes from the hypervariable loop regions connecting the core structural elements. Even with the limited number of structures currently available, differences in shape and surface properties arising from sequence diversity in
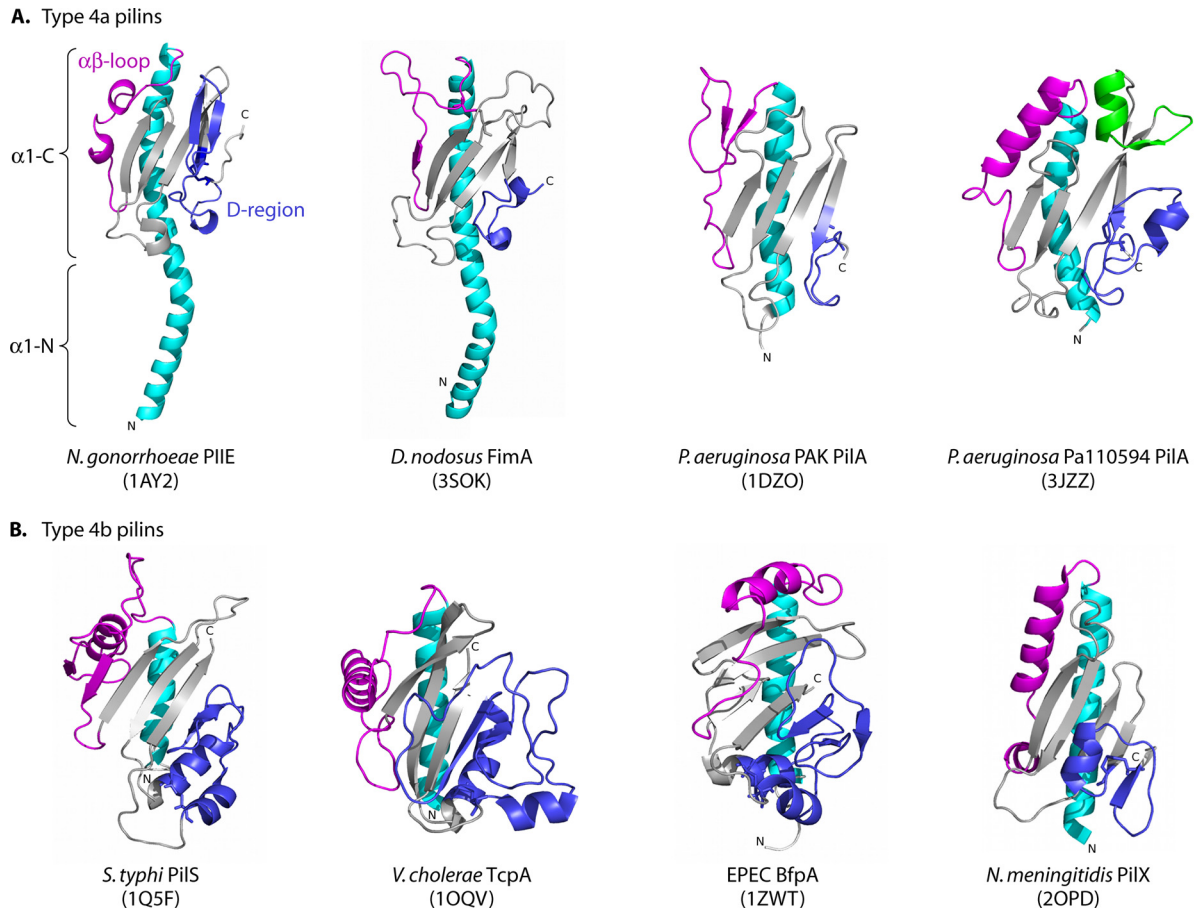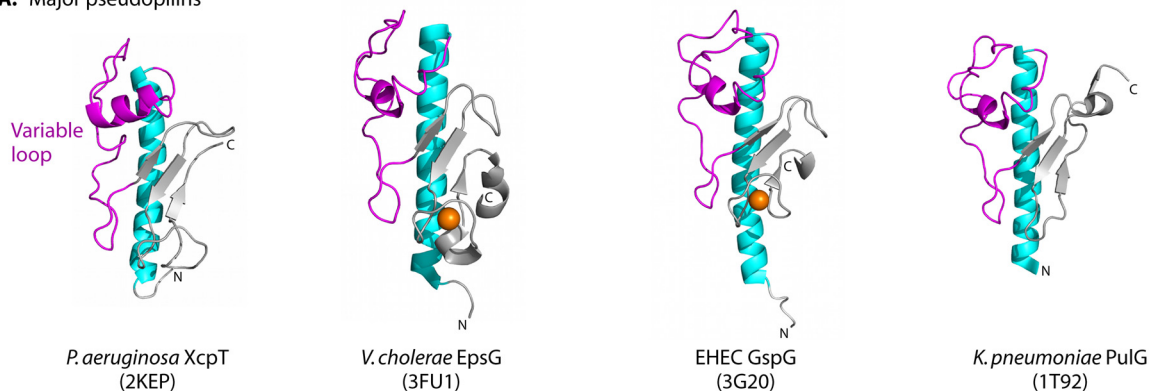
A. Type 4a pilins

N. gonorrhoeae PIIE
(1AY2)

D. nodosus FimA
(3SOK)

P. aeruginosa PAK PilA
(1DZO)

P. aeruginosa Pa110594 PilA
(3JZZ)

B. Type 4b pilins

S. typhi PilS
(1Q5F)

V. cholerae TcpA
(1OQV)

EPEC BfpA
(1ZWT)

N. meningitidis PilX
(2OPD)

FIG 2 Structures of T4 pilins and minor pilins. Type IV pilins are characterized by conserved N-terminal α-helices (cyan) connected to a β-sheet (gray) by the αβ-loop (magenta). The N-terminal α-helix is divided into two subdomains, α1-N (amino acids ~1 to 28) and α1-C (amino acids ~29 to 52), as indicated on the *N. gonorrhoeae* PilE structure. In most pilins, two cysteine residues form a disulfide bond to form the variable D region (blue). Although the *Dichelobacter nodosus* FimA protein lacks these characteristic residues, its C terminus retains a similar architecture through alternative interactions (169). (A) T4a pilins, represented by *Neisseria gonorrhoeae* PilE (Protein Data Bank [PDB] accession no. 1AY2), *Dichelobacter nodosus* FimA (PDB accession no. 3SOK), *Pseudomonas aeruginosa* PAK PilA (PDB accession no. 1DZO), and *P. aeruginosa* Pa110594 PilA (PDB accession no. 3JZZ), share a shallow S-shaped N-terminal helix and a four-stranded continuous antiparallel β-sheet. The architectures of the αβ-loop and the D region of T4a pilins vary, with defined secondary structure in the GC pilin structure (top left) and the Pa110594 PilA structure (top right). Pa110594 PilA has an extended loop region, with an additional α-helix and β-strand between β3 and β4 (green) differentiating it from other T4a pilins. (B) T4b pilins, represented by *Salmonella* Typhi PilS (PDB accession no. 1Q5F), *Vibrio cholerae* TcpA (PDB accession no. 1OQV), and enteropathogenic *E. coli* BfpA (PBD accession no. 1ZWT), have a different protein fold, with discontinuous β-strands making up the β-sheet. The D region is embedded in the protein, with the C terminus forming the last strand of the β-sheet. The αβ-loop contains an α-helix oriented roughly 90° relative to α1. The structure of the noncore minor pilin PilX from *N. meningitidis* (PDB accession no. 2OPD) is similar to that of the major pilins but has two α-helices in the αβ-loop. The D region contains a short α-helix with a hook implicated in function (176). Figures were prepared using MacPymol (DeLano Scientific).

loop regions are readily apparent. Evolution of loops is constrained, however, by the need to accommodate multiple protein-protein interactions with neighboring subunits during fiber assembly. Expression of even closely related pilins in a single background yields only fibers of homologous composition (308), providing strong evidence that interactions among or a lack of steric clashes between loop regions is important for polymerization. There are 3 important hypervariable regions: the loop connecting the conserved N-terminal α-helix to the remainder of the protein, the loops between β-strands of the β-sheet, and the C-terminal loop.

The loop connecting the main N-terminal α-helix to the C-terminal β-sheet is called the αβ-loop in pilins and the variable loop in pseudopilins. In the T4a pilins, the αβ-loop can contain a minor β-sheet like that in the pilin of *P. aeruginosa* strain PAK, α-he-

lices such as those of *N. gonorrhoeae* PilE and *P. aeruginosa* Pa110594 PilA$_V$ (the latter of which has a loop region with helical character like that of the *P. aeruginosa* strain K122-4 pilin), or it can be largely unstructured, as in *Dichelobacter nodosus* FimA (Fig. 2) (26, 114, 115, 169, 172, 219, 306). The αβ-loop of *N. gonorrhoeae* PilE has a short, 1-turn α-helix that is O-glycosylated at Ser63, whereas PilA$_V$ of *P. aeruginosa* Pa110594 has a 3-turn unmodified α-helix (114, 143, 297, 306). This α-helix is reminiscent of the one in *N. meningitidis* PilX, which is a 4-turn α-helix in the corresponding region. In addition, there is a prominent α-helix in the αβ-loops of most T4b pilin structures solved to date (36, 114, 176, 326, 420), although its ~90° angle relative to the extended α1-helix differs from that of the T4a proteins (Fig. 2). The exception is CofA of *E. coli*; its αβ-loop has a 10-residue insertion that forms an irregular loop containing a short 3$_{10}$ helix (234).

A. Major pseudopilins

P. aeruginosa XcpT
(2KEP)

Vibrio cholerae EpsG
(3FU1)

EHEC GspG
(3G20)

K. pneumoniae PulG
(1T92)

B. Minor pseudopilins

V. cholerae EpsH
(2QV8)

V. vulnificus EpsI
(2RET)

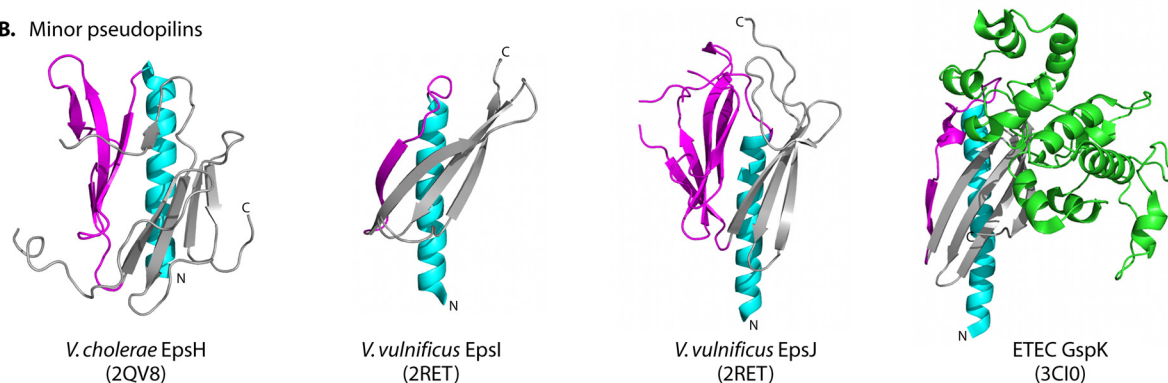*V. vulnificus* EpsJ
(2RET)

ETEC GspK
(3CI0)

FIG 3 Structures of T2S pseudopilins and minor pseudopilins. T2S pseudopilins are structurally similar to the T4 pilins, with an N-terminal α-helix (cyan) connected to a β-sheet (gray) by a variable loop (magenta). (A) The major pseudopilins, represented by *Pseudomonas aeruginosa* XcpT (PDB accession no. 2KEP), *Vibrio cholerae* EpsG (PDB accession no. 3FU1), enterohemorrhagic *E. coli* GspG (PDB accession no. 3G20), and *Klebsiella pneumoniae* PulG (PDB accession no. 1T92), have variable loops with a helical character followed by a 3-stranded β-sheet. Near the C terminus is a calcium-binding motif (with a calcium ion shown in orange) in EpsG and GspG, although calcium binding is expected to occur in all major pseudopilins (235). (B) The minor pseudopilins, represented by GspH from *V. cholerae* (PDB accession no. 2QV8), GspI and GspJ from *V. vulnificus* (PBD accession no. 2RET), and GspK from enterotoxigenic *E. coli* (PDB accession no. 3CI0), vary in architecture, with a large α-domain insertion (green) in GspK. Figures were prepared using MacPymol (DeLano Scientific).

In contrast to the T4 pilins, all of the major pseudopilin structures solved thus far have a mostly unstructured loop with a single helix connecting the N-terminal α-helix and β-sheet domains (Fig. 3). Although referred to as a variable loop, this region has a conserved motif (LPXDPWGXXY) that is found only in major pseudopilins (16, 233, 236). Until recently, the major pseudopilins were thought to be a single protein class, since they were among the few functionally interchangeable components of T2S systems from different species. However, Durand et al. (127) showed that the HxcT major pseudopilin from *P. aeruginosa* could not complement a *P. aeruginosa* mutant lacking XcpT, the major pseudopilin from its predominant T2S system. Biochemical and mutagenesis studies confirmed that the Hxc (homolog of Xcp) system is the archetype of a similar but separate T2S subfamily. The lack of conservation in sequence and secondary structure of the variable loops in minor pseudopilin orthologues across species may reflect a role in selection of unique sets of secreted substrates, a hypothesis supported by the recent demonstration of direct interactions between minor pseudopilins and effector proteins in *P. aeruginosa* (124). The minor pseudopilin GspI (EpsI) from *Vibrio vulnificus* has a single β-strand in the variable loop, whereas GspI from enterotoxigenic *E. coli* has a short α-helix followed by a β-strand, similar to that found in the variable region of GspK (236, 421, 422). The variable regions of GspH and GspJ are sub-stantially larger than those of other type IV pilin proteins and are composed mainly of β-strands (236, 422). In particular, the variable segment of GspH forms a five-stranded antiparallel β-sheet, although the strands are not continuous (Fig. 3) (422). The structures of the orthologous proteins GspJ (from enterotoxigenic *E. coli*), EpsJ (from *V. vulnificus*), and XcpW (from *P. aeruginosa*) vary in this region, but they are mostly β-strands (244).

Loops connecting the β-strands that form the C-terminal β-sheet of type IV pilins and pseudopilins are mainly unstructured, though shorter loops can form β-turn motifs. Exceptions include PilA$_V$ from *P. aeruginosa* Pa110594 and the minor pseudopilin GspK, where defined secondary structure elements appear in specific loops. Pa110594 PilA$_V$ has an α-helix and a β-strand between β$_3$ and β$_4$, whereas the loop region between β$_2$ and β$_3$ of GspK has a massive α-domain insertion composed of 12 α-helices and 4 short β-strands (Fig. 2 and 3) (236, 297). The α-domain of GspK also contains a disulfide bond that likely stabilizes the domain, as well as a dinuclear metal binding site predicted to contain calcium.

The loop regions may participate in interactions with neighboring subunits, although the β$_3$-β$_4$ loop of Pa110594 PilA$_V$—located at the "top" of the structure, adjacent to the αβ-loop—has also been hypothesized to interact with its dedicated accessory protein, TfpZ (112, 113, 115, 297). Interestingly, *D. nodosus* FimA has a

large unstructured β_1-β_2 loop that occupies a position similar to that of the β_3-β_4 loop in PilA$_V$ (169) and is coexpressed with a TfpZ-like accessory protein, FimB (220). For T4b pili, limited interactions between subunits (251) suggest only minimal contact between loops of adjacent monomers in the assembled fiber. However, two recent studies (207, 255) showed that specific residues in the loop regions of T4b pilins play critical roles in interfiber bundling and aggregation, as mutations at those positions affect lateral interactions between filaments. These data suggest that surface topography created by variable loops can affect pilus function in ways that are independent of effects on interactions between adjacent subunits in the same fiber.

