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Motility and adhesion through type IV pili in Gram-positive bacteria

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Abstract

Type IV pili are hair-like bacterial surface appendages that play a role in diverse processes such as cellular adhesion, colonization, twitching motility, biofilm formation, and horizontal gene transfer. These extracellular fibers are composed exclusively or primarily of many copies of one or more pilin proteins, tightly packed in a helix so that the highly hydrophobic amino-terminus of the pilin is buried in the pilus core. Type IV pili have been characterized extensively in Gram-negative bacteria, and recent advances in high-throughput genomic sequencing have revealed that they are also widespread in Gram-positive bacteria. Here, we review the current state of knowledge of type IV pilus systems in Gram-positive bacterial species and discuss them in the broader context of eubacterial type IV pili.

Introduction

Pili or fimbriae are hair-like appendages present on the surface of bacterial cells. They are universally oligomeric fibers composed of protein subunits called pilins. They can be assembled noncovalently through β-strand insertion (chaperone–usher pili or the recently characterized Bacteroidia FimA family) or through subunit interactions (curli or type IV pili) or covalently through sortase linkage (cell wall-linked pili) [1–4]. In type IV pili, the pilin subunits are arranged in a repeating helical pattern. Each pilin consists of an N-terminal helical domain composed primarily of hydrophobic amino acids (bearing a strong resemblance to a transmembrane helix) and a soluble ‘headgroup’ domain. In the assembled pilus, the subunits are arranged such that the hydrophobic N-termini are in the interior of the fiber with the soluble headgroups forming the exterior [5,6]. The noncovalent association of pilin N-termini provides the energy for pilus assembly and the soluble portions are monomeric [5].

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

Type IV pili serve a variety of functions including motility along solid surfaces [7,8], adhesion to eukaryotic host cells [9,10], microcolony/biofilm formation [11–13], and horizontal gene transfer [14]. All of these functions are dependent on one or more of three basic activities: (i) extension, lengthening the pilus through polymerization; (ii) adhesion, the ability of one or more pilus subunits to bind to target surfaces or biomolecules; and (iii) retraction, shortening the pilus through depolymerization. For example, twitching motility, across smooth, dry solid surfaces, involves all three — pili extend, bind weakly to the surface, and as the pilus is retracted, the bacterium is pulled toward the point of attachment.

Type IV pili in Gram-negative bacteria have been divided historically into two classes, types IVa and IVb. This classification is based on evolutionary relationships inferred from the length of an N-terminal signal peptide (the prepilin leader sequence), which is shorter for type IVa pilins, and the identity of the first residue of the mature protein, phenylalanine for type IVa with another hydrophobic residue for type IVb. Type IVa pilins are typically smaller than their type IVb counterparts, forming thinner pilus fibers, and there is less variation in genetic organization among the type IVa pilus systems than their type IVb counterparts [4]. Functionally, type IVa pili are frequently implicated in eukaryotic cell adhesion [9,10] and horizontal gene transfer [15] and less frequently in biofilm formation [11,12], whereas type IVb pili promote bacterial self-association (i.e. microcolony formation or auto-aggregation) [16,17].

Despite the evolutionary gulf between Gram-positive and Gram-negative bacteria, many of the components in the type IV pilus system appear to be conserved; the questions before us in this review are (i) what can we learn from the differences between type IV pili in Gram-positive and Gram-negative bacteria? and (ii) how are diverse functions accomplished through the flexible molecular architecture of type IV pili?

Type IV pilus biogenesis

While type IV pili are composed almost entirely of repeating units of a single protein, called either simply the pilin or the major pilin, other proteins, commonly referred to as minor pilins, can be incorporated into the pilus in smaller numbers. Additionally, there are several intracellular proteins required for pilus assembly, extension, and retraction that are typically the most conserved components of type IV pilus systems.

Pilin proteins

The three type IV pilus systems highlighted in the figures of this review were chosen as representatives of different classes of type IV pili; *Streptococcus pneumoniae* R6 contains a Com operon, a DNA-uptake system found in a wide range of Gram-positive and Gram-negative bacteria [18], *Clostridium difficile* R20291 produces classical type IV pili similar, in some respects, to those of type IVb pilus systems [19,20], and *Ruminococcus albus* 8 uses a largely uncharacterized and unusual pilus system to adhere to cellulose [21,22]. Also discussed here are unique type IV pilus systems from related bacterial species *Clostridium perfringens* and *Streptococcus sanguinis*.

