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A tale of two pili: assembly and function of pili in bacteria

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Abstract

Bacterial pili have long been recognized as mediators of initial host-pathogen interactions important for the progression of Gram-negative bacterial diseases. An appreciation for the role of pili on Gram-positive bacteria in virulence, as well as the unique properties of their biogenesis is a rapidly emerging area of research. In this review, we focus on recent advances in one of the longest-studied Gram-negative pilus systems, the chaperone/usher assembled pili, along with the newcomer to the field, the sortase-assembled pili of Gram-positive bacteria. In both systems, a wealth of new structural and molecular details has emerged recently. In light of this, we explore similarities between chaperone/usher and sortase-assembled pilus biogenesis and highlight paradigms unique to each, with an eye toward using knowledge of each system to raise new questions and inform future studies of the other.

Pili in bacteria

Bacterial pili are defined as non-flagellar, proteinaceous, multi-subunit surface appendages involved in adhesion to other bacteria, host cells, or environmental surfaces [1, 2]. Pili were first recognized by electron microscopy on Gram-negative bacteria more than 50 years ago [3] and on Gram-positive bacteria 40 years ago [4]. Since that time pili have been implicated in critical host-pathogen interactions, colonization, tropism determination, biofilm formation, invasion, and signaling events [5, 6]. Non-flagellar organelles of Gram-negative bacteria include the well-characterized chaperone/usher-assembled pili, type IV pili, and curli. Gram-positive bacteria express pili assembled by sortase enzymes as well as type IV pili similar to those of Gramnegative organisms. In each of these systems, bacteria have evolved mechanisms to efficiently assemble highly stable fibrillar structures on their surface while preventing the aggregation and/or premature assembly of highly interactive subunits within the cell interior. In this review we focus on one of the longest studied and best understood pilus systems, the chaperone/usher assembled pili of Gram-negative bacteria, along with the newest pilus system to be described, the sortase-assembled pili of Gram-positive bacteria. The diversity of chaperone/usher pathways across a number of bacteria provides an interesting parallel to the diversity of sortase-assembled pili now characterized. Both pilus pathways are pathogenic determinants for a variety of organisms and are proving to be promising targets for anti-virulence therapeutics [7, 8]. Thus, we highlight recent advances in understanding of the pilus structure and biogenesis of both pilus types and we stress paradigms that are similar between them, with the goal of highlighting how increased understanding of one system might shed light on the other (Table 1).

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Chaperone/usher pili

In Gram-negative bacteria, the chaperone/usher (CU) pathway includes S and Dr pili, and is exemplified by the type 1 and Pap (P) pilus systems of uropathogenic *Escherichia coli* (UPEC) [9]. Although a single *E. coli* genome can encode up to 12 CU pili [10], the assembly machinery and fiber components are encoded by gene clusters distinct for each type of CU pilus, which contain the pilin subunits, the adhesin, and the dedicated chaperone and usher (Figure 1). We focus on P and type 1 pili as model CU pili in this review, based on the wealth of structural and molecular information available for each. The CU molecular machine functions to regulate the ordered secretion, folding and assembly of tens of thousands of pilin subunits into pilus fibers on the surface of the bacterium.

Chaperone-mediated donor strand complementation

Newly synthesized pilin subunits are translocated across the cytoplasmic membrane via the sec translocase where they form complexes with periplasmic chaperone proteins (PapD for P pili, FimC for type 1 pili; Figure 1). Pilus chaperones consist of two immunoglobulin-like (Ig-like) domains stabilized by a conserved salt bridge. This arrangement was first seen in the crystal structure of the prototype chaperone PapD [11] and later also shown for other chaperones including FimC [12, 13]. Each subunit of CU pili consists of an incomplete Ig-like fold and an N-terminal extension (Nte). Periplasmic chaperones for P and type 1 pili couple subunit folding with the capping of the interactive subunit assembly sites in a process termed donor strand complementation (DSC) [14–16]. In DSC, the G1 beta strand lining the interdomain cleft of the chaperone transiently serves as the C-terminal beta strand of the subunit, thus providing the steric information necessary for folding by completing the Ig-like fold of the subunit (Figure 2a).

Donor strand exchange

Once formed, each chaperone-subunit complex is targeted to the outer membrane usher (PapC for P pili, FimD for type 1 pili; Figure 1) where the chaperone-pilin complex then dissociates by an unknown mechanism and the subunit is added to the base of the growing pilus. The order of subunit assembly is based on affinity for the usher and for each other [17–20]. In the case of P pili, PapD-PapG (chaperone-adhesin) complexes bind first to empty PapC sites, followed by PapD-PapF (chaperone-minor pilin subunit) binding, which initiates tip assembly (Figure 1). The chaperone is then released from the PapD-PapG complex, as the Nte of PapF is zippered into the newly opened groove of PapG to complete the Ig-like fold of the PapG pilin domain (Figure 2a). This process is termed donor strand exchange (DSE) (Figure 2b), and results in a head to tail linear array of non-covalently assembled pilin subunits. The joining of PapG to PapF is then followed by multiple rounds of PapD-PapE (chaperone-minor pilin subunit) binding and DSE reactions to form the bulk of the pilus tip. The binding and incorporation of PapK terminates the addition of PapE subunits to the growing fibrillum and nucleates the incorporation of PapA major subunits into the fiber. Since PapH is unable to undergo DSE, pilus growth is terminated when PapH is incorporated at the base of the fiber because it lacks the open groove that would be necessary to join to any additional subunits to it [21, 22]. It has also been proposed that PapH mediates membrane attachment of the pilus fiber, although the mechanism for this is unknown [21, 22].