The fiber surface generated by loops of adjacent subunits can create contiguous cavities or grooves that provide binding sites for other molecules. For example, the cystic fibrosis transmembrane regulator protein, a host receptor for *S.* Typhi T4bP, is proposed to bind in a pocket of complementary charge formed by adjacent PilS subunits (35). Where T4P are involved in competence, binding of extracellular DNA can be facilitated by positively charged furrows on fiber surfaces (115, 396). In the case of the minor pseudopilins, the large, arrowhead-shaped α-domain insertion of GspK and the organization of the GspIJK heterotrimer with the α-domain at the top of the complex led to the suggestion that the insertion domain may be positioned at the tip of the pseudopilus, where it could induce secretin opening or interact with secreted proteins (142, 236). These hypotheses were recently strengthened by elegant studies showing that minor pseudopilins equivalent to GspH, GspI, and GspK from the Xcp system of *P. aeruginosa* interact directly with the T2S substrate, elastase (124), and with the periplasmic portion of the secretin (330).

The last important hypervariable region of type IV pilin proteins is located at the C terminus. In this region, most major pilins and some minor pilins have a conserved structural element known as the disulfide-bonded loop (DSL), or D region (Fig. 2, shown in blue). In the majority of major pilins characterized to date, two Cys residues near the C terminus form a disulfide bond that staples the C terminus of the protein to the β-sheet. An important disulfide is also present in the ComGC competence pseudopilin, involved in formation of the DNA uptake system in *Bacillus subtilis* and other Gram-positive species (94). Interestingly, the recently characterized structure of *D. nodosus* FimA from serotype A (169) revealed a D-region architecture similar to that of other major pilins, even though it lacks the typical C-terminal disulfide bond (Fig. 2). Instead, a noncovalent network of hydrogen bonds maintains the orientation of the loop. There are two Cys residues in FimA, but they form a disulfide bond linking the αβ-loop—which contains a large unstructured region—to the start of β2; a similar bond is present in the *P. aeruginosa* K122-4 pilin, which has the more typical C-terminal disulfide as well (26).

The length and secondary structure of the D region vary between pilins of different species, even among pilins from different strains of the same species (242). In the *P. aeruginosa* PAK and K122-4 pilin structures, the D region is short and has a type I β-turn followed by a type II β-turn (26, 172). Although the Pa110594 PilA$_V$ pilin has a larger D region, with an additional α4-helix, the structure of a type I β-turn followed by a type II β-turn is conserved (297). The D region of the *N. gonorrhoeae* pilin contains a prominent β-hairpin (306) whose sequence is among the most hypervariable among antigenic variants.

The D region of major pilins has a structural role, as mutations

that lead to a loss of C-terminal disulfide bond formation—including mutations in both general and dedicated disulfide bond isomerases—prevent pilus assembly (94, 390, 403, 427, 429). For some species, a lack of disulfide bond formation was reported to result in pilin instability. This finding could be due to a loss of reactivity with pilin-specific antisera, as the D region is an important immunodominant epitope. In *P. aeruginosa*, mutation of either of the Cys residues or deletions within the loop region impact twitching motility by impairing assembly (170). Because the D region contacts adjacent subunits in the fiber, altering its conformation could impede subunit-subunit interactions. The D region continues to have a key role after polymerization of the subunits, because treatment of assembled pili with reducing agents leads to their rapid disintegration (250). In *B. subtilis*, mutation of the Cys residues in ComGC to Ser resulted in a loss of transformation capacity, a loss of higher-order ComGC complexes, and a marked decrease in detectable membrane levels of monomeric ComGC, suggesting instability of the mutant proteins (94). It will be interesting to determine if mutations that disrupt the hydrogen bond network that stabilizes the FimA C-terminal region (169) will similarly affect stability or pilus assembly in *D. nodosus*.

A number of studies suggested that the D region of the T4a major pilin can function as the adhesive component of the pilus, as specific antibodies or competitive peptide inhibitors were specifically able to block pilus-mediated binding to a variety of surfaces (156, 249, 374). To explain how D regions with diverse sequences can provide similar functions, their adhesive properties were attributed to main chain- rather than side chain-based interactions (172). Those data led to a focus on development of anti-*P. aeruginosa* vaccines containing peptides corresponding to the D region (71, 72, 75, 162, 216). Newer information showing that the minor pilins are present in sheared pilus fractions (154, 416) and that the orthologous PilC1 or PilC2 (*Neisseria* and *Kingella*) and PilY1 (*Pseudomonas*) proteins are potentially pilus associated and required for adherence to—and manipulation of—the host (174, 204, 217, 288, 289, 384) suggests that additional studies are needed to unequivocally identify all T4aP adhesins. Given the wide range of surfaces to which T4P bind, there may be multiple players that contribute to adherence under specific circumstances.

The T4b pilins have substantially larger D regions than those of the T4a pilins, containing more defined secondary structure elements (36, 114, 176, 326, 420). The connectivity between strands of the β-sheet varies, and the characteristic disulfide bond joins residues that are more distant from one another in the primary sequence than the case with the T4a pilins. In *V. cholerae*, point mutations in a portion of the TcpA D region that alter its conformation affect pilus morphology or stability, whereas mutations in the area involved in lateral interactions between fibers have effects on pilus-mediated aggregation and host cell colonization (207, 227, 255).

The D region in the noncore *N. meningitidis* minor pilin, PilX, is small but has defined secondary structure (176). Deletions within this region severely impair pilus-mediated aggregation and adhesion, supporting a key role for the region in PilX function (176). The PilX D region has a hook-like conformation, proposed to protrude from assembled fibers. Upon retraction of adjacent but antiparallel fibers, the hook-like domains of PilX were hypothesized to catch upon one another, antagonizing pilus retraction and thus contributing to cell-cell aggregation (176). Although sequence analyses of other core and noncore minor pilins revealed
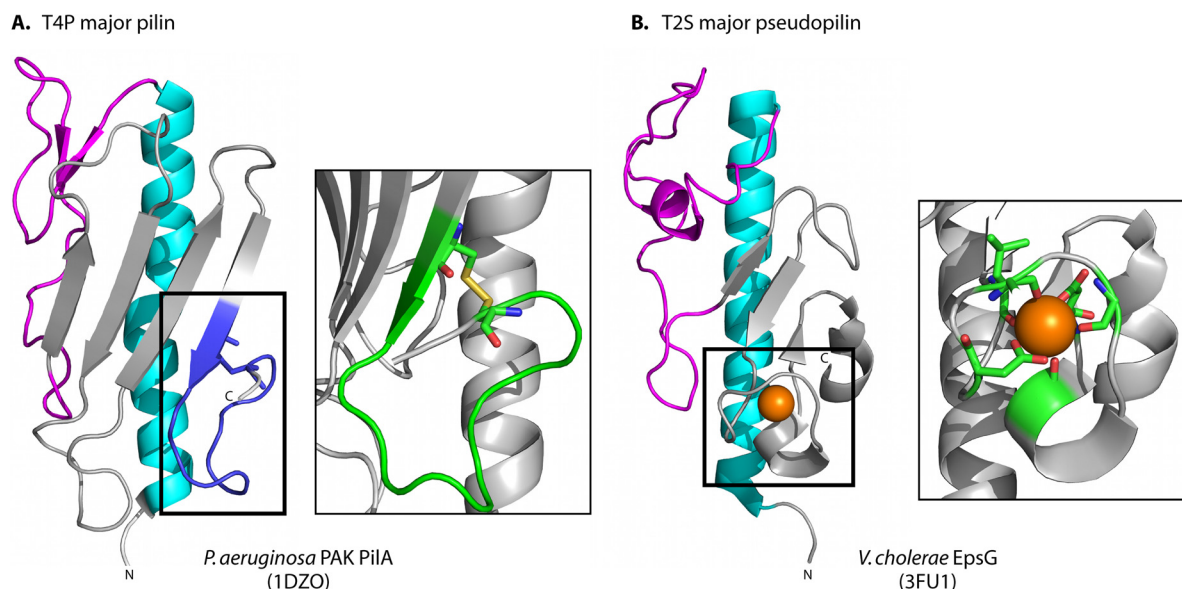
A. T4P major pilin

B. T2S major pseudopilin

P. aeruginosa PAK PilA
(1DZO)

V. cholerae EpsG
(3FU1)

FIG 4 Comparison of the T4 pilin D region and the T2S pseudopilin calcium-binding domain. One of the defining differences between T4 pilins (left) (*P. aeruginosa* PAK PilA [PBD accession no. 1DZO]) and T2S pseudopilins (right) (*V. cholerae* EspG [PBD accession no. 3FU1]) is found at the C terminus, where the majority of T4 pilins have a disulfide-bonded loop (D region) and the pseudopilins have a calcium-binding motif (with a calcium ion shown in orange) (235). The disulfide bond creates a loop with two β-turns (left inset), while calcium binding by both main chain and side chain residues stabilizes the corresponding region in the pseudopilins (green, right inset). Both structural motifs are implicated in function. Figures were prepared using MacPymol (DeLano Scientific).

that many have two or more Cys residues near the C terminus, there are no structures yet available to confirm the formation of predicted disulfide bonds, nor have their roles in minor pilin stability or function yet been examined.

Unlike most major T4 pilins, major pseudopilins lack a stabilizing disulfide bond near the C terminus. Instead, crystal structures of the major pseudopilins from *V. cholerae*, *V. vulnificus*, and enterohemorrhagic *E. coli* (EHEC) revealed a calcium-binding site in this region (Fig. 4) (235). Calcium is coordinated in an octahedral manner by different residues in the *V. cholerae* and EHEC proteins, suggesting that they are the archetypes of at least two structural subclasses. Both contribute carboxylates from two conserved Asp residues, but in the *V. cholerae* pseudopilin, four main chain carbonyl oxygens complete the Ca^{2+} coordination site, while the same function is provided by two main chain carbonyl oxygens and a Thr/Ser side chain oxygen in the case of EHEC (235). Between the conserved Asp residues involved in calcium binding, there is an additional α-helix in the *Vibrio*-like pseudopilins that is not present in EHEC-like proteins. Although structures of the major pseudopilins from *Klebsiella oxytoca* and *P. aeruginosa* have been solved by X-ray crystallography and NMR spectroscopy, respectively, a calcium-binding site was not identified (16, 233). However, a β-strand swap between protomers in the dimeric structure of GspG from *K. oxytoca* may have disrupted the coordination site (233, 235). Loss of secretion upon mutation of the Asp residues involved in calcium binding suggested that they are integral to T2S system function (235). Based on the importance of the D-region conformation to pilus assembly, loss of secretion was likely related to the inability of mutant pseudopilins to form a pseudopilus (see below).

FROM SUBUNIT TO FIBER

A defining functional characteristic of type IV pilin proteins is their ability to reversibly assemble into polymeric fibers, which in

the case of pili can further interact to form higher-order bundles or tangled aggregates. In all cases examined to date, the subunits assemble in a helical manner. Several T4P fiber models have been proposed, including both right- and left-handed single-start (172, 219, 306) and multistart (114, 115, 251) helical models. Although under native conditions T2S pseudopili are predicted to be only long enough to span the periplasm of Gram-negative bacteria, overexpression of the major pseudopilin leads to the formation of long, surface-exposed pseudopili that are amenable to structural analyses (77, 128, 349, 400). In the case of archaeal flagella, only limited structural information on the fibers is available, but thus far, right-handed filament models predominate (378). In some archaeal species, the flagella are made of multiple subunit types, making model building more challenging.

Type IV Pilus Models

Crystallographic and cryo-electron microscopy (cryo-EM) evidence suggests that the pilin monomers of the best-characterized T4a pilus from *N. gonorrhoeae* are arranged in a 3-start left-handed helix, which can alternately be viewed as a 1-start right-handed or 4-start right-handed helix, with a predicted 3.6 subunits per turn (Fig. 5) (115). The fiber has an outer diameter of ~60 Å (115), consistent with the ~65-Å-diameter opening of the T4aP secretin through which the fiber passes to the cell's exterior (49, 103, 104). The fiber is stabilized by hydrophobic and electrostatic subunit-subunit interactions between the N-terminal α-helices, which form the central core of the pilus. The methylated, positively charged N-terminal residue of one subunit is thought to neutralize the charge of the Glu +5 residue of the adjacent subunit and to act as a means of registration to ensure the correct degree of vertical displacement between one subunit and the next (115, 306).

The N-terminal α-helices form staggered helical bundles that spiral along the length of the pilus. A single α-helix participates in
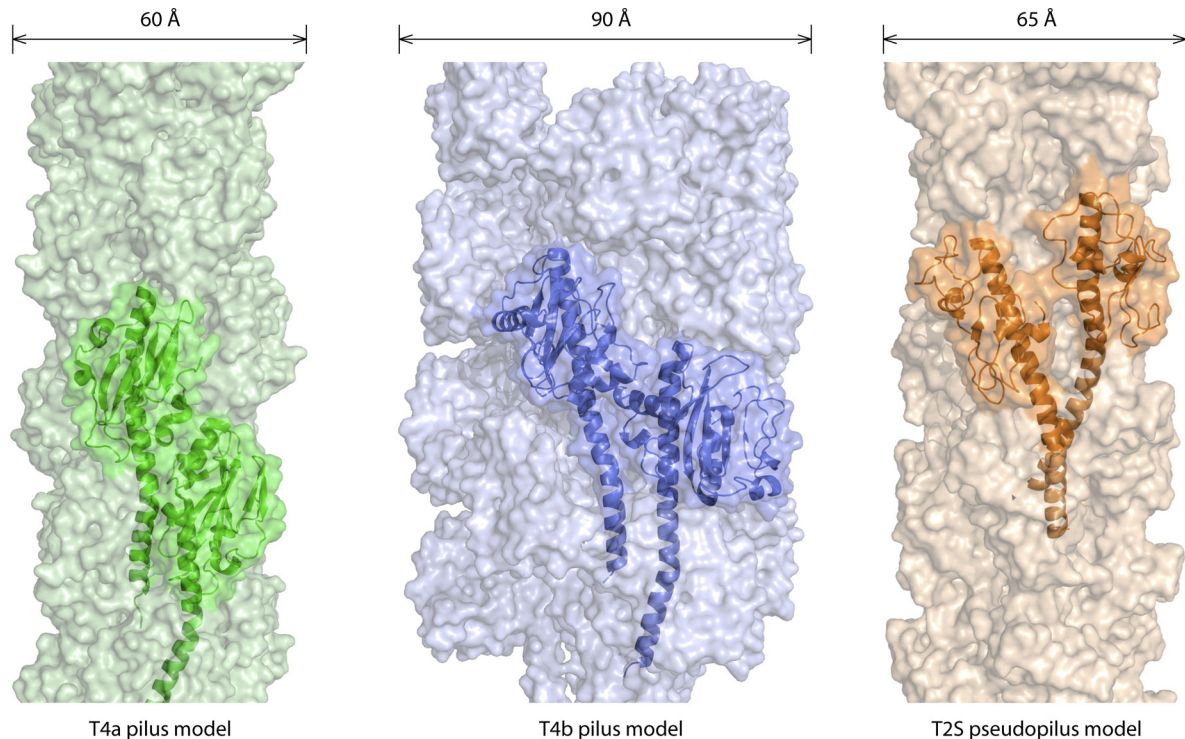
FIG 5 Models of pilus and pseudopilus fibers. Using structural subunit information, low-resolution EM data, and biochemical data, fiber models of the T4aP (left), T4bP (center), and pseudopilus (right) were generated. The T4aP model can be viewed as a one-start or four-start right-handed helix or a three-start left-handed helix (one strand is pictured, with two subunits shown as a green cartoon) with a diameter of approximately 60 Å. Tight packing of the N-terminal helices holds the structure together, with additional polar interactions between the C-terminal head groups. The three-start left-handed helix T4bP model has a larger diameter, at approximately 90 Å, due to the larger subunit size along with the more loose packing of the subunits, exposing portions of the N-terminal helix and producing deep grooves and bulges along the fiber (model kindly provided by Lisa Craig). The right-handed one-start helix T2S pseudopilus model is slightly larger than the T4aP model, at 65 Å, with hydrophobic and electrostatic interactions stabilizing subunit interactions (model kindly provided by Olivera Francetic). Figures were prepared using MacPymol (DeLano Scientific).