Figure 1A shows the putative pilin genes for *C. difficile* R20291, *S. pneumoniae* R6, and *R. albus* 8. Each of these genes was identified based on three criteria: (i) the presence of a signal peptide, (ii) a recognition site for a prepilin peptidase, GFxxxE (see below), and (iii) a transmembrane-like α-helix in the predicted protein product. The known major pilin genes (*pilA1* for *C. difficile* and *R. albus*, *comGC* for *S. pneumoniae*; colored orange) are the closest putative pilin genes to their common promoters, consistent with their higher expression levels. Both *C. difficile* and *C. perfringens* contain multiple clusters of type IV pilin genes as well as multiple copies of genes encoding pilus biogenesis proteins PilB, PilC, and PilM. The presence of multiple *pilB* genes in particular implies that *Clostridia* produce more than one type IV pilus or homologous secretion system, as is the case for several strains of *Escherichia coli* as well as *S. sanguinis* [23–25]. For *C. difficile*, only those genes predicted to be involved in the production of PilA1 pili are included in Figure 1.

The genes colored blue (*pilK* for *C. difficile* and *comGG* for *S. pneumoniae*) likely encode initiator pilins that form the template upon which polymerization begins. These proteins lack a conserved glutamate residue at position 5 of the mature protein, which is thought to form a salt-bridge with the N-terminus of the previously incorporated pilin (and hence is not required for the first subunit) [26], and are much larger than their cognate major pilin proteins. Proteins with these two features can be found in most type IV pilus systems and appear analogous to GspK of the *E. coli* type II secretion system [27]. EGC03637.1 from *R. albus* 8 and BAB81985.1 from *C. perfringens* str. 13 are similarly large and may represent equivalent proteins.

The role of the other genes encoding putative pilins, depicted in gray in Figure 1A, remains to be determined, but we expect that they can be conceptually divided into two classes: those which form a complex with the initiator pilin before the major pilin is polymerized and hence are found primarily at the tip of the pilus and those which are incorporated sporadically throughout the pilus in place of one of the major pilin subunits. The latter category, which we refer to as ‘intercalated pilins’ in Figure 1C, is only sparsely characterized at present and presumably provides no benefit in terms of stabilization; one possible function then is to provide additional adhesive activities to pili into which they are incorporated.

Prepilin processing

Any protein that can be incorporated into a type IV pilus is first incorporated into the plasma membrane by the hydrophobic α-helix conserved among all pilins. This insertion is dependent on a signal peptide at the N-terminus [28]. However, before extraction from the membrane and insertion into the pilus, the signal peptide must be cleaved by a prepilin peptidase (Figure 1C). Although poorly conserved, prepilin peptidase genes can be identified clearly in the genomes of *C. difficile* R20291, *S. pneumoniae* R6, and *R. albus* 8 as shown in Figure 1B, as well as in *C. perfringens* str 13 (WP_043013013.1) and *S. sanguinis* 2908 (CEL91498.1). Notably, all strains of *C. difficile* contain two genes encoding putative prepilin peptidases, one of which, *pilD2*, is similar to the *pilD* gene of *C. perfringens* with the other, *pilD1* (which is listed in Figure 1B), being more similar to the *pilD* genes of Gram-negative bacteria [23].

Pilus extension and retraction

All type IV pilus and type II secretion systems utilize cytoplasmic hexameric AAA+ family ATPases to extend the pilus or pseudopilus, respectively. These proteins are universally required for pilus biogenesis and easily identifiable by their sequence similarity. They are typically denoted as PilB (type IV pili), GspE (type II secretion), or ComGA (competence pili). WP_011010862.1 of *C. perfringens* and CEL91506.1 of *S. sanguinis* are the equivalents to those PilB proteins listed in Figure 1C. Additionally, an integral membrane protein is also required, typically called PilC, GspF, or ComGB (WP_003451114.1 in *C. perfringens* and CEL91511.1 in *S. san-guinis*). How the extension ATPase and the integral membrane protein interact to extend the pilus beyond the plasma membrane remains an area of intense investigation.

In addition to the extension ATPases, many but not all type IV pilus systems include additional related hexameric ATPases (commonly denoted PilT and/or PilU) that are not required for pilus biogenesis and extension but rather for retraction [29]. Correspondingly, Δ*pilT* mutants are typically hyperpilated as the pili extend but do not retract [30]. Putative retraction ATPases can be found in *C. perfringens* (WP_003451114.1), *C. difficile*, *R. albus*, and *S. sanguinis* (CEL91511.1) but not in *S. pneumoniae* (Figure 1B). Competence pili [sometimes referred to as type IV filaments rather than type IV pili] in other organisms also lack *pilT* homologs raising the obvious question of how DNA bound by these appendages is taken up by the cell. However, we note that some bacteria without *pilT* genes are nevertheless capable of retracting their pili [31,32]. The mechanism for this retraction remains uncharacterized but given the homology between PilB and PilT, it is not unreasonable to propose that an ancestral ATPase was capable of facilitating both extension and retraction, and that some AAA+ ATPases retain the ability to do so.

Known functions of type IV pili

The basic architecture of type IV pili is used for a wide variety of functions including secretion [33], DNA uptake [34], surface motility [14], eukaryotic cell adhesion [10], microcolony formation [35], and even electrical conductance [36] and, in archaea, flagellar motility [37]. We expect, therefore, that in Gram-positive bacteria, the functions of type IV pili will be similarly broad. To date, we can divide the known functions into two classes, adhesion and motility.

Adhesion

With the possible exception of the electrically conductive nanowires of Geobacteraceae, all type IV pili have some adhesive activity. Each of the bacterial species discussed here utilizes type IV pili for adhesion in a different fashion; for bacterial self-association, adherence to abiotic surfaces or DNA-binding (shown in Figure 2).

The formation of microcolonies through bacterial self-association, as a prerequisite for the formation of biofilm or other bacterial aggregates, is a well-characterized function of type IVb pili in enteropathogenic *E. coli*, enterotoxigenic *E. coli*, and *Vibrio cholerae* [17,32,38]. The type IVa pili of *Pseudomonas aeruginosa* have also been suggested to play a role in

biofilm formation by promoting the initial attachment of bacteria [39], and the type IVa pili of *Neisseria gonorrhoeae* have been shown to form microcolonies on host cell surfaces in a type IV pilus-dependent manner [40,41].

Varga and colleagues demonstrated that type IV pili were required for native levels of biofilm formation by *C. perfringens* and more recently, the type IV pili of *C. difficile* have been also been shown to mediate bacterial autoaggregation in static liquid cultures and microcolony formation as part of *in vitro* biofilm growth [42–45]. The potential ability of *C. difficile* to form biofilms *in vivo* may explain the high rate of recurrence of *C. difficile* infections as *in vivo* biofilms have been shown to form reservoirs of drug-resistant bacteria that can subsequently expand [46]. The mechanism by which an increase in biofilm occurs remains unclear, but two possibilities are shown in Figure 2A: (i) in the cell-attachment model, type IV pili bind to bacterial surface proteins to form a web that prevents cells from moving independently and (ii) in the pilus bundling model, individual type IV pili from neighboring cells interact with each other from bundles that tie their respective cells together. Additionally, the major pilin of the *C. perfringens* type IV pili, PilA2, has been shown to mediate adhesion to host eukaryotic cells when expressed by *N. gonorrhoeae* [47], raising the possibility that the same is true for other infectious clostridial species, including *C. difficile*, *C. tetani*, and *C. botulinum*.

Type IV pili mediate adhesion to abiotic surfaces both in a semi-permanent manner [48] and transiently as part of twitching motility (see below). Rakotoarivonina et al. [21] demonstrated that in *R. albus*, type IV pili directly mediate the attachment to crystalline cellulose (Figure 2D). It remains unclear whether the major pilin subunit (PilA1 or GP25) is the adhesin, but immunogold microscopy demonstrated that cellulose was able to bind along the entire length of the pilus rather than simply at the tip.