3 different helical bundles, contacting neighboring subunits at residues 1 to 13, 4 to 19, and 24 to 39 (115). Packing of the α-helices is facilitated by the S-shaped curvature created by residues Pro22 and Gly42 (113). The C-terminal domains form subunit-subunit contacts, mainly in loop regions. The outer surface of the pilus, formed by the C-terminal domains of the subunits, is characterized by deep grooves separating the head groups (115). From the 3-start left-handed helix view, there are polar interactions between the αβ-loop of one subunit and the D region of the next subunit, while in the 4-start right-handed helix view, there are interactions between the loop regions connecting strands of the β-sheets (Fig. 5) (115). Li et al. (250) reported that stacking of conserved aromatic residues, particularly the mature N-terminal Phe with Tyr24 and Tyr27 of the α1-N domain of the adjacent subunit, likely contribute additional stabilizing interactions in the pilus core. Combined, these features allow for a highly flexible structure that can withstand the piconewton-scale forces required for twitching motility (263).

While a 70-Å-diameter opening was reported for the T4bP secretin, T4bP fiber models predict a wider pilus of approximately 90 Å (114, 115). Current models and experimental data support a 3-start left-handed helix (114, 250, 251). The C termini of T4b pilin monomers are bulkier in three dimensions than their T4a pilin counterparts, and they protrude outward. Like T4aP, T4bP is held together by tight interactions of the subunits' N-terminal helices in the core of the pilus; however, hydrogen-deuterium-

exchange mass spectrometry (MS) data comparing monomeric with assembled subunits suggested that the C-terminal head groups are loosely packed (251). The only interactions of the head groups occur via polar and hydrophobic interactions between the αβ-loop and a portion of both α1-N and α3, not α4 of the D region as previously thought. This minimal level of interaction leaves the rest of the pilin subunit—including the majority of the D region—solvent exposed, and it results in the presence of bulges and deep grooves that expose an amphipathic portion of the N-terminal helix, previously thought to be buried completely in the fiber's core (251). The loose packing of the pilins' C termini is thought to underlie the reduced resistance of T4bP to heat, proteases, and chaotropic agents compared with that of T4aP (250).

Due to technical and computational constraints, pilus models are usually derived from X-ray diffraction or cryo-electron microscopy analyses of straight segments of assembled pili, into which crystal or NMR structures of individual subunits are fitted. There are a number of caveats to this approach. At the moment, only major subunit structures are considered during the model-building process, although it is becoming clear that minor subunits may also be part of the fiber (100, 154, 416). Pronounced flexibility is an inherent property of T4P, and the interactions between pilin subunits in the straightened segments of fibers used to collect diffraction or cryo-EM data may be different from those that occur in acutely bent segments. The resolutions that can be
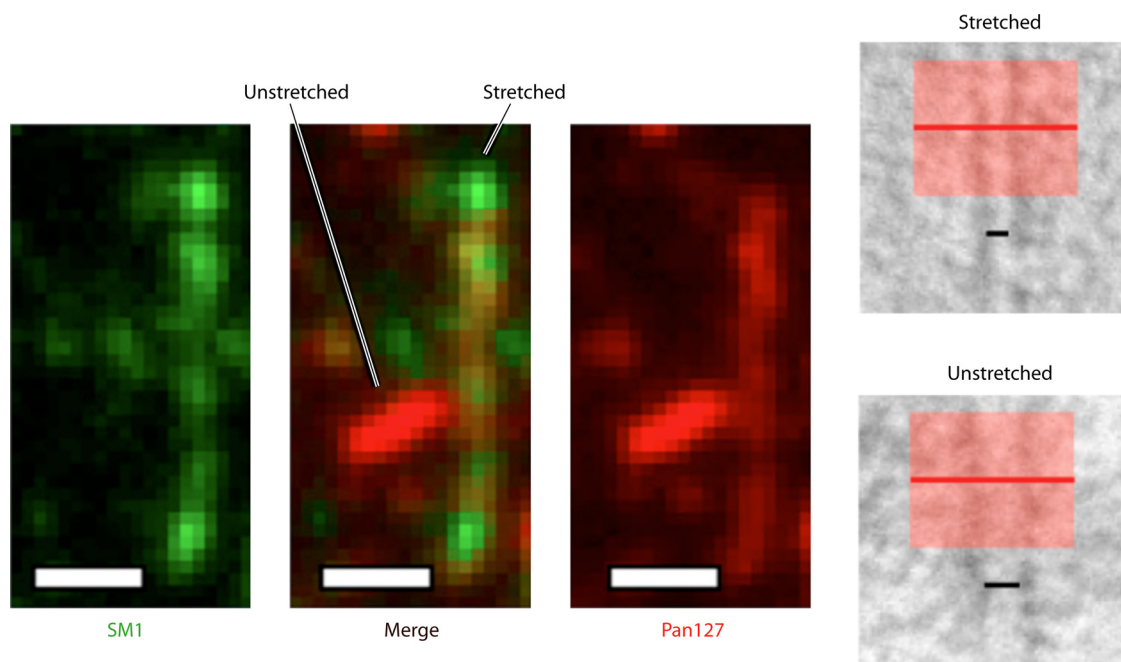
FIG 6 Force-dependent conformational changes in T4aP fibers. Biais et al. (46) showed that applying pulling forces just below those that cause *Neisseria* pili to break can lead to stretching of the pili in their long dimension, making them ~40% narrower, with only 2/3 the mass per unit length of normal fibers (right). This conformational distortion was reversible and exposed epitopes (left) for the SM1 monoclonal antibody (green) that were hidden in normal fibers (red). (Left panel reprinted from reference 46 with permission of the publisher; right panel courtesy of Nicolas Biais.)

obtained are low, presumably because the filaments are not uniform (250).

Interestingly, Biais and colleagues (46) demonstrated that the T4a pili of *N. gonorrhoeae* can undergo dramatic and reversible conformational changes upon application of threshold levels of force, stretching in their longer dimension to become ~40% narrower than unstretched pili, with a concomitant reduction of ~2/3 of the mass per unit length (Fig. 6). These changes alter the nature of interactions occurring between subunits, as they expose epitopes on the pilins that are normally occluded in unstretched fibers. In some cases, the deformations occur in localized regions, implying that there are multiple types of interactions that can occur between pilin subunits in a single intact fiber. The ability to stretch without breaking was suggested to provide a buffer against force fluctuations along the length of a fiber, preventing detachment during transient increases in shear forces (46). The ability to undergo deformation was recently linked to the presence of the *Neisseria*-specific minor pilin, PilX (63). Pili from PilX-expressing *N. meningitidis* underwent the structural transition that exposes the epitope for the monoclonal antibody (MAb) SM1 upon binding to host cells, while *pilX* mutants or strains expressing mutant forms of PilX did not show this change. In light of PilX's proposed function as a retraction antagonist (175, 176), it is possible that deformations are initiated when PilX-PilX interactions between adjacent but antiparallel fibers that are being retracted in opposite directions temporarily increase local forces. Affinity differences in the subunit-subunit interaction interfaces of PilE-PilE versus PilE-PilX may allow for initiation of deformation within a specific fiber.

The propensity of T4P to form bundles that mediate bacterial aggregation has been well established (365, 411). However, the ability of such bundles to cooperatively generate retraction forces that exceed those produced by single filaments was not appreciated until recently. Biais et al. (47) developed a clever assay in which they used micropillars made from an elastic hydrogel whose stiffness could be varied from 100 to 500 pN/μm. Piliated *N. gonorrhoeae* was inoculated onto a single micropillar and incubated until the pili became attached to adjacent micropillars, whose lateral displacement upon pilus retraction was monitored by video microscopy. By manipulation of the growth medium, retraction events were biased toward those generated by single pili or by laterally associated bundles. Bundles of pili, containing up to 10 individual fibers, generated retraction forces 8 to 10 times higher than those generated by a single pilus, suggesting that T4P can retract in a cooperative manner and impose substantial forces. Further studies by Holz et al. (184) confirmed that cooperative retraction of multiple pili increases the persistence of *Neisseria* movement.

Type II Secretion Pseudopilus Models

The structure of the pseudopilus in the T2S system has been more challenging to elucidate, since native fibers are short and not exposed on the cell surface. However, some major pseudopilins are capable of forming long, surface-exposed fibers when overexpressed (127, 128, 349, 400). Such hyperpseudopili measure 6 to 9 nm in diameter. This dimension is consistent with initial electron microscopy analysis of the T2S secretin, which predicted a 95-Å-diameter opening (49). However, more recent cryo-EM data from the *V. cholerae* system suggest that the secretin channel varies in diameter, with a 75-Å opening that constricts to 55 Å followed by a chamber of 100 Å (330).

Initial analysis of the pseudopilus suggested a one-start left-

handed helical arrangement of pseudopilins (233); however, recent computational models calculated from sparse data suggest otherwise (76, 77). Instead of docking the crystal structure of the pseudopilin subunit within high-resolution cryo-EM data (as was done to generate the T4aP fiber model), Campos et al. (76, 77) used low-resolution EM data, conformational restraints, and molecular modeling to generate a suite of models. On average, the pseudopilus fiber model is similar to the GC pilus model, with a diameter of 65 Å and a one-start right-handed helical arrangement (Fig. 5). Taking the restraint energy into account during structure calculations confirmed the right-handed helical character, as no models with a left-handed helix were generated. The smaller size of pseudopilins than of pilins is consistent with the observation of ∼4.25 subunits per turn for the pseudopilus, compared to ∼3.6 for the pilus. There are several subunit interactions mediated by hydrophobic and electrostatic contacts, where each subunit interacts to various extents with three upper and three lower subunits (77). Intermolecular salt bridges help to stabilize the structure, with interactions between Asp44-Arg88 and Asp48-Arg87 being key to pilus assembly and secretion. Asp44 and Asp48 are part of the α1-C domain, while Arg87 and Arg88 are in the variable loop region. A positive patch within the variable loop is involved in interactions with the crucial calcium-binding motif, characterized by two conserved Asp residues (77, 235). Interestingly, only 18% of the models generated included a potential salt bridge between Glu5 and the positively charged N-terminal residue, as was predicted for T4P subunits; instead, the majority of structures showed interaction between Glu5 and the side chains of Lys residues in the N-terminal helix. In support of this alternative model, mutation of Lys28 abolished piliation and reduced secretion (77).

How Do Individual Subunits Assemble into a Fiber?

Among the (many) unresolved mysteries in the field is how type IV pilins are extracted from the membrane and assembled into (and then subsequently disassembled from) a fiber. How is the process initiated, and by which components? Is the rate of assembly similar to the rate of disassembly, which is estimated to be between 1,000 and 1,500 subunits per second (281, 358)? How might disassembly rates be affected by bundling of pili or by binding of extraneous molecules such as DNA? Do pilins interact directly with the motor ATPases that power assembly/disassembly or with membrane-bound components that function to transduce the mechanical energy generated by ATP hydrolysis to the subunits (285)? How is the incorporation of core and nonconserved minor subunits (154, 175, 176, 416) into the fibers controlled? Although many of these questions remain unanswered, substantial progress is being made on some fronts.

While early studies suggested that pilin dimers were the building blocks of the pilus (409), more recent models suggest that single pilin monomers are added to the growing pilus fiber (115). The discovery that minor subunits are present outside the cell in assembled fibers (154, 416) also meant that the order and stoichiometry of subunit incorporation needed to be considered. The structure of a heterotrimeric T2S core minor pseudopilin complex, which revealed a bulky α-domain insertion in GspK, implied that the complex was likely to form the tip of the fiber; it was difficult to envision how additional subunits could fit above it without significant steric hindrance (142, 236). If the above hypothesis was true, the core minor subunit complex would form first, and subsequent polymerization of major subunits beneath the complex would lead to fiber elongation. Based on this idea, GspK, and possibly its core minor pilin equivalent, PilX (in *Pseudomonas*) or PilK (in *Neisseria*), would be the first component to be extracted from the membrane. In both T2S and T4P systems, this particular subunit is unique in that it lacks the highly conserved Glu5 residue present in other type IV pilin proteins, instead having a nonpolar residue at position +5. The absence of a charged side chain may improve the ability of GspK orthologues to leave the membrane during assembly initiation. Core minor pilin/pseudopilin gene clusters can readily be identified due to the presence of the gene encoding this atypical subunit.

Recent data from an exciting study by Cisneros and colleagues (100) supported the idea that the minor subunits form an initiation complex that primes subsequent fiber assembly. They showed that GspI, GspJ, and GspK orthologues (PulI, PulJ, and PulK) from *Klebsiella* were important for efficient expression of pseudopili in an *E. coli* overexpression system (349) and in a strain missing all of the minor pseudopilins, that GspI and GspJ alone were sufficient for assembly to proceed. Pilus assembly in spheroplasts was examined to rule out the possibility that the minor pseudopilins participate in assembly by opening the secretin; pseudopili were observed only when the minor pseudopilins were expressed, regardless of secretin expression. Bacterial two-hybrid and cysteine cross-linking studies showed that GspI and GspJ (as well as GspI and GspK) interact in the membrane and that due to the resulting conformational changes, their α1-N segments become displaced vertically by ∼1 nm relative to their initial side-by-side orientation. This displacement is equivalent to the rise between subunits in an assembled fiber, hinting at the formation of a priming complex. Molecular dynamics simulations further supported the formation of a staggered, pseudohelical GspI-GspJ-GspK complex, from which GspK protruded and deformed the membrane. This complex was proposed to provide a stable nucleus, templating the subsequent addition of major subunits beneath it.

The core minor pilins of the T4aP system could similarly form an initiation complex that primes pilus assembly, leading to their incorporation into the fiber (154, 416), although further studies are needed to test this idea. Data from overexpression studies suggested that the stoichiometry of the *P. aeruginosa* GspK equivalent, PilX (called PilK in *Neisseria*), and GspH equivalent, FimU (called PilH in *Neisseria*), relative to other minor pilins was important for the control of pilus length, since mutants with excess PilX or FimU had extremely short but functional pili (154). Interestingly, interactions of PilX with other components of the T4aP assembly system were implied by cross-complementation studies using two strains of *P. aeruginosa* (PAO1 and PA14) encoding heterologous sets of minor pilins. Replacement of the entire set of minor pilins of one strain with those of the other did not restore pilus assembly until the native PilX protein was also provided (155).

Tad/Flp systems differ from other T4aP, T4bP, and T2S systems in that they lack obvious homologs of the 4 core minor pilins (393). Typically, they have one major subunit and two minor subunits that have not been detected in the fibers. Do those proteins function to initiate Tad pilus assembly? The lack of a retraction ATPase in Tad systems may preclude the need for a core minor pilin complex, as studies of the T4aP system showed that all minor pilins may be dispensable for pilus assembly in retraction-deficient backgrounds (79, 154, 416). However, this possibility needs

to be tested formally, as T2S systems, which also lack a retraction ATPase, need the minor pseudopilins to function. This is a rapidly moving area of research, and further studies are necessary to determine whether T4P assembly is primed or optimized by one or more minor pilins.

FUNCTIONS OF TYPE IV PILIN PROTEINS

The relationship between the diverse sequences and structures of pilin proteins and their broad range of functions is among the fascinating but poorly understood aspects of type IV pilin protein biology. This puzzle persists because although proteins with widely divergent sequences can play similar roles—suggesting that substantial sequence variation can be tolerated—in some cases even single residue changes in otherwise identical proteins can markedly affect function (4, 424). Well-characterized roles include adherence to living and nonliving surfaces, including other bacteria; twitching motility; modulation of biofilm architecture; DNA uptake (competence) and exchange (conjugation); secretion of exoproteins; and bacteriophage susceptibility. More exotic functions include swimming motility and binding of sugars (both archaeal traits), electron transfer (in *Geobacter*), and manipulation of host cell biology (20, 327). Some systems have multiple functions; for example, the T4P of *Neisseria* are adhesins and motility organelles and are required for competence, while the T4P system of *V. cholerae* is involved in both adherence and protein secretion. Some bacteria express multiple types of T4P or both T4P and T2S systems. A recent bioinformatic analysis of sequenced genomes by Imam and colleagues (195) showed that the distribution of genes with the potential to encode pilin-like proteins is far broader than previously appreciated, and thus the list of processes in which they are involved is ripe for expansion. This section gives brief examples of the ongoing research into the functions of type IV pilin proteins.