Finally, the type IV pili of *S. pneumoniae* have been shown to directly bind DNA [49] (Figure 2B). Type IV pili are also required for DNA uptake in many Gram-negative bacteria, and in the case of *Neisseria meningitidis*, a minor pilin, ComP, has been shown to bind DNA directly [50,51]. It remains unclear whether the mechanism of DNA uptake is the same for type IVa pili in *N. meningitidis* and *Acinetobacter* as it is for the Gram-positive competence pili of *S. pneumoniae* and *Bacillus subtilis*. Notably, the retraction ATPase PilT (which has no homolog in *S. pneumoniae*) is required for DNA uptake by *Acinetobacter baumannii* [14,34].

Motility

In the two most widely studied Gram-positive type IV pilus systems, those of *C. difficile* and *C. perfringens*, mutants lacking type IV pili have been shown to be deficient in some form of surface motility. Purcell et al. [44] demonstrated that type IV pili mediate twitching motility in *C. difficile* in a manner dependent on the extension ATPase PilB1; particularly on agar firmer than that typically used for twitching motility assays (1.8% vs. 1.0%; Figure 2C). Varga et al. [52] demonstrated that in *C. perfringens*, gliding motility (which occurs at an agar–air interface rather than an agar/plastic interface) is dependent on type IV pili with Δ*pilC* and Δ*pilT* mutants being nonmotile.

Like *S. pneumoniae*, *S. sanguinis* is naturally competent and contains a homologous type IV pilus system (see above). In addition, *S. sanguinis* has recently been shown to produce a more conventional type IV pilus system, capable of twitching motility [24,53]. In particular, Gurung et al. note that while Δ*pilT* mutants are nonmotile, the deletion of a gene of unknown function, *pilK*, results in cells that are pilated and motile but move only perpendicularly to the long axis of the bacterial chains (rather than along it in a 'train-like' motion as the wild-type cells do). Unusually, two major pilin proteins are expressed, PilE1 and PilE2; it remains unclear whether these proteins form separate homopolymers or are combined into a single heteropolymer.

Type IV pilin structure

There are many high-resolution structures available for the soluble portion of both major and minor type IV pilins from Gram-negative bacteria. Recently, the structures of two pilin proteins from *C. difficile* have been resolved [19,20], allowing us to compare type IV pilins from a Gram-positive bacterium with their counterparts from Gram-negative species.

With a single exception of the only available structure of an initiator pilin [31], all known high-resolution structures of the soluble portion of type IV pilins from Gram-negative bacteria are homologous. They show a single domain consisting of a β-sheet sandwiched against an α-helix with a loop of varying length (the αβ loop) connecting the two. The type IV pilins from Gram-negative bacteria also universally contain disulfide bonds, commonly, though not exclusively, at the C-terminus [54–56]; the loop bounded by a C-terminal disulfide bond in a type IV pilin is termed the D-loop.

The structure of PilA1, the *C. difficile* major type IV pilin, shows a similar overall fold in spite of its lack of sequence similarity (the closest known homolog, TcpA from *V. cholerae*, has a sequence identity of 14%). Notably, PilA1 lacks cysteine residues and, in the place of the C-terminal disulfide bond typically found in type IV pilins, PilA1 from *C. difficile* R20291 and NAP08 strains form an antiparallel β-sheet, which we hypothesize increases stability in a similar manner (Figure 3A). However, a recently discovered divergent strain, *C. difficile* CD160, exhibits an alternate conformation for one of the β-strands, preventing it from forming a β-sheet. Instead, a network of water-mediated hydrogen bonds between the two strands takes the place of the direct hydrogen bonds found in the R20291/NAP08 β-sheet (Figure 3B). These novel stabilization strategies suggest that type IV pilins from Gram-positive organisms may show a greater variability in their C-terminal structures than those from Gram-negative bacteria.