Adherence and Aggregation

The most commonly reported function of T4 pili is adherence to a diverse range of surfaces, from metal, glass, plastics, and rocks to plants and various host tissues (48, 51, 123, 156, 164, 209, 217, 313, 341, 391, 393, 419, 431). T4P have repeatedly been shown to contribute to the infectivity of pathogens—even intracellular pathogens such as *Francisella tularensis* (344)—firmly establishing them as important virulence factors.

Although T4P promote adherence, the biophysical aspects of their adhesive mechanisms are not well characterized. In other words, how exactly do T4P stick to diverse surfaces, and with what affinities? Does adherence occur only at the tips of the fibers, or are additional points of contact made along the fiber length? A series of studies aimed at measuring the forces generated upon pilus retraction was carried out (47, 101, 281), which required, of course, that the pili be stuck to a surface. However, it is not possible to calculate binding affinities from studies that measure the maximum pulling forces that pili can withstand, as release could happen for a number of reasons beyond loss of cohesion with a surface. Alternatives include breakage of the fiber (disruption of subunit-subunit interactions) or the forcible—or possibly deliberate—separation of the fiber from the cell at its base. The latter mechanism has been proposed to explain shedding of *S.* Typhi pili (385).

The major subunits of T4P can act as the adhesive component, as has been reported for *P. aeruginosa* pilins (249), *Neisseria* pilins

(351), the bundlin subunit of EPEC bundle-forming pili (194), and the PilS subunit of *S.* Typhi T4bP (394). For *P. aeruginosa*, the ability of pilins of diverse sequence to bind to the proposed receptor, the glycosphingolipid asialo-GM1, on host cells was suggested to occur through main chain rather than side chain interactions (172). However, the identity of a specific host receptor for *P. aeruginosa* has been called into question by a study showing that asialo-GM1 does not colocalize with piliated bacteria bound to epithelial cells (132). The major pilin from *Neisseria*, PilE, was reported to hemagglutinate erythrocytes (351) and to mediate binding to host proteins on endothelial cells (110). The receptor for *Neisseria* pili was proposed to be CD46 (214, 413), although newer evidence suggests otherwise (224).

Characterization of the binding mode of *E. coli* bundlin showed that it is a lectin that recognizes *N*-acetyllactosamine (LacNAc) moieties on host cells (191, 194). Interaction of bundlin with *N*-acetyllactosamine induces pilus retraction and the upregulation of virulence gene expression (192). A similar phenomenon has been reported for the T4aP of *M. xanthus*, where binding of T4P to its self-produced exopolysaccharide (EPS) matrix induces pilus retraction and helps to coordinate social motility for fruiting body formation (253).

Not all pilins are lectin-like, however; PilS from *S.* Typhi was reported to bind to the first extracellular loop of the cystic fibrosis transmembrane conductance regulator (CFTR) protein on intestinal epithelial cells (or peptide mimetics thereof), while its equivalent from *S. enterica* serovar Typhimurium does not (394). This specific interaction was thought to explain why only *S.* Typhi is capable of causing human epidemics. However, these data were recently challenged by immunofluorescence studies that did not find colocalization of bacteria and CFTR, although the pili were required for epithelial cell invasion (62). In a final example, expression of the PilA2 subunit from the Gram-positive bacterium *Clostridium perfringens* in a nonpiliated *N. gonorrhoeae* mutant provided the recombinant strain with the novel ability to bind to myoblast cell lines, similar to *C. perfringens* itself (333).

Alternatively, T4P can display minor pilins or other adhesive proteins that mediate binding. The *N. gonorrhoeae* noncore minor pilin PilV is critical for host cell adherence (415), while PilV from *N. meningitidis* binds the β-adrenergic receptor of endothelial cells. In *Neisseria*, the large (nonpilin) PilC1 and PilC2 proteins have been reported to act as cell contact-dependent pilus-associated adhesins (384), although the evidence is indirect and therefore somewhat controversial. *N. meningitidis* isolates use PilC1 and PilC2 to adhere to the uropods of neutrophils in order to escape phagocytosis (362). *P. aeruginosa* expresses an integrin-binding PilC homolog, called PilY1, that requires the T4P system for surface localization, although its direct association with pili has not yet been demonstrated convincingly (13, 53, 174, 204). Mutation of PilY1 homologs in plant pathogens has also been associated with defects in motility and biofilm formation (252, 310). T4P that are involved in DNA transfer via conjugation (below) display lectin-like adhesive proteins whose sequences dictate the range of potential recipients (198).

The propensity of T4P to aggregate laterally into bundles of fibers promotes microcolony formation, an important virulence trait for pathogens such as *Neisseria*, *V. cholerae*, *S.* Typhi, and enteropathogenic *E. coli* (227, 255, 258, 266, 290), and it increases the retraction forces generated by piliated cells (47). In *Neisseria*, pilus-mediated aggregation was linked to the noncore minor pi-

lin, PilX, which was proposed to inhibit retraction of adjacent, antiparallel pili through interaction of D-region protrusions of PilX molecules on opposing fibers (175, 176). However, pilus-mediated aggregation must occur in a controlled manner. Mutations in the major subunit that do not affect pilus assembly but preclude bundling diminish the pathogenicity of *V. cholerae* (255), as do mutations that lead to formation of pilus bundles and bacterial aggregates that cannot subsequently separate (243). In *E. coli*, remodeling of bundled pili into even thicker bundles via the action of the retraction ATPase BfpF was required for microcolony dispersal (232), suggesting that the particular mode of pilus-pilus interaction affects the subsequent behavior of bacteria. A recent study of *V. cholerae* binding to intestinal epithelia revealed that aggregates of T4P enveloped the bacteria, potentially protecting them during the infection process (238). In *S.* Typhi, bundling of pili is negatively controlled by expression of one of two variants of the minor pilin, PilV (note that this is a different component than the *Neisseria* protein of the same name). When PilV is not expressed due to increased supercoiling of the encoding DNA under low oxygen tension—such as would be found in the small intestine—the pili are able to self-associate and mediate microcolony formation, enhancing pathogenicity (290).

Motility

The ability to generate surface-associated twitching motility makes T4P unique among bacterial pili and fimbriae (305). Bradley (59–61) discovered that T4P retract by his studies of pilus-specific filamentous bacteriophages. He saw that pili became shorter after phage attachment, bringing the phage in contact with the cell surface, and from there, he inferred that pilus retraction was responsible for flagellum-independent motility. Twitching, also known as crawling motility (108), or social motility in *Myxococcus* (213), arises from the alternate polymerization (extension) and depolymerization (retraction) of pili that translocate the bacteria along surfaces. The ability to retract pilus fibers is essential for motility, as mutants lacking the retraction ATPase PilT are nonmotile. Twitching motility has so far been associated exclusively with T4aP, as the ability of T4b to retract does not appear to be a universal property; many T4b systems lack a PilT homolog. In fact, some of the differences in the two classes of assembly systems may be related in part to the evolution of T4aP into an efficient motility apparatus. There are a few reports providing evidence that the T4b bundle-forming pili of EPEC (which has a PilT homolog) can retract (21, 192, 428), but they are not involved in twitching motility. Other modes of T4aP-dependent movement, including "walking" and "slingshot" motilities, have recently been reported for *P. aeruginosa*; all modes depend on pilus retraction (108, 153).

Twitching has been characterized extensively for species that express T4aP, including *M. xanthus*, *N. meningitidis*, *N. gonorrhoeae*, *P. aeruginosa*, and *D. nodosus*. There are also reports of T4P-mediated "gliding" motility in the clostridia, notably *C. perfringens* and *Clostridium beijerinckii* (397). Motility can be random or directed; for example, the cyanobacterium *Synechocystis* uses its T4P to move toward light sources, while *M. xanthus* and *P. aeruginosa* twitch up phospholipid gradients (54, 66, 284). Motility, or pilus retraction, is an important trait for many pathogens, as piliated but retraction-deficient mutants are attenuated in virulence (48, 107, 166, 280, 428). In some cases, loss of virulence in the absence of pilus retraction was linked to an impaired ability to

engage the needle-like type III secretion system for the injection of toxins due to loss of intimate host cell contact (107, 215, 428), and toxicity could be restored by expression of alternative adhesins (375).

Pilus retraction is also an important virulence attribute in parasites of bacteria, such as *Bdellovibrio* (277). This small bacterium preys on other Gram-negative bacteria by ramming into them at high speed and then using its T4P to enter the periplasm, where it digests them from the inside (134). *Bdellovibrio pilT* mutants have an attenuated predation capacity (277). For detailed discussions of twitching motility that are beyond the scope of this review, the reader is directed to previous references (68, 69, 112, 273).

Archaeal flagellins are more similar to T4 pilins than to eubacterial flagellins, including a requirement for processing of their type III secretion signal by dedicated peptidases prior to assembly (388). However, archaeal flagellar filaments are functionally similar to bacterial flagella, providing a propulsive force through rotation, rather than retraction, of the fibers. It was recently proposed that these unique structures be called "archaella" to distinguish them from eubacterial flagella (199). Although many components of the archaellar assembly system have been identified by knockout studies, the mechanism by which the system generates torque by use of a pilus-like fiber remains elusive (247). For a comprehensive overview of archaeal flagellin assembly and function, we suggest some recent reviews (151, 199).

Biofilm Formation and Remodeling

All classes of T4P, and even the T2S hyperpseudopili produced upon overexpression of the major pseudopilin, can participate in biofilm development in a wide variety of bacterial and archaeal species by promoting initial (and often highly tenacious) adherence to surfaces, as well as cell-cell interactions (122, 128, 208, 209, 328, 398, 400). O'Toole and Kolter (304) first reported that *P. aeruginosa* mutants lacking T4P are deficient in biofilm formation. Since then, similar findings have been reported for a number of bacteria and archaea, including (but not limited to) *V. cholerae*, *A. actinomycetemcomitans*, *Aeromonas caviae*, *Shewanella oneidensis*, *Legionella pneumophila*, *Caulobacter crescentus*, *Deinococcus geothermalis*, *C. perfringens*, and *Sulfolobus acidocaldarius* (40, 44, 102, 133, 178, 342, 389, 398, 408, 431).

In addition to promoting adherence and aggregation in biofilms, T4aP participate in remodeling of biofilm architecture through twitching motility (see above). Studies by Klausen et al. (228, 229) showed that twitching motility allows bacteria to move to specific zones in biofilms. Chiang and Burrows (95) showed that twitching motility mutants formed abnormal biofilm architectures. More recently, pilus mutants were shown to form aberrant biofilms due to loss of efficient detachment (108). Biofilm formation by *Shewanella* requires not only pilus expression but also pilus retraction (350). The role of twitching motility in biofilm biology is complex, however, as stimuli that increase twitching motility during early development can reduce the extent of irreversible attachment, which is necessary for further stages to proceed. Studies of *P. aeruginosa* showed that stimulation of twitching through increased rhamnolipid synthesis, caused by the chelation of iron or treatment with small molecules, peptides, or complex natural products such as ginseng, decreased biofilm formation (118, 158, 356, 412, 417).

Manipulation of Host Cells

Although attachment via T4P is the first step in infection for many pathogens, the participation of pili in virulence does not necessarily stop there. The ability of some bacteria to manipulate host cell biology via their T4P is a very interesting feature. Both *N. meningitidis* and *N. gonorrhoeae* can modulate host cell function to promote infection and persistence, but they have distinct tissue tropisms and cause different symptoms; for example, *N. meningitidis* is capable of crossing the blood-brain barrier to cause meningitis (293). *N. meningitidis* can bind to and traverse both epithelial and endothelial cell layers, in a T4P-dependent manner (376), and it recruits a variety of host proteins to do its bidding. The bacteria bind to cells and form microcolonies in a manner that requires retractable T4P and the noncore minor pilins PilV and PilX, as well as the pilus-associated PilC1 and PilC2 proteins (63, 111, 225, 283, 287, 288, 362, 415).

T4P-mediated adherence and retraction lead to the rapid formation beneath the microcolonies of cortical plaques (179, 280, 303), structures enriched in the cytoskeletal proteins actin and ezrin and in signaling proteins such as caveolin, epidermal growth factor receptor (EGFR), CD44, and ICAM-1 (52). *Neisseria* causes cytoskeletal rearrangements that block bacterial uptake, a phenotype also caused by *E. coli* expressing bundle-forming pili (52), and induces membrane ruffling, enveloping adherent microcolonies and protecting them from shear forces (283). *Neisseria* also alters cell signaling and cytokine production (97, 314, 383, 399) and impairs adherens and tight junction integrity, allowing bacteria to traverse polarized cell layers, including those forming the blood-brain barrier (110, 111, 248, 334). Pilus retraction is a key facet of the *Neisseria*-host interaction, as infection with retraction-deficient mutants impairs cortical plaque formation and cytoprotective signaling (119), leading to higher levels of host cell apoptosis (179). For further information on *Neisseria*-host cell dynamics and the role of T4P, we suggest previous reviews and the references therein (80, 110, 180, 206, 294, 402).

Competence and Conjugation

Studies of bacterial evolution have revealed the extensive contribution of horizontal gene transfer to the shaping of bacterial genomes. T4P have long been recognized as important systems for the acquisition of exogenous DNA in both Gram-negative and Gram-positive bacteria, both by acting as receptors for transducing bacteriophages and by their involvement in competence and conjugation (28, 93). Because of these key functions, T4P genes are often identified and/or annotated in genome sequences as *com* or competence genes. Well-studied examples include those of *Haemophilus*, *Neisseria*, *Legionella*, *Bacillus*, and *Thermus* (8, 33, 82, 150, 338, 367). DNA uptake occurs by a series of steps that require both inner and outer membrane components of the T4P assembly system, some of which have been shown to bind DNA directly (24, 245). The exact mechanism by which T4P participate in DNA uptake is not yet clear, but mutants lacking pili or that are incapable of pilus retraction lose competency (70). Some bacterial families, such as the *Neisseriaceae* and *Pasteurellaceae*, exhibit a preference for DNA with particular repetitive uptake sequences (34, 141), for reasons that are not entirely clear. Other bacteria, such as *V. cholerae*, become competent in response to specific environmental cues (279).

In addition to playing a role in DNA uptake, T4P can participate in the transfer of self-encoding DNA to recipient cells by conjugation. The best-characterized example of such a system is the IncI1 plasmid R64's thin pilus, involved in DNA transfer under liquid growth conditions. The plasmid encodes 12 proteins required for biogenesis of a T4bP that binds to the lipopolysaccharide (LPS) of recipient cells by using 1 of 7 variants of the PilV adhesin, generated by a shufflon mechanism that randomly swaps the C-terminal domains while maintaining the highly conserved N-terminal α-helix region (198, 343, 426). Similar genes are found on a number of conjugative plasmids and on self-transmissible genomic islands in human and animal pathogens, such as enterotoxigenic *E. coli*, *S. enterica*, *S.* Typhi, *Yersinia pseudotuberculosis*, and *P. aeruginosa* (83, 105, 364). The frequent appearance of transposon-carried drug and toxin resistance genes in such transmissible elements suggests that they contribute to the spread of antibiotic resistance. In some cases, they have been implicated in pathogenesis, as deletion of the entire locus reduces virulence in animal models (105). For further information on the role of T4P in DNA transfer and uptake, we recommend several comprehensive reviews (28–30, 70, 93).