In contrast with the conservation between PilA1 and TcpA, the fold of the minor pilin PilJ is markedly distinct, containing two domains. The first domain contains the typical pilin α-helix at the N-terminus, with a very helical αβ-loop followed by a two-stranded β-sheet (pilins and pseudopilins typically have at least three strands in their central β-sheet). A loop then leads into the second domain that has another helix at a ~70° angle to the N-terminal domain α-helix, followed by a five-stranded β-sheet (Figure 3C). PilJ is the first pilin shown to exhibit this two-domain fold, although there are many similarly sized pilins including CPE2280 from *C. perfringens*, implying that it may be the first example of a broader class of

similar minor pilins. This unique combination of two pilin-like folds (Figure 3D) suggests a gene-duplication event, although there is no sequence similarity between the N- and C-terminal domains [19]. Although the function of PilJ remains to be determined, the presence of a second pilin domain that is superfluous for pilus formation strongly implies that it serves an adhesive function, and that this dual-pilin fold is a mechanism by which novel adhesive molecules can be introduced into type IV pili.

Conclusion

Despite the ~2 billion years of evolution that separate Gram-negative and Gram-positive bacteria, the structure of the type IV pilus is remarkably well conserved. The strength of the type IV pilus architecture rests on its stability, ease of extension and retraction, and perhaps most importantly, ability to incorporate multiple subunits, which allow a single appendage to perform such distinct functions. However, despite nearly 40 years of study, basic mechanisms of pilus biogenesis and DNA uptake remain mysterious. The study of type IV pili in Gram-positive bacteria provides the opportunity to examine the structure and function of type IV pili in a new context, the potential to shed light on fundamental mechanisms conserved throughout eubacteria, and the possibility of structure-based rational design of novel narrow-spectrum drugs targeting Gram-positive pathogens.

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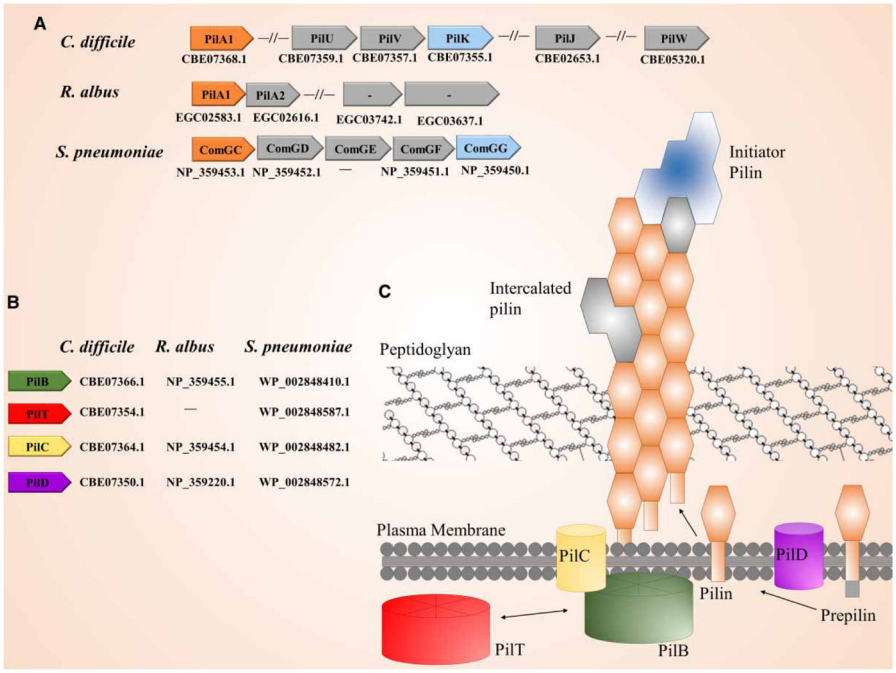

Figure 1. Components of type IV pilus systems

(**A**) Putative pilin genes; major pilins are colored orange, initiator pilins in blue, and all others in gray. Below each gene is the NCBI accession number for the protein it encodes. (**B**) Type IV pilus biogenesis proteins; accession numbers are listed for PilB, PilT, PilC, and PilD homologs for each species. (**C**) Type IV pilus biogenesis in Gram-positive bacteria; a schematic showing the proposed organization of type IV pilus proteins in Gram-positive bacteria.