Electron Transfer

T4P of the environmental bacteria *Geobacter sulfurreducens* and *Geobacter metallireducens* have the fascinating property of electroconductivity, giving them the nickname "nanowires" (264, 327). The bacteria use their pili to make contacts with terminal electron acceptors (both nonliving and living, in the case of biofilms) external to the cell, allowing them to thrive in anaerobic environments. Their ability to reduce insoluble iron and manganese oxides and to reductively precipitate other potentially toxic metals, such as uranium, makes them important players in biogeochemistry and bioremediation (261). There is considerable interest in developing *Geobacter*-based "bio-batteries," inspiring research efforts to understand the physicochemical basis for pilus conductivity (260). *Geobacter* pili were reported to belong to the T4a class, and the bacteria of these genus have the characteristic *pilMNOPQ* genes of the T4aP system in their genome, but the subunits that have been characterized are unusually small (~61 residues) and lack most of the characteristic C-terminal domain, similar to Flp pilins (332). This unique pilin structure is suggested to have arisen during its evolution into an efficient conductor, because the pilins lack other motifs associated with electron transfer in cytochromes or photosynthetic reaction centers. Feliciano et al. (137) suggested that the conserved N-terminal α-helix has the necessary geometry for electronic coupling, with alignment of peptide bond dipole moments and an electrostatic field along the helix, from the positively charged N terminus to the negatively charged C terminus. Truncation of the C terminus compared with those of other T4a pilins is thought to prevent the neutralization of moving electrons before they jump to the next subunit. Specific charged and aromatic residues with delocalized electrons are conserved throughout pilins of the *Geobacter* family and may form a relay, allowing electrons to move easily along the pilus (137). Two start sites in the same open reading frame (ORF) result in 9-kDa and 7-kDa products, both of which are processed at the same site by the prepilin peptidase. Although the mature products are identical after processing, mutation of the gene to eliminate one or the other start site affects pilin function. Klimes et al. (230) reported that some *Geobacter* strains continue to produce pilus-like filaments in the absence of PilA but that the fibers are lost when minor pilin genes

are inactivated. It is possible that *G. sulfurreducens* produces multiple classes of T4P and that some assembly components are shared.

Protein Secretion

The T2S system is broadly distributed in eubacteria, where it exports fully folded proteins and protein complexes from the periplasm across the outer membrane, where they remain associated with the cell surface or are released (98, 99, 205, 237). This pathway allows for the secretion of proteins folded with cofactors or associated with chaperones or inhibitory peptides that are released upon secretion. It was originally called the main terminal branch of the general secretory pathway, because its substrates are first exported to the periplasm by the Sec or Tat pathway; this function led to the Gsp designation of this system (319). The nature of the secreted substrates varies between systems, and they are usually not interchangeable, suggesting that compatibility between substrate and assembly system components is necessary. Well-studied examples of T2S systems include the *Klebsiella oxytoca* pullulanase (Pul) system; the *V. cholerae* Eps system, responsible for secretion of cholera toxin; the *P. aeruginosa* Xcp system, which secretes elastase, exotoxin A, and lipases; the Out system of *Erwinia*; the Exe system of *Aeromonas hydrophila*; and the Xps system of *Xanthomonas campestris* (140, 320, 339, 346). Since many T2S substrates are virulence factors in diseases of plants and animals, there is significant interest in understanding T2S function from a therapeutic standpoint.

The relatedness of the T2S and T4P systems was recognized early (183) and has only been strengthened by a wealth of data, including structural studies revealing that even proteins with poor sequence identity have similar architectures (31, 205, 236, 275, 322, 345). Current models of T2S function that invoke a piston-like action pushing substrate proteins through the secretin were informed by models of the extension and retraction of T4P and by studies showing that T2S pseudopilins could form pilus-like fibers under specific conditions (128, 190, 349, 400). Furthermore, there are several examples of proteins that are secreted by T4P systems, including the TcpF colonization factor of *V. cholerae* (226), proteases of *D. nodosus* (167), and virulence factors of *Francisella tularensis* (19, 163), showing that the systems are functionally similar.

The T2S system is the only system for which structures of all the major and minor pseudopilins are available, alone and in subcomplexes. Those data have been invaluable for the refinement of theories about the mechanism of fiber assembly and function. Other key information came from protein-protein interaction studies that showed the specific pattern of interactions, both stable and transient, between various minor pseudopilins, between pseudopilins and components of the assembly system, and even with the T2S substrates (124, 125, 236, 237, 244, 329). Among the important questions that remain to be answered is how the substrates of T2S (and T4P, where they are involved in secretion) are recognized against the background of myriad periplasmic proteins. No linear secretion signal has been identified, and protein folding is required for secretion competency, suggesting that the signal may be a three-dimensional one (145, 407). However, comparison of substrates for which structures are available does not suggest an obvious candidate(s), and thus studies aimed at identifying the elusive secretion signal continue.

REGULATION OF PILIN AND PSEUDOPILIN EXPRESSION

Although the pseudopili of the T2S systems are typically shorter than a dozen subunits, T4P and archaeal flagella can be several micrometers long, corresponding to thousands of subunits. Making a large amount of protein that is exposed on the cell surface—subject to accidental loss or, in some cases, deliberate shedding (385)—is metabolically expensive. The need for control of pilus biogenesis in response to environmental signals has contributed to the evolution of complex regulatory mechanisms, ranging from transcriptional to posttranslational mechanisms. Pilin gene expression is responsive to environmental cues that are relevant to the lifestyle of the specific organism. In many species, surface contact—or, perhaps, a local increase in viscosity or mechanical strain on membranes—leads to upregulation of pilin expression (43). In *Haemophilus influenzae*, alkaline pH is required for optimal pilus expression (33), while some archaea produce T4P in response to UV irradiation (7, 147). For many pathogens, T4P are made only in response to growth under specific culture conditions or at specific temperatures and/or in response to host cell contact (102, 254, 274).

Regulation of Amino Acid Composition

Although pili are strong (250, 281), they can be lost under conditions of strong shear forces. Smith and Chapman (361) showed that bacterial proteins destined to be surface exposed or extracellular tend to contain a preponderance of "cheap" amino acids (in terms of the number of ATP molecules used in their production), such as Gly, Ala, Ser, Glu, Thr, Gln, and Asn, and fewer "expensive" amino acids, such as Trp, Phe, Tyr, His, and Met. Inspection of the composition of representative major pilins from *P. aeruginosa* supports this idea, as the content of Gly, Ala, Ser, and Thr (GAST) in mature pilins ranges from ~38% to 47%. The GAST contents of *P. aeruginosa* minor pilins are slightly lower, ranging from ~31% to 37%. In contrast, the GAST content of the major pseudopilin, XcpT, is ~26%, consistent with those of other proteins predicted to remain intracellular, such as the cytoplasmic PilB, PilT, and PilU ATPases, all of which have GAST contents of ~27%. Similarly, the XcpUVWX minor pseudopilins that remain periplasmic have GAST contents of ~24% to 32%.

Transcriptional Regulation

The ability to reinsert subunits from existing fibers back into the cytoplasmic membrane upon depolymerization, where they can be available for another round of assembly, is an amazing bacterial example of recycling. This strategy conserves energy and provides a mechanism by which bacteria can monitor the amount of available subunits. In many T4P-expressing species, including *M. xanthus*, *D. nodosus*, *G. sulfurreducens*, *Kingella kingae*, *Xanthomonas axonopodis*, and *P. aeruginosa*, the major pilin subunit is expressed from a σ^{54}-dependent promoter (Fig. 7) (182, 197, 218, 239, 418, 423). The complex formed by RpoN and RNA polymerase (RNAP) binds such promoters but remains stalled in a closed configuration. Conversion of the RNAP-σ^{54}-DNA complex to a transcription-competent open form requires an enhancer binding protein, which in the case of pilin gene expression is PilR, the response regulator of the two-component PilR-PilS system.

In *P. aeruginosa*, PilS is a membrane-bound histidine kinase that is autophosphorylated in response to an as yet unknown signal and, in turn, phosphorylates PilR, promoting pilin expression. PilS levels are critical to the control of PilA levels, as both lack of
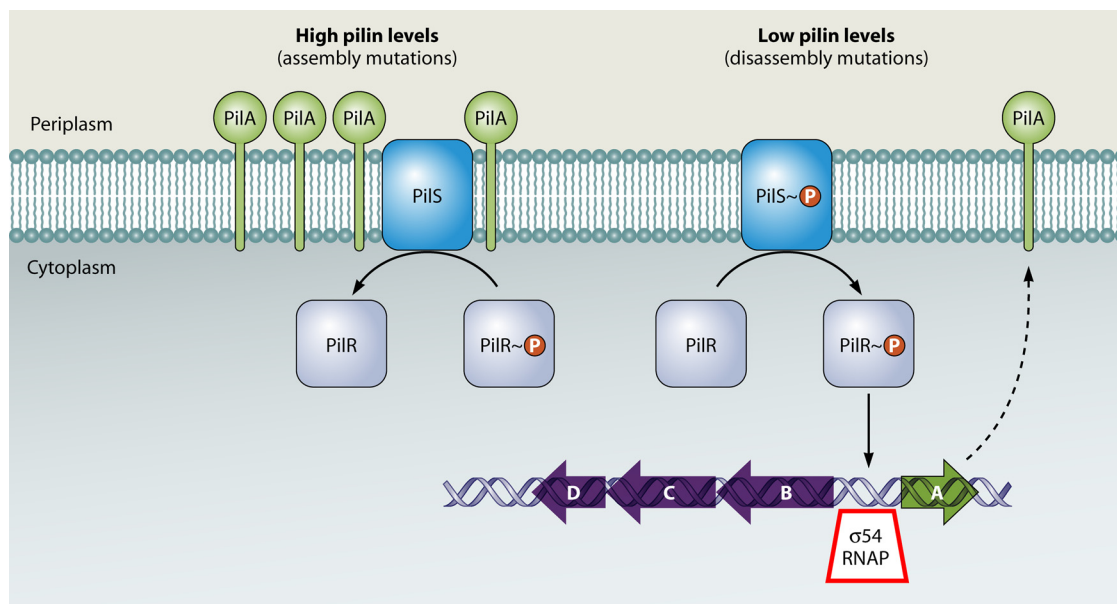
FIG 7 Proposed model of T4a pilin autoregulation by intramembrane interactions with the PilS sensor kinase. The PilR-PilS two-component system regulates major pilin biosynthesis in many T4aP-producing bacteria. Because PilS lacks a typical periplasmic sensor domain and controls the expression of pilins with diverse C-terminal sequences, it may instead detect the highly conserved N-terminal domain of PilA orthologs via intramembrane interactions (11). High intracellular levels of PilA, as in pilus assembly mutants, may inhibit *pilA* transcription in a manner that depends on sequences in the pilin's N terminus, possibly through modulation of PilS dimerization or stimulation of phosphatase activity of PilS on the response regulator, PilR (37, 338). When pilin levels are low—as in retraction-deficient mutants, where the pilins are trapped outside the cell—PilS kinase activity is stimulated, phosphorylating PilR and upregulating PilA transcription. The organization of the *pilABCD* genes in *P. aeruginosa* is shown. *pilA* is transcribed by σ^{54}, RNAP, and phospho-PilR from a divergent promoter that also controls expression of PilB (the pilin polymerase), PilC (the platform protein), and PilD (the prepilin peptidase).

PilS and its overexpression had a detrimental effect on pilin gene transcription (58). Boyd and Lory (58) suggested that PilS overexpression titrated a crucial interaction partner, leading to decreased pilin gene transcription. In some species, including *M. xanthus*, PilR is required for pilin gene expression, but PilS is a negative rather than positive regulatory component (418). The pathway of major pilin regulation in *M. xanthus* has additional layers of complexity, since it can be upregulated in a *pilRS*-independent manner under nutrient-rich conditions. *N. gonorrhoeae* encodes a PilR-PilS hybrid, named Rsp, that binds to the major pilin promoter, but its inactivation has no effect on piliation, suggesting that its regulatory function was lost during evolution of that pathogen (81). In keeping with this regulatory rewiring, expression of the *N. gonorrhoeae* pilin is driven by σ^{70} (although a functional σ^{54} promoter is present), and the gene encoding σ^{54} is truncated in pathogenic *Neisseria* species.

Wu and Kaiser (418) showed that pilin mutants of *M. xanthus* had increased activity from a *pilA* transcriptional reporter, a finding that was corroborated by a recent study in the Engel lab (43), using *P. aeruginosa*. These data suggest that PilA negatively regulates its own expression, possibly through direct interactions with PilS (Fig. 7). In a *P. aeruginosa* mutant lacking the retraction ATPase PilT, the reporter was similarly activated, although an *M. xanthus pilT* mutant had only mildly elevated levels of *pilA* expression. In a *pilT* background, pilin levels in the cytoplasmic membrane would presumably be low due to the inability of the cells to retract their pili. In contrast, *P. aeruginosa* mutants lacking the assembly ATPase PilB had low levels of reporter activity (43). Such mutants accumulated pilins in the inner membrane due to the blockade in pilus assembly. The phenotypes of assembly and re-

traction mutants are consistent with the hypothesis that high levels of PilA in the inner membrane suppress *pilA* transcription. Similar findings have been reported for *N. gonorrhoeae*, where inactivation of *pilT* markedly increased the levels of transcription of the major subunit gene, *pilE*, while mutations that block assembly (in *pilD*, encoding the prepilin peptidase, or *pilF*, encoding the pilin polymerase) decreased its expression (120).

When *pilA* or *rpoN* was inactivated in *P. aeruginosa*, levels of specific pilus assembly system components were reduced (32). Interestingly, when *pilR* or *pilS* was inactivated, levels of the assembly system components were similar to those in the wild type, suggesting that even though there was no PilA expression in either of those mutants, the cells were unable to sense its absence and respond accordingly. In *M. xanthus*, *pilB* mutations that caused the accumulation of pilins in the inner membrane led to the downregulation of EPS synthesis, a phenotype that could be relieved partially by inactivating *pilA* in the *pilB* background (425). In contrast, mutations that led to the accumulation of PilA in the cytoplasm (signal sequence mutations) did not repress EPS synthesis. Both T4P and EPS are required for *M. xanthus* social motility, and these data suggest that they are coregulated in response to the levels of pilin present in membrane pools. In general, PilR-PilS may regulate other components in the cell in response to alterations in the amount of pilin detected. Bioinformatic studies of *G. sulfurreducens* promoters suggested that PilR might regulate the production of pili, flagella, secretion components, and proteins involved in cell envelope biogenesis (239).

The sensor kinase PilS, which has an atypical 6 transmembrane domains but no periplasmic sensor domain, could monitor levels of pilin through direct intramembrane interactions with the con-

served hydrophobic N terminus of PilA (Fig. 7). High PilA levels could drive PilA-PilS interactions that, depending on the particular species, could either promote or prevent kinase activity. As pilin levels decrease, PilS might adopt an alternate conformation and act as a PilR phosphatase. Such a model for control of PilS activity is not unprecedented; regulation of sensor kinase activity by intramembrane interactions with hydrophobic peptides was recently reported for a number of systems (12). For example, Lippa and Goulian (256) showed that PhoPQ, a two-component system controlling important virulence pathways in many pathogens, is negatively regulated by the 47-residue hydrophobic peptide MgrB, in that direct interaction between PhoQ and MgrB inhibits the expression of PhoP-activated genes.

Szurmant et al. (381) showed that the YycH and YycI proteins of *Bacillus subtilis*—like PilA—each have a single TM domain, with the bulk of the proteins outside the cytoplasmic membrane. Short fragments from the N terminus of either protein regulated the activity of the YycG sensor kinase in a manner that could be disrupted by acidic point mutations within the hydrophobic TM domains (380). Wu and Kaiser (418) showed that mutation of the highly conserved Glu5 residue in the N-terminal helix of the major pilin to Lys recapitulated the phenotype of a *pilA* mutant; a reporter construct showed that pilin expression was upregulated, even though the E5K mutant protein was stable and present at wild-type levels in the cell. This particular residue is highly conserved among major pilins (and some minor pilins) in most species, suggesting that this proposed mechanism of regulation could accommodate diverse pilin alleles.

Regulation through Pilin Stability

Pilins are quite stable, and protein levels remain elevated even after cells are transferred to growth conditions (such as broth culture) that do not promote *pilA* expression (43). A reduced rate of pilin turnover is consistent with an energy conservation strategy in which pilins are retained in the inner membrane under conditions where twitching motility is not optimal but are ready for deployment if conditions become favorable. For T4P, there appears to be a minimum threshold of pilin expression that is necessary for motility to occur. In *M. xanthus*, Jelsbak and Kaiser (201) expressed the major pilin subunit from an inducible promoter and showed that motility was similar to that of uninduced strains until a level of ≥50% of the wild-type level was produced. The amount of motility was related to the number of surface pili rather than to total PilA levels. A similar phenomenon was seen in *P. aeruginosa* using an arabinose-inducible promoter; without inducer, the low level of PilA synthesized was insufficient for motility (170). When 0.1% arabinose or more was used, motility was restored to wild-type levels. Whether this phenomenon is related to pilus length is not clear, since even mutants with very short pili can be motile (154).