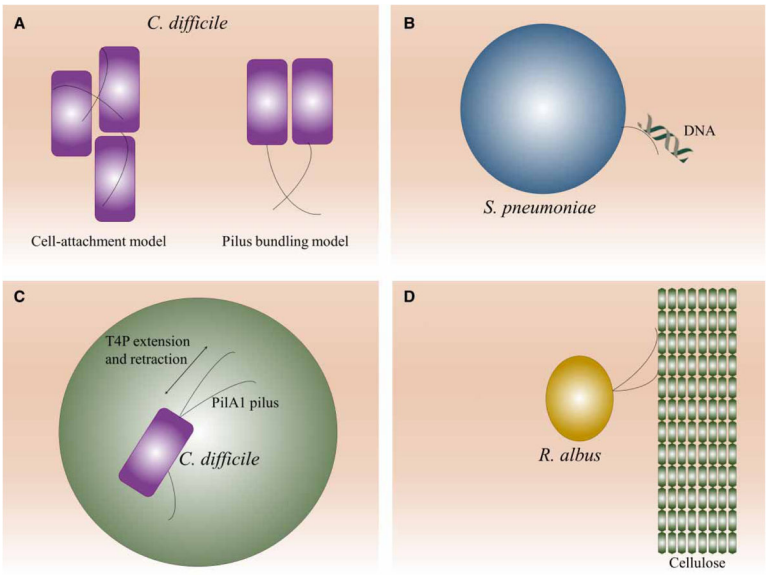

Figure 2. Functions of type IV pili in Gram-positive bacteria

(**A**) Proposed mechanisms for bacterial self-association through *C.* difficile type IV pili. (**B**) DNA-binding by *S. pneumoniae* type IV pili. (**C**) Twitching motility in *C.* difficile. (**D**) Adhesion to crystalline cellulose by the type IV pili of *R. albus*.

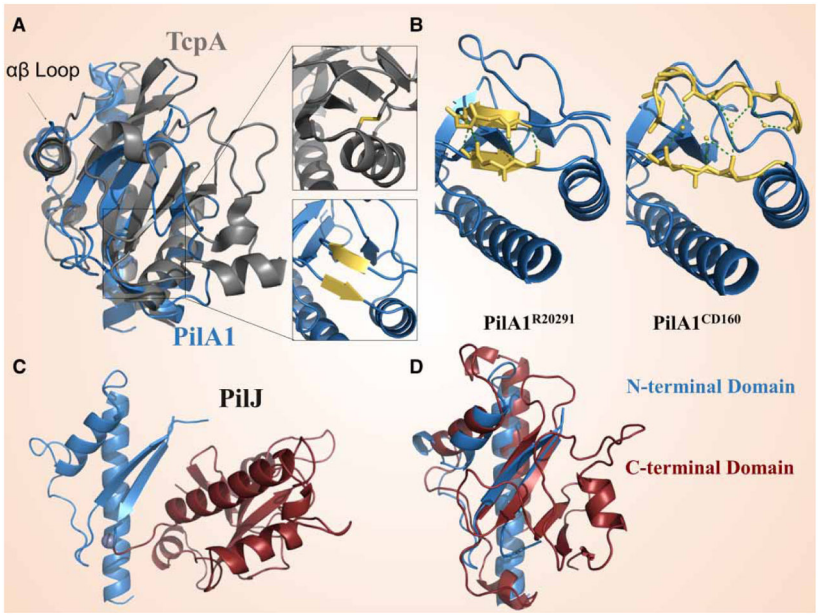

Figure 3. Structures of type IV pilins from Gram-positive bacteria

(**A**) Superimposition of *C.* difficile R20291 PilA1 (blue) with *V. cholerae* TcpA (gray), panels show the C-terminal disulfide bond of TcpA and β-sheet of PilA1. (**B**) Comparison of the PilA1$^{R20291/NAP08}$ β-sheet with the equivalent region of PilA1^{CD160}.

(**C**) Structure of *C.* difficile PilJ with the N-terminal domain in blue and the C-terminal domain in red and the zinc-binding site.

(**D**) Superimposition of the two PilJ pilin-like domains.