Minor Pilin Regulation

Regulation of minor pilin expression has not been well characterized. Because T4a (unlike T4b) genes are not clustered in the genome, it is possible to differentially regulate subsets of pilus genes. In *P. aeruginosa*, the core minor pilin operon is positively regulated by the AlgR-FimS two-component system (41, 257, 414), and *algR* mutants are consequently nonmotile. Although AlgR controls other operons as well, provision of the minor pilin genes in *trans* is sufficient to restore twitching motility. Inactivation of

individual genes in the minor pilin operon of *P. aeruginosa* resulted in increased expression of others, implying a positive-feedback loop in the absence of specific components (53, 154). In *Neisseria*, the genes encoding the core minor pilins PilHIJK are clustered with the gene for the noncore minor pilin, PilX (called PilL in some *N. gonorrhoeae* strains [416]), while the loci encoding other noncore proteins (PilV and ComP) are unlinked. PilV and ComP are functionally connected to one another, however, since ComP-dependent DNA uptake is modulated in a reciprocal manner by PilV levels, because PilV is an antagonist of ComP trafficking (2, 64).

The minor pilin genes for T4b and T2S systems are generally clustered with those encoding the other components of the system and therefore are likely to be coregulated. For the T2S system, only a few major subunits are needed to produce a pseudopilus of sufficient length to cross the periplasm; therefore, coregulation of major and minor subunit expression to maintain their stoichiometry is likely straightforward. In the T4b system, the number and stoichiometry of minor components vary depending on the species and, in general, have not been well characterized.

PILIN DIVERSITY

Intra- and Interspecies Diversity of Pilin Proteins

Identifying functionally significant sequence differences among type IV pilin proteins is a challenge because of their immense diversity. As surface-exposed structures, they are likely under diversifying selection for variants that attach more or less persistently to various surfaces or receptors; for increased or decreased bacterial cell-cell aggregation, influencing dispersal and formation of biofilms; as receptors for bacteriophages; for uptake of DNA in competent species, which depends in part on pilin surface chemistry; or, in the case of pathogens, for avoidance of host immune recognition while retaining the ability to affect host biology. Although some bacteria can assemble heterologous pilins, a critical reading of the literature reveals that in most cases, (i) it is not possible to recapitulate all of the functions of the native protein and (ii) heterologous fibers are produced only in retraction-deficient backgrounds, suggesting that sequence differences reflect a balance between assembly dynamics and function.

The sequences of type IV pilins can undergo variation through horizontal gene transfer between species, by shufflon or other recombination events within species and strains, and by point mutation. Horizontal acquisition of new pilin-encoding DNA fragments whose 5′ sequences, encoding the highly conserved N-terminal helix, are very similar increases the probability of recombination with existing genes. In the case of pseudopilins, their diversity might be driven in part by their interactions with specific substrates secreted by the T2S system (124). The minor pseudopilins form functional complexes (100), suggesting that diversity may also reflect the coevolution of protein-protein interaction interfaces. In this section, we consider pilin diversity and its sources at the protein level; additional diversification can occur through decoration with various posttranslational modifications (PTMs) (see below). Type IV pilins are highly immunogenic and provoke protective serological responses, although typically only to homologous or closely related antigens (17). Therefore, a thorough understanding of pilin diversity and the extent to which major versus minor subunits are conserved is important for the design of broadly protective pilus-based vaccines (87). Detailed

studies of type IV pilin protein diversity have so far been confined to a small number of well-characterized species, but the advent of inexpensive genome sequencing and identification algorithms will allow for comparisons among a larger pool of sequences.

Diversity in *Neisseria* Pilins

For obligate human pathogens such as *Neisseria*, which are continually exposed to immune surveillance, the ability to diversify their pilus composition is critical for survival. Because of their restricted host and niche range, they have limited opportunity to acquire new pilin genes by horizontal gene transfer. To circumvent this problem, *Neisseria* organisms have evolved an ingenious strategy involving intragenic recombination of a number of silent pilin gene cassettes (*pilS*) with a single expressed major pilin locus (*pilE*), in a process called gene conversion (181). Studies in male volunteers showed that strains recovered from infection with a single piliated strain had pilin sequences that were different from those of the parental strain (354, 377). Sequence analyses showed that during the course of infection, some or all of a *pilS* locus recombined into the active *pilE* locus (377). This recombination event can cause a hyperpiliated phenotype or, more commonly, a nonpiliated strain, where a frameshift in the *pilE* sequence creates a premature stop codon (116). A more recent study of isolates recovered from a scientist accidentally infected with a single *N. meningitidis* strain (302) showed that even with the limited number of *in vivo* cell divisions (~25) estimated to have occurred before resolution of the infection by antibiotic therapy, at least 7 different pilin variants with different adhesive capacities arose, showing rapid diversification. *Neisseria* is naturally competent for DNA uptake, in a T4P-dependent manner, and can undergo intergenic recombination with DNA scavenged from its environment. Provision of exogenous DNA increased antigenic variation of the pilus in *N. meningitidis* (11), while addition of DNase during infection was shown to decrease antigenic diversity (152).

Most *Neisseria* have a single *pilE* locus, but in some cases, tandem alleles (*pilE1* and *pilE2*) have been reported (6). The number of *pilS* alleles varies between strains, as 17 silent copies were identified in *N. gonorrhoeae* strain MS11, while 19 different *pilS* loci at 6 distinct chromosomal locations were identified in *N. gonorrhoeae* strain FA1090 (161, 165). Each *pilS* allele lacks a promoter, a ribosome binding site, and an approximately 150-bp region from the 5′ end of *pilE* (116, 161). These features prevent the expression of *pilS* alleles until they recombine with the functional *pilE* allele. Each *pilS* allele contains a semivariable region (5′ end) and a hypervariable region (3′ end). Within the variable regions are 1- to 34-bp sections that are conserved between *pilS* and *pilE* and predicted to allow for crossover between the two genes (116, 181). The combination of a series of repetitive elements between *pilS* genes, a putative recombinase site upstream of *pilE*, and a conserved Sma/Cla repeat downstream of *pilE* permits the nonreciprocal transfer of DNA from *pilS* to *pilE* though homologous recombination. Furthermore, recombination can occur between *pilS* alleles, adding to potential diversity.

The mechanism by which the initiation of high-frequency pilin gene conversion occurs in *N. gonorrhoeae* was recently discovered (74). Homologous recombination was previously shown to mediate pilin antigenic variation (231, 278, 353, 357), but no specific genetic element capable of triggering the recombination event had been identified. A *cis*-acting 16-base guanine-rich sequence—designated the G4-RIS or "recombination initiation sequence"—located upstream of *pilE* on the lagging strand, was shown to form a guanine quartet (G4) which facilitates nick formation within the G4 DNA (74). Mutation of any of the 12 G-C base pairs in the 16-bp region abolished pilin antigenic variation without affecting pilin expression, while mutation of the other 4 residues had no effect. Cahoon and Seifert further showed that the position, sequence, and orientation of the G4 structure were required for antigenic variation, as alterations in any of these factors prevented nicks from occurring on the G-rich strand (74). Although other predicted G4 structures were encoded in the gonococcal genome, only the specific sequence upstream of *pilE* was capable of promoting antigenic variation. Interestingly, this structure is conserved in pathogenic but not commensal species of *Neisseria*.

In addition to the pilins described above, which are referred to as class I pilins, specific clonal complexes of *N. meningitidis* express a second T4aP pilin type, designated class II (6, 88). Class II pilins are smaller (15 to 17 kDa) and do not bind the monoclonal antibody SM1, which recognizes both *N. gonorrhoeae* and *N. meningitis* class I pilins. Genetic and immunological studies suggest that some commensal species of *Neisseria* express class II pilins, suggesting the possibility of horizontal transfer between pathogenic and nonpathogenic species. Class II pilins are not subject to conversion and therefore are more antigenically stable than class I pilins (177). Class II pilins lack the C-terminal hypervariable region, although loss of this domain does not preclude class II pilin-expressing meningococci from causing invasive disease (88).

The impressive ability of *Neisseria* species to undergo rapid and frequent variation of their pilins has led to the abandonment of PilE (in particular, class I) as a vaccine candidate. However, *Neisseria* pili were also recently shown by a number of investigators to contain minor pilins, whose low abundance relative to that of the major subunit does not necessarily reflect their important contributions to pilus function and pathogenicity (63, 154, 175, 248, 283, 415, 416). To determine whether the level of protein diversity among minor pilins was lower than that among major pilins, and thus whether they would potentially make better vaccine candidates, Cehovin et al. (88) examined the prevalence and sequence similarity among specific minor pilin genes in a large set of pathogenic isolates of *N. meningitidis*. The *pilX*, *pilV*, and *comP* genes (encoding minor pilins involved in aggregation, adherence to host cells, and DNA uptake, respectively) were each present in all of the isolates tested, though sequence conservation among the predicted gene products varied from 64% identity for PilV orthologues to 97% for ComP orthologues, with that for PilX orthologues being intermediate, at 73%. The potential utility of the minor pilins as candidate antigens was supported by the identification of anti-minor-pilin antibodies in convalescent-phase patient sera (87). A more detailed characterization of their functions and proof-of-principle vaccination studies will be needed to show if they will be effective as protective antigens.

Diversity in *Pseudomonas* Pilins

Unlike *Neisseria*, *Pseudomonas* species occupy a broad range of environmental niches and therefore have access to a wider pool of DNA as a source of diversity. The best-characterized species, *P. aeruginosa*, can express multiple types of pili and fimbriae, including T4a, T4b, and Tad pili (42, 83, 395), but T4aP are the most commonly observed fibers on laboratory-grown cells. A recent examination of intraspecies diversity of the T4a pilin in *P. aeruginosa* and other *Pseudomonas* species provided evidence for a

genomic pilin island—approximately 36 kb in the PAO1 reference strain—that encodes both the T4a major and minor pilins as well as the two-component PilRS system that controls major pilin expression (155). Remarkably, in at least one strain of *P. aeruginosa* (PA14), a set of genes expressing a separate, self-transmissible T4bP system as part of *P. aeruginosa* pathogenicity island 1 (PAPI-1) (83) is inserted within the T4aP pilin island, at three tandem tRNA genes (155). In other *Pseudomonas* species, the same locus is occupied by DNA insertions of up to ~100 kb, suggesting that this position is an active hot spot for recombination.

Five different groups of T4a major pilins have been identified in *P. aeruginosa*, classified by sequence length, the size of the D region, and the identity of the pilin accessory protein encoded downstream of the pilin (242). The first identified groups were groups I and II (86). The common laboratory strains PAO1, PAK, and PA103 express group II pilins; such strains lack accessory genes between *pilA_{II}* and the downstream tRNA^{Thr} gene. In contrast, strain 1244, the Liverpool epidemic strain (LES), and strain PA2192 have a slightly larger group I pilin and an additional ORF, between the *pilA_I* and tRNA^{Thr} genes, involved in glycosylation of group I pilins at Ser148 (85, 106, 242). In 1995, the prototype of the group III allele was identified in a single *P. aeruginosa* isolate. Spangenberg et al. described the group III pilin of 173 amino acids—longer than the previously identified pilins—and an open reading frame of unknown function, now named *tfpY*, carried downstream of the *pilA* gene (242, 363). Additional isolates with group III alleles (including the well-characterized PA14 strain), as well as isolates with two new pilin alleles (groups IV and V), were subsequently identified in a survey of >300 *P. aeruginosa* strains (242). Since analysis of this relatively small sample size readily led to identification of novel pilins, examination of a greater number of isolates from the broad spectrum of environments colonized by this species will likely reveal additional diversity. Group V strains encode pilins and accessory proteins (TfpZ) most similar to, but distinct from, those of group III, as polyclonal antisera raised against pilins of the two groups do not cross-react. Group I alleles were most common, followed by groups II, III, and V (242), although a study by Römling et al. (335) found that clone C, the most common strain of *P. aeruginosa* distributed around the globe, belongs to group II. To date, group IV contains only two isolates, Pa5196 and PA7, which encode the TfpW and TfpX accessory proteins via genes downstream of *pilA_{IV}* (242, 336). TfpW is a large membrane-bound glycosyltransferase that adds one or more D-arabinofuranose sugars to the pilin (241, 405). TfpX is a predicted pilus accessory protein most similar in sequence to TfpZ, the group V accessory protein. The specific function of the related accessory proteins TfpX, TfpY, and TfpZ is currently unknown, but mutagenesis studies show that they promote efficient assembly of their cognate pilin (23).

As in *Neisseria*, the minor pilins of *P. aeruginosa* were shown by Western blotting of sheared pili, as well as by immunogold labeling and transmission electron microscopy, to be associated with external pilus fractions. Using available *P. aeruginosa* genomes and additional sequencing of the minor pilin genes from selected strains, three unique sets of minor pilins were identified (23, 155). Similar to the case for *Neisseria*, there is less diversity among the minor pilins of *P. aeruginosa* than among the major pilins. However, the observed sequence diversity has functional implications, as the group II and III PilX proteins are not interchangeable (155).

A thorough assessment of minor pilin diversity among other *Pseudomonas* species has not yet been done, in part because the reduced level of sequence identity between orthologous proteins has hampered their accurate annotation.

Moraxella and *Dichelobacter* Pilin Diversity

Moraxella bovis is the causative agent of infectious bovine keratoconjunctivitis (IBK), a highly contagious and economically significant disease that can cause permanent blindness in cattle (65), while *Moraxella lacunata* causes a similar disease in humans. Only piliated strains are able to bind to corneal tissue and cause IBK, highlighting the significance of T4P as a virulence factor. Two different pilin proteins have been identified in *Moraxella*, termed Q (quick) and I (intermediate) (148), and these form a total of 7 serogroups as determined by immunological cross-reactivity studies (286). The expression of these proteins is mutually exclusive, and switching between one or the other is dependent on the inversion of a 2.1-kb segment of DNA, as only one promoter is present. The inversion occurs in the N-terminal region, and the process is reversible, as Q strains can become I strains and vice versa (269). Further sequence analysis revealed that a 26-bp region including the sequence encoding the first 6 amino acids of PilQ was responsible for inversion of the pilin genes and subsequent expression of the leading gene (148). The Q pilin is associated with increased binding to corneal tissue compared with the I pilin (337). Recent studies of *Moraxella catarrhalis*, an emerging human pathogen responsible for a large proportion of otitis media and other upper respiratory tract infections, showed a low level of pilin diversity (262). All strains tested expressed either clade 1 (PilA1; 42%) or clade 2 (PilA2; 58%) pilins, with PilA1 having an 11-residue deletion at the C terminus compared with PilA2. PilA1 pilins from various strains showed 78 to 86% identity, while PilA2 pilins showed 88 to 99% identity, with 59 to 66% identity between the two types. Unlike the case for other *Moraxella* species, no phase variation was observed. The limited diversity of pilin types in *Moraxella* has allowed the pilins to be used successfully as vaccine antigens for IBK (17).

Dichelobacter (formerly *Bacteroides*) *nodosus* is the pathogen responsible for foot rot in sheep. The pili of this organism are important virulence factors and vaccine antigens, and the major pilin, FimA, is used to serotype isolates (220). Genome and microarray analyses (291) suggested that the *fimA* locus has a nucleotide composition that is atypical for the species and is divergent, suggesting hypervariability. Although *D. nodosus* pilins have been used successfully as vaccines (130), their ability to protect against homologous but not heterologous antigens and the presence of multiple strains on individual animals (430) mean that vaccines have to be multivalent, including the most prominent serotypes.

Tandem Chromosomal Pilin Loci

A number of groups have documented tandem major T4a pilin subunit genes on the chromosome. Initially, this arrangement suggested that the promoter-distal *pilA* allele might be used in antigenic variation, similar to the case for the *Neisseria pilS* loci. However, in species where the role of each allele has been investigated, the promoter-proximal *pilA* gene is required for surface piliation, while the second *pilA* gene appears to have little effect on surface piliation or motility.

Ruminococcus albus has two *pilA* loci, *pilA1* and *pilA2* (325). The

pilA2 gene is located ~115 bp downstream of the *pilA1* stop codon, with a putative hairpin loop separating them. The two genes are 73% identical, but analysis of mRNA levels revealed that *pilA2* was expressed at a level 73-fold greater than that of *pilA2*. While transcripts can be monocistronic (*pilA1* only) or dicistronic, the former is predominant (325). Localization studies showed that while the pilus is composed of PilA1, PilA2 is confined to membrane fractions. Addition of anti-PilA2 sera did not block T4P-mediated adhesion, suggesting that PilA2 either was not incorporated into the pilus or was not the adhesin.

More than one potential major pilin gene is carried by the periodontal pathogen *Eikenella corrodens*, in a three-gene cluster of *pilA1*, *pilA2*, and *pilB* (homologous to the pilin accessory gene *fimB* in *D. nodosus* and the *tfpY* gene from group III strains of *P. aeruginosa*). Similar to the *R. albus* system, a hairpin loop is located between the *pilA1* and *pilA2* genes, and transcripts were found to be both monocistronic (*pilA1* only) and polycistronic (*pilA1*-*pilA2*-*pilB*) (401). *E. corrodens* can modulate the expression of surface pili, which manifests as an observable change in colony morphology; piliated bacteria form small (or S-phase) colonies, while nonpiliated bacteria form large (or L-phase) colonies. This unique form of antigenic variation in *E. corrodens* is unidirectional, where an S-phase colony can become an L-phase colony but not the reverse (401). The reason for loss of surface pili in the L-phase variant is unknown, as both PilA1 and PilA2 are expressed. Although a small decrease in the *pilA1*-*pilA2* transcript level in L-phase colonies was observed compared to the S-phase colonies, it was not considered significant enough to cause the nonpiliated phenotype (401). The authors attributed this transcript decrease to morphological differences between the variants; however, it is possible that a feedback loop resulting from higher PilA1 levels in the nonpiliated strain led to the overall decrease of *pilA1* transcripts.

The cyanobacterium *Synechocystis* sp. PCC6803 has two morphologically different types of pili, termed thick and thin pili (45). Thin pili (2 to 3 nm in diameter and 500 to 1,000 nm long) are distributed around the entire surface of the cell, and their expression was unaffected in a *pilA1* mutant. Thick pili (6 to 8 nm in diameter and 1,000 to 2,000 nm long) were found in asymmetrical clusters around the cell, and motility and surface expression of these pili were abolished in a *pilA1* mutant (45). Mutation of the *pilA2* gene did not affect surface piliation or motility. Analysis of the sequence directly upstream of *pilA1* and *pilA2* showed a Shine-Dalgarno (SD) sequence upstream of *pilA1* but not *pilA2* (45). The lack of an SD sequence for *pilA2* suggests either that a polycistronic transcript is made, similar to the case for *E. corrodens* and *R. albus*, or that *pilA2* is not expressed or expressed at levels below detection.

A tandem arrangement of pilin genes was also described for *Pseudomonas stutzeri*, with *pilAI* and *pilAII* being cotranscribed; however, *pilAII* expression was only 1/10 that of *pilAI*. Mutation of *pilAI* abolished piliation, while a *pilAII* mutant expressed surface pili and had increased transformability (159). Interestingly, the hypertransformation phenotype was maintained in a *P. stutzeri pilAI pilAII* double mutant expressing pilins from nonnaturally transformable species such as *P. aeruginosa* (159). These data suggest that while *pilAI* and *pilAII* share 55% identity, their functional roles are distinct.

Diversity among T4b Pilins

The best-characterized T4b pilins with respect to diversity are the BfpA (bundlin) subunits of EPEC bundle-forming pili and the TcpA subunits of the toxin-coregulated pili of *V. cholerae*. In general, there is less diversity in T4b pilins than in T4a pilins. This finding may be related to the inability of the assembly systems to recognize heterologous subunits. For example, McNamara and Donnenberg (276) showed that TcpA could not be assembled into fibers by the *E. coli* Bfp system.

All EPEC strains carry one of two major subtypes, named α and β, which are further divided into numbered sequence variants (e.g., α1) (50, 138). The α proteins are 97% identical, while the β proteins are ~80% identical to the α-bundlins. As is typical for pilins, sequence differences are concentrated near the C terminus. In terms of pilus formation, the two forms of bundlin are interchangeable and even form mixed fibers (138, 194), a phenomenon not observed when heterologous T4a pilins are coexpressed (308). However, there are functional differences between the two BfpA subtypes with respect to receptor interactions that mediate the important localized adherence phenotype required for EPEC colonization of intestinal epithelium. A number of studies (191–194) have reported the interaction of bundlin with the carbohydrate receptor *N*-acetyllactosamine (LacNAc). Mutagenesis studies which systematically targeted the surface-exposed sequence of α-bundlin (137-GENNI-141) and replaced it with the respective β-bundlin sequence (136-SPDST-140) and vice versa showed the LacNAc–α-bundlin binding to be specific. Conversion of the α-bundlin to the β-bundlin sequence caused loss of binding, while β-bundlin conversion to the 137-GENNT-141 sequence resulted in preferential binding to a fucosylated derivative of LacNAc, the Lewis X antigen (191). The specificity of the LacNAc–α-bundlin interaction could potentially explain its high level of sequence conservation compared with β-bundlin.

Most cases of epidemic cholera are caused by *V. cholerae* serogroups O1 and O139, with sporadic outbreaks caused by strains of other serogroups that express Tcp. The O1 serogroup contains the genetically similar classical and El Tor biotypes, which vary at only a few loci, including that for the major pilin subunit, TcpA (387). Comparison of TcpA sequences between those two serogroups showed that they were ~80% identical, with the N terminus being the most highly conserved region. The primary mechanism responsible for generating TcpA diversity was proposed to be recombination rather than mutation (387). Even small sequence changes in TcpA have profound effects on pilus function, as single point mutations in the D region caused a loss of pilus bundling, a phenotype important to pathogenesis. Minor sequence differences between the El Tor and classical TcpA proteins generated different patterns of pilus bundling (255). TcpA proteins from classical strains formed large intertwined rope-like bundles, while those from El Tor strains formed a cross-hatched pattern. To understand the structural basis for these differences, Lim et al. (255) solved the structure of the El Tor subunit for comparison with the previously determined classical version (114) and used it to build a fiber model. Key residues that differed between the two forms were located on the protruding D regions and in the cavities between subunits, generating a combined "knob and hole" interaction surface. Mutagenesis of specific nonconserved residues to disrupt or switch the stereochemical complementarity of these

interaction interfaces supported a role for them in pilus-pilus interactions leading to aggregation.

Boyd and Waldor (56) showed that despite sequence divergence of up to 33% from the classical biotype protein, TcpA variants from non-O1/non-O139 serogroup isolates supported infectivity and cholera toxin phage susceptibility, suggesting that they retained the functions of the well-characterized O1 and O139 TcpA proteins. Polymorphic sites were clustered in nonstructural regions of the proteins that were predicted to be surface exposed in the fiber. The diversity in *tcpA* was much higher than that in other loci, showing that it is a hypervariable locus subject to frequent horizontal gene transfer.

POSTTRANSLATIONAL MODIFICATIONS

All type IV pilin proteins identified to date undergo a unique and essential PTM, which is removal of the type III signal sequence by dedicated prepilin peptidases. Blocking of this step prevents the subunits from assembling into fibers (301, 312, 371, 373). The proteins are subject to additional modifications, including N-terminal methylation following removal of the signal sequence (373) and, depending on the species and strain, glycosylation or addition of other moieties to solvent-exposed regions. *Neisseria* is the current champion of pilin PTMs, with several different forms of modification identified, and sometimes multiple types of modification on a single subunit (1). With some exceptions that are detailed below, these decorations are not essential for fiber formation but can have important effects on filament function. Advances in mass spectrometry techniques that cause less damage to samples and increase sensitivity with respect to detection of nonstoichiometric modifications will likely lead to the identification of additional, potentially novel, PTMs.

N-Terminal Cleavage and Methylation

Although the mechanistic details of pilin N-terminal cleavage and methylation have been characterized most extensively using the *V. cholerae* (TcpJ and VcpD) and *P. aeruginosa* (PilD; also called XcpA) prepilin peptidases, homologs of these enzymes are found in all systems with type IV pilin proteins. Proteolysis and methylation were initially proposed to depend on four Cys residues in an N-terminal cytoplasmic loop, but it was later discovered that some PilD orthologues lacked the relevant Cys residues and had proteolytic activity even in the presence of sulfhydryl-reactive inhibitors. XcpO—a PilD homologue in *Xanthomonas campestris*—lacks all four Cys residues but complemented a *P. aeruginosa pilD* mutant (189), suggesting that the Cys residues are not part of the PilD active site. Further studies showed that two highly conserved Asp residues at the membrane boundary of a predicted C-terminal cytoplasmic loop are essential for protease activity; therefore, the enzymes were reclassified as aspartyl proteases (246). Prepilin peptidases are members of a larger family of aspartyl proteases, including mammalian presenilins, that catalyze the intramembrane proteolysis of their substrates. The first structure of a member of this protease family was solved in 2011 (188).

The discovery of similar peptidases associated with archaeal flagellar systems strengthened the hypothesis that these flagella are more similar to T4P than to eubacterial flagella (9, 10, 39). The enzymes FlaK (*Methanococcus maripaludis* and *Methanococcus voltae*) and PibD (*Sulfolobus solfataricus*) cleave archaeal type IV pilin-like proteins. FlaK and PibD have conserved C-terminal Asp residues, suggesting that the archaeal peptidases may likewise

function as aspartyl proteases. FlaK peptidase activity was detected in *M. voltae* membrane fractions (109), and site-directed mutagenesis of the conserved C-terminal Asp residues abrogated peptidase activity (39). The recently solved FlaK crystal structure is proposed to show an inactive conformation, because the two Asp residues are relatively far apart (188).

P. aeruginosa PilD cleaves the leader peptide of PilA between Gly (−1) and Phe (+1) to form the mature pilin, and subsequently adds a single methyl group to the new N-terminal residue, using *S*-adenosylmethionine (AdoMet) as the methyl donor (37, 369). Other pilin-like proteins in the *P. aeruginosa* type IV pilus system (FimU, PilV, PilW, PilX, and PilE) and type II secretion system (XcpT, XcpU, XcpV, XcpW, and XcpX) are similarly cleaved by PilD, although subsequent methylation has been confirmed only for the minor pseudopilins (38, 154, 300, 372). The N-terminal processing of pilin subunits precedes and is independent of pilus assembly, as mutants lacking key components of the assembly machinery—such as the pilin polymerase, PilB—accumulate pools of processed subunits in the inner membrane. PilD has high specificity for particular residues within the leader peptide, as a complete loss of peptidase activity was observed when Gly (−1) was mutated, while mutation of Phe (+1) resulted in a reduced rate of proteolysis (369, 370). *In vitro* studies of the prepilin peptidase showed that peptidase and methylase activities are independent; methylation could be blocked without affecting peptidase activity, indicating that these processes occur at two distinct active sites (312, 372, 373). Site-directed mutagenesis of the two putative active sites demonstrated that while peptidase activity was essential, loss of *N*-methyltransferase activity did not affect surface piliation (312). Although methylation of type IV pilin proteins in T4P and T2S systems has been demonstrated in a number of species, this modification has not been reported for the Com or Flp pilins, archaeal flagellins, or pili. The FlaK and PibD peptidases are smaller by 54 to 60 residues than PilD, which may account for their lack of methylase activity.

In *A. actinomycetemcomitans* and *P. aeruginosa*, the *fppA* gene is located downstream of the *flp-tad-rcp* locus, whose gene products are responsible for assembly of Flp pili. At 160 amino acids, FppA is smaller than PilD (290 amino acids), with 4 predicted TM domains versus 6 for PilD (117). Sequence alignments of FppA and PilD showed that the active site Asp residues are conserved; however, FppA lacks the N-terminal domain containing the conserved Cys residues and the methyltransferase box found in PilD.

While cleavage of the type III signal is necessary for pilin function, it can also be used as a regulatory mechanism. *V. cholerae* can express 3 different kinds of type IV pili in different environmental niches: chitin-regulated pili, mannose-sensitive hemagglutinin (MSHA) T4a pili, and toxin-coregulated (Tcp) T4b pili. MSHA pili are important for persistence of *V. cholerae* in the environment as biofilms on zooplankton (96), while Tcp pili are involved in colonization of mammalian hosts. Hsiao et al. (187) found that the major subunit of MSHA, MshA, could be processed by the Tcp prepilin peptidase, TcpJ. However, this processing event led to degradation of MshA, preventing expression of MSHA and resulting clearance from the host, when the Tcp genes were activated by the regulator, ToxT, in response to host cues.

Because it is dispensable for fiber assembly, the specific role of pilin N-terminal methylation is so far unclear. It may enhance the stability of pilin subunits, extending their life span for recycling. Other possibilities include increased hydrophobicity of the N ter-
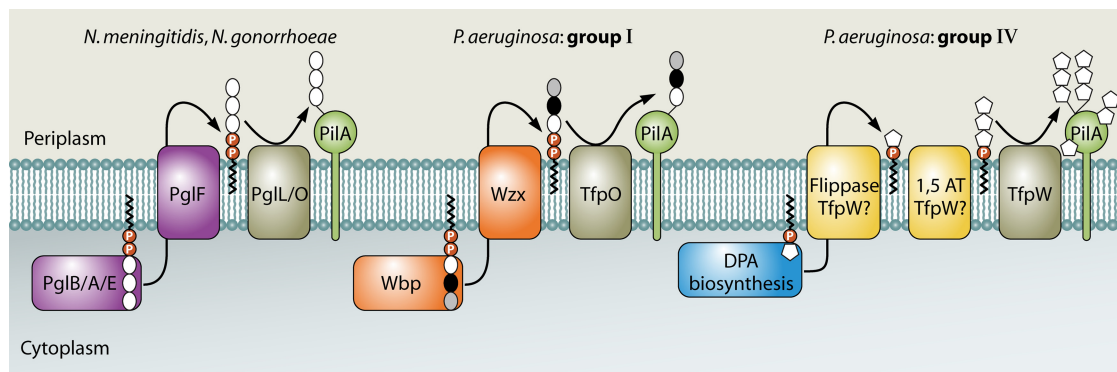
FIG 8 Type IV pilin glycosylation systems. Three different type IV pilin O-glycosylation systems have been characterized to date, one from *Neisseria* and two from *Pseudomonas aeruginosa*. The Pgl system (purple) from *Neisseria* generates undecaprenol-linked di- or trisaccharides via the action of cytoplasmic enzymes PglA, PglB, and PglE and translocates them via the activity of the flippase PglF (purple) to the periplasmic face of the membrane, where they are attached to Ser63 of PilE by one of two oligosaccharyltransferases, PglL or PglO (tan) (199, 256, 257). In *P. aeruginosa*, group I pilins are modified at the C-terminal Ser148 by TfpO (PilO) (tan) with an undecaprenol-linked O-antigen subunit generated by the Wbp enzymes of the lipopolysaccharide pathway (orange) and are flipped to the periplasmic face of the membrane by the O-unit flippase, Wzx (orange) (67, 87, 150). Group IV pilins are modified at multiple Ser and Thr residues by monomers, dimers, and longer polymers of D-arabinofuranose synthesized by cytoplasmic enzymes on a lipid carrier to form decaprenol-arabinofuranose (DPA) (blue), polymerized into α1,5-linked homopolymers, translocated to the periplasm by an unknown enzyme(s), and attached to the pilin by TfpW (tan) (241, 242, 405). TfpW is significantly larger than TfpO (694 versus 461 amino acids) and may be responsible for both the flippase and α1,5-arabinosyltransferase (AT) functions (yellow) in the group IV system.

minus, to allow subunits to be extracted more easily from the cytoplasmic membrane by the assembly machinery during fiber formation. Similarly, methylation may contribute to increased rates of depolymerization by increasing the propensity of the subunits to reinsert into the inner membrane. Interactions with the transmembrane domains of assembly proteins could be improved by methylation. Since the N termini of pilins are buried within the core of assembled fibers, methylation could contribute to the interactions that give assembled fibers their impressive capacity to resist disassembly under force.

In addition to methylation of the mature N terminus, C-terminal methylation of pilins was recently reported for the cyanobacterium *Synechocystis* (223). Using high-field Fourier transform ion cyclotron resonance (FT-ICR) MS—a form of MS that reveals small changes in mass in large proteins—a trimethylated Lys residue (versus a potentially acylated species of nearly identical mass) was identified at the C terminus of the major pilin subunit, PilA1. This unusual PTM was important for function and possibly stability of the subunits, as site-directed mutagenesis of the C-terminal Lys to Gln (K168Q) disrupted pilus-mediated gliding motility, reduced the intracellular levels of pilins, and resulted in the formation of fewer and significantly shorter pili on the cell surface than those of the wild type.

Production of Soluble Pilins

When pilus assembly is impaired, membrane-bound *N. gonorrhoeae* pilins can undergo an additional posttranslational cleavage event between residues 39 and 40 of the mature subunit to form shorter, soluble (S) pilins that are secreted (4, 160). Essentially, this cleavage event—carried out by an as yet unidentified protease—removes the hydrophobic N-terminal helix portion of the protein. Point mutations adjacent to the cleavage site prevented formation of S pilins and, depending on the nature of the mutation, blocked pilus assembly but did not affect competence for DNA transformation (4). A *Neisseria*-*Pseudomonas* chimera in which residues 37 to 43 from *P. aeruginosa* PilA replaced the cor-

responding residues in PilE was defective for assembly and competence, even though native *P. aeruginosa* pilins support competence in *Neisseria* (4, 5). The S pilins bind to human cells, but not animal cells, in a CD46-specific manner (341). Pilin glycosylation (below) was required for production of S pilins by *N. meningitidis*, but *N. gonorrhoeae* can produce nonglycosylated S pilins (although glycosylation increased the rate of S-pilin production) (268). The specific role that S pilins play in the pathogenesis of *Neisseria* disease is obscure at present, and truncated soluble forms of pilin have not been reported for other bacteria.

Glycosylation of Type IV Pilin Proteins

In addition to the variety in type IV pilin proteins arising from differences in their primary sequences, PTMs of the subunits with sugars or other moieties can increase antigenic and functional diversity. After processing of the N terminus, the most frequently identified PTM of type IV pilin proteins is O-glycosylation. Only T4a pilins have thus far been reported to have this modification (Fig. 8) (3, 73, 92, 136, 185, 186, 241, 318, 352, 405). Also, only major subunits are modified, although their relative abundance may simply make such modifications easier to detect. In addition to contributing to diversity, the presence or absence of glycosylation and the nature of the sugars that are attached to the subunit can modulate function. Kim and coworkers (222) inactivated a predicted acyl- or nucleotidyltransferase gene, *sll0899*, altering the *Synechocystis* O-linked pilin glycan. The *sll0899* mutant produced pilins of increased mass compared with the parent strain but was significantly impaired for motility, even though the amount of surface-recoverable protein was similar. Mass spectrometry analysis revealed that the mutant pilins were modified with glycans containing the deoxyhexose rhamnose in addition to the xylose and fucose residues found on wild-type pilins. The mechanism by which this change alters function is unknown, but it may affect retraction dynamics, interactions with the surface necessary for motility, or even interactions with minor components involved in adherence.

Neisseria Pilin Glycosylation

Type IV pilin glycosylation was first identified in *N. meningitidis*, when mass spectrometry analysis of the major pilin PilE revealed a covalent Gal(β1-4)-Gal(α1-3)-2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) trisaccharide modification (366). Soon after, the crystal structure of PilE from *N. gonorrhoeae* MS11 revealed covalent modification of Ser63 with Gal(α1-3) GlcNAc (306), with similar findings for *N. meningitidis* strain 8013SB (267). Pilins of *N. gonorrhoeae* strain N400 are modified with a hexose attached to 2,4-diacetamino-2,4,6-trideoxyhexose (HexDATDH) (173). Together, these studies suggested that more than one type of sugar could be attached to the pilins in *Neisseria*, but the glycan biosynthetic pathways, the mechanism by which glycans were added to PilE, and the extent of structural diversity among the sugars were unknown. Since those initial discoveries, T4P glycosylation has been identified in other bacterial species and in archaea.

Although S-pilin processing, glycosylation, and other posttranslational modifications have been studied extensively in *Neisseria*, the order in which these events happen is still being clarified. Long and colleagues put various forms of *pilE* (with and without a modifiable Ser63 residue) under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and followed pilus-related phenotypes across a range of induction levels (259). S pilins released into the supernatant showed the same distribution of glycosylated and nonglycosylated forms as full-length pilins. Together, these data suggest that glycosylation likely occurs before proteolysis.

Studies of both *N. gonorrhoeae* and *N. meningitidis* led to the identification of the *pgl* (pilin glycan) locus, which encodes synthesis of the pilin glycan, its translocation to the periplasmic face of the inner membrane, and its covalent attachment to the pilins (173, 203, 212, 316, 317) (Fig. 8). The Pgl proteins work in tandem to sequentially add sugars to an undecaprenol lipid carrier to form the glycan, which is subsequently flipped across the inner membrane and transferred to the periplasmic C-terminal domain of an individual pilin monomer (318). Changes in composition of the pilin glycan occur through phase variation of the gene encoding the O-oligosaccharyltransferase (*pglL* or *pglO*) and through specific alleles of the sugar biosynthesis genes. *Neisseria*'s glycosylation system is not specific to pilins, as it was recently shown to modify other surface proteins with the glycan derived from the Pgl pathway (240).

In addition to providing antigenic variation, the pilin glycan was recently shown to play a role in host cell invasion (202). In what has been called a "sticky and sweet" model (211), only glycosylated pili induce a high-affinity conformation of the I domain of the CR3 receptor on primary human cervical epithelial cells, which is normally present at low levels on uninfected cells.

Even though the minor pilins of *Neisseria* have not been reported to be glycosylated, they play an important role in control of other PTMs, as does the size of the glycan decorating the pilins (18). In particular, the abundance of the noncore minor pilin PilV controls the extent and type of alternate phospho-form modifications (see below) (1).

Pseudomonas Pilin Glycosylation

Two distinct pilin O-glycosylation systems have been identified in *P. aeruginosa*. In the first, glycosylation is mediated by TfpO (PilO), an O-oligosaccharyltransferase that (in another clever example of bacterial economy) adds an O-antigen unit synthesized by the LPS biosynthetic pathway (Fig. 8) to the C-terminal Ser of group I pilins (PilA$_I$) (84, 106, 185). *tfpO* mutants still express surface pili and exhibit twitching motility, suggesting that glycosylation is not necessary for function. Instead, this PTM was suggested to provide a pathogenic advantage, since *tfpO* mutants were outcompeted by their wild-type parent in an acute mouse pneumonia coinfection model (360).

TfpO is a large protein with multiple membrane-spanning domains and is encoded immediately downstream of the *pilA$_I$* gene. A stem-loop transcriptional terminator is present between the genes and likely functions to attenuate the level of *tfpO* expression from the strong *pilA* promoter (84). The very low abundance of TfpO required concentration of membranes before the protein could be detected by Western blotting (324). Together, these data suggest that the resulting stoichiometry would be such that a single TfpO enzyme could glycosylate multiple pilins. Transfer of the *pilA$_I$-tfpO* gene cassette to strains of other LPS serotypes resulted in modification of the pilins with the corresponding O-antigen units of the new host, showing that TfpO has broad glycan specificity (121). Since only group I pilins that end in Ser are glycosylated by TfpO, the properties of PilA$_I$ that make it suitable for modification were investigated. Only Thr could substitute for the C-terminal Ser, and compatibility of the surface charge of the pilin's D region with TfpO was important for modification (185). In a pilin with an incompatible D-region charge, extension of the length of the C-terminal tail improved accessibility of the target residue and led to robust glycosylation. It is interesting that Cys-to-Ala mutations of the group I pilin from *P. aeruginosa* 1244 did not block glycosylation of the protein, suggesting that either the conformation of the D region was not crucial for substrate recognition by TfpO or other interactions maintained the architecture needed for TfpO activity (185). Pilins were modified in a *pilD* mutant, showing that processing of the N terminus was not required for glycosylation and that glycosylation can precede processing.

The second pilin glycosylation system in *P. aeruginosa* was identified in group IV strains Pa5196 and PA7, which lack *tfpO* (Fig. 8) (241, 242, 405). An O-antigen mutant of strain Pa5196 expressed glycosylated pilins, suggesting that the pilin glycan was derived from another pathway (405). Mass spectrometry and nuclear magnetic resonance studies revealed that the major pilin PilA$_{IV}$ was modified at multiple Thr and Ser residues by monomers, dimers, trimers, and potentially longer homo-oligosaccharides of α-1,5-linked arabinofuranose, a glycan typically found as part of the cell wall polymers of mycobacteria. Further studies showed that TfpW, the large protein encoded downstream of PilA$_{IV}$, was required for pilin glycosylation (241). Mutants lacking TfpW produced unmodified pilins and few surface pili, suggesting that glycosylation of PilA$_{IV}$ is important for its assembly or stability. More recently, the D-Ara*f* biosynthetic pathway in *Pseudomonas* was defined by reconstitution of the pilin glycosylation system in a laboratory strain of *P. aeruginosa* (171). Based on the genes required, the sugar precursor is synthesized as an unusual polyprenyl-phosphate intermediate, similar to that in mycobacteria. D-Ara*f* biosynthetic mutants had the same phenotype as *tfpW* mutants, with unmodified pilins and few surface-exposed fibers even in retraction-deficient backgrounds, showing a profound assembly defect in the absence of glycosylation.

Since the Pa5196 assembly system is not specific for glycosylated pilins, the lack of glycosylation probably affects PilA$_{IV}$ conformation, making it incompetent for assembly in the absence of PTM. The inability of TfpW to glycosylate other *P. aeruginosa* pilins—even those with Ser or Thr at similar positions to those modified in PilA$_{IV}$—suggested that recognition of the atypical conformation of unmodified PilA$_{IV}$ could underlie TfpW's substrate specificity (171).

Glycosylation of Archaeal Flagellins and Pilins

Glycosylation of some archaeal flagellins and pilins has been reported. However, unlike the O-linked glycans identified on bacterial pilins, the glycans on archaeal subunits are N-linked. For *M. voltae*, glycosylation of 14 of 15 possible Asn residues on each of the four structural flagellin proteins (FlaA, FlaB1, B2, and B3) was reported (404). Mass spectrometry showed that a unique trisaccharide (β-Man*p*NAcA6Thr-(1-4)-β-Glc-*p*NAc3ACA-(1-3)-β-Glc*p*NAc) was linked to both flagellin and S-layer proteins, suggesting a common glycosylation system. Insertional inactivation of genes potentially involved in flagellin glycosylation showed two to be essential not only for modification but also for flagellin assembly (90). These genes, termed *agl* (for archaeal glycosylation) genes, encode a glycosyltransferase (AglA) with sequence similarity to the glycosyltransferases in *P. aeruginosa* and *Thermus* spp. and an oligosaccharyltransferase (AglB) similar to the STT3 homologue in *Campylobacter* spp. (PglB), responsible for transfer of glycans to a protein acceptor (90, 295, 382). Such similarities between the archaeal and bacterial systems suggest that archaea may have homologues of other members of the *pgl* locus. Some of the genes thought to be involved in glycan synthesis and translocation have been identified by bioinformatics and mutant analysis (89).

Other Forms of Pilin Posttranslational Modification

Besides glycosylation, modification of pilins with phosphate, phosphoethanolamine (PE), phosphocholine (PC), or phosphoglycerol (PG) has been reported, although thus far only for *Neisseria* species (143, 173). In the first example of such a modification, crystallographic analysis of the *N. gonorrhoeae* PilE protein revealed an electron density peak corresponding to a phosphate group at Ser68 (143).

In *N. gonorrhoeae*, the phase-variable PptA enzyme is responsible for transfer of both PC and PE to specific Ser residues of the pilin, PilE (1, 292). PptA is related to enzymes involved in PE modifications of LPS and is sufficient for PE but not PC transfer to PilE when expressed in a heterologous host, *P. aeruginosa*. It is possible that PE is converted to PC after attachment (292). As with the *Neisseria* pilin glycosylation system, the phospho-form modification system has additional periplasmic targets of unknown function. There is interplay between the glycosylation and phospho-form modification systems, as mutations that block glycosylation of substrate proteins or decrease the size of the glycan attached to the pilin at Ser63 result in PE/PC microheterogeneity (18). This change is separate from that mediated by altered PilV abundance, since *pilV* mutants exhibit no change in glycosylation.

Hegge et al. (173) suggested that *N. gonorrhoeae* modulates its pilin biochemistry through regulation of *pilV* levels. Wild-type strains have only PE modifications, while *pilV* null mutants have either PE or PC at Ser68 and Ser156 of PilE. Overexpression of PilV substantially reduced PE modification (173). Changing from a PE pilus to a PE/PC pilus could potentially modulate infection,

as PC can function as a receptor for both C-reactive protein and platelet activating factor (355).

An elegant study by Chamot-Rooke et al. (91) recently provided an interesting biological rationale for modification of *N. meningitidis* pilins with PG. Exposure of *N. meningitidis* to host cells upregulated the enzyme PptB, responsible for the addition of PG at Ser69 and Ser93. Modification at Ser69 was similar in the presence or absence of host cell contact, while the increase in PptB expression associated with host cell contact led to Ser93 being the primary site of modification. The PG modification reduced the pI of the pilins, with the negative charge on Ser93 specifically reducing interfiber interactions (bundling) normally mediated by a patch of 5 Lys residues in the same region. Bundling of *Neisseria* pili is an important facet of their function, enhancing bacterial aggregation and substantially increasing force generation compared to that produced by single fibers (47). Both S93A and *pptB* mutations increased pilus-mediated aggregation, while overexpression of PptB blocked aggregation in a manner that could be reversed by the S93A substitution. Together, these data show that PG modification of Ser93 is a primary driver of pilus disaggregation. PG modification did not affect initial adherence or microcolony formation but was important for subsequent detachment and transmigration of cells across epithelial monolayers, thus favoring bacterial dissemination and invasion of host cells.

CONCLUSIONS AND FUTURE DIRECTIONS

The incredibly broad distribution of type IV pilin proteins in eubacteria and archaea from every environment suggests that they are highly adaptable functional modules built from a common blueprint. Their signature domain, a highly conserved, multifunctional N-terminal alpha helix that contains the site for processing by dedicated bifunctional prepilin peptidases/N-methylases, makes them easy to identify, especially now that specific algorithms are available (195). The C-terminal domains of type IV pilin proteins exhibit wide sequence diversity, even in different strains of the same species, reflecting both their broad range of functions and evolutionary responses to environmental pressures.

These versatile molecular modules participate in a remarkable number of processes, including protein secretion, DNA uptake/competence, phage infection, adherence to both living and nonliving surfaces, twitching and swimming motility, and electron transport. It is highly likely that additional functions will be discovered in the coming years. Areas of research that need further study include the role of minor subunits in assembly and function of the fibers, how the subunits are recognized and polymerized/depolymerized by their respective assembly systems, how these proteins contribute to DNA uptake and thus horizontal gene transfer, how Flp pilins and their assembly systems differ in structure from the canonical T4a and T4b systems, and many more.

The expression of type IV pilin proteins by many pathogenic bacteria, as well as their potential utility as antivirulence drug targets and vaccine candidates, makes them interesting from a medical standpoint. Their small size and ability to mediate motility or transduce electrons highlight the potential for their exploitation for industrial purposes, including formation of nanotubes with emergent properties (25, 27), movement of nanoscale cargo (410), and powering of bacterial batteries (261). Their ability to tolerate bulky modifications such as sugars (55, 73, 131, 323, 405) or peptides (400) makes them amenable to synthetic biology approaches (272) for development of completely novel uses, once a compre-

hensive understanding of their functional logic is available. The use of filamentous phages that have T4P-like assembly systems, such as M13, has revolutionized many aspects of molecular biology (406), and the type IV pilins are equally suited to take us in new directions.

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