Usher-catalyzed assembly across the outer membrane

The PapC and FimD usher proteins serve as assembly sites and pores in the outer membrane allowing export of the growing fiber to the surface of the cell [23]. The N-terminal domain of the usher has been implicated in the initial binding of the chaperone-subunit complexes in the periplasm [24]. Once bound by initiating chaperone-subunit complexes, ushers undergo

a conformational change, which is then maintained throughout fiber assembly [20]. Recently solved structures for the N-terminal domain of the usher FimD with a complex of the FimC chaperone and FimH adhesin pilin domain, as well as a FimD-FimC-FimF complex gave insight into usher surfaces responsible for binding to the chaperone-subunit complexes [25, 26]. A new structure of the central portion of PapC revealed that the usher is a dimer, in which each PapC monomer forms a 24 strand β barrel with a central channel able to export fully folded polymerized pilin subunits [27]. Cryo-electron microscopy (EM) studies showed that only one of the PapC dimer pores is used for extrusion of the assembled pilin subunits [27]. In the absence of subunits in the crystal structure, the pore in each monomer is blocked by a plug domain, which originates from within the PapC barrel. The plug is stabilized by interactions within the barrel and it is proposed that the conformational change that occurs upon binding of initiator chaperone-subunit complexes results in destabilization of those interactions and displacement of the plug, such that the polymerized subunits can transit the channel. It is also proposed that the dual usher structure facilitates ordered stepwise interaction and handoff of each subunit-chaperone complex at the periplasmic face of the usher during pilus biogenesis.

The process of polymerization and extrusion of the subunits through the outer membrane does not require energy input [28], rather it is likely driven by the energy stored in the 'primed' chaperone-subunit complexes [29–32]. Upon DSE, topological rearrangements of the subunit and condensation of the hydrophobic core form a polymer held together by non-covalent interactions, which are stronger than those in the stable chaperone-subunit complexes. In addition, once on the surface of the cell, the PapA pilus fiber packs into a tight right-handed helical cylinder, which is thought to provide additional force for outward growth of the organelle [23].

Chaperone/usher pilus adhesins

Bacterial attachment is a key event in the colonization of mucosal surfaces. CU pili contain adhesins that mediate attachment to a specific receptor. While the pilin subunits are single domain proteins, tip adhesins are composed of two domains. The C-terminal domain of tip adhesins is a typical Ig-like pilin domain that participates in DSE to join the adhesin to the tip of the pilus [12]. Crystal structures for the PapG, FimH and other CU adhesins show that each N-terminal ligand-binding domain also shares a similar β barrel jellyroll fold [12, 33–36]. However, each ligand-binding domain is variable with respect to its length and molecular organization. In addition, each adhesin uses a different surface to bind to its cognate ligand. The P pilus tip adhesin PapG binds to Galα1–4Gal-containing glycolipids [37] and is required for UPEC to cause pyelonephritis [38]. The PapG ligand site is on the long side of the ligand-binding domain (Figure 2c), but is made up of residues that are distal to the core Ig-like domain shared with the other receptor binding domains [33]. Type 1 pili contain the FimH adhesin at their tip which makes critical host-pathogen interactions necessary for UPEC pathogenesis [39–42]. FimH binds to mannosylated receptors on the surface of the bladder epithelium and the ligand site is located at the distal tip of the ligand-binding domain [34] (Figure 2d). Mannose is bound in a deep negatively charged pocket at the tip of the receptor-binding domain, which consists of an elongated eleven-stranded β barrel. Mutation of FimH residues essential for mannose-binding such as Q133 [34] results in attenuated virulence in a murine urinary tract infection model [43]. Scanning and high-resolution EM of UPEC in a mouse cystitis model revealed that adhesive type 1 piliated bacteria are able to interact with bladder superficial facet cells leading to binding, invasion, and formation of intracellular bacterial communities with biofilm properties [39, 44]. This process is dependent on the FimH adhesin and allows UPEC to gain a foothold in the acute stages of infection. Thus, these binding domains serve as the functional end of the pilus, providing specific affinity for host ligands and influencing bacterial tropism for host tissues.

Sortase-assembled pili

Pili in Gram-positive bacteria are composed of a single major pilin subunit and usually 1–2 accessory subunits, the genes for which are all clustered in genetic loci. Pilin loci always contain a sortase gene (or genes), which encodes the enzyme that catalyzes the polymerization of these sortase-assembled (SA) pili. Several Gram-positive organisms such as *Corynebacterium diphtheriae* and *Enterococcus faecium* encode more than one pilin locus [45, 46], although genes of each locus encode components specific for the assembly of a unique pilus type [47].

Accessory factors for SA pili

Gram-positive organisms lack a membrane-bound periplasm but, nevertheless, secrete many virulence factors that require post-secretion modification [48]. It has been proposed that the space between the cell membrane and cell wall in Gram-positive bacteria provides a protected environment for folding and processing of secreted proteins [49–52], raising the possibility of a role for secreted chaperones in pilus biogenesis. In fact, in addition to pilin- and sortase-encoding genes, some pilus gene clusters of *Streptococcus agalactiae*, *Streptococcus suis, Streptococcus pneumoniae*, and *Streptococcus pyogenes* are also genetically linked to type I signal peptidase homologues. The pilin signal peptidase homologue in *S. pyogenes* lacks canonical signal peptidase active site residues and therefore does not act as a signal peptidase. Instead, it has been proposed to act as a chaperone to prevent premature subunit interactions during biogenesis, analogous to the chaperone activity required in CU pilus biogenesis, since it is required for pilus fiber formation and interaction with the major pilin subunit [53, 54]. However, the precise role for pilin locus-associated signal peptidase homologues in fiber formation remains to be determined.

Sortase-mediated pilus assembly

Each pilin locus contains at least one associated Class C sortase enzyme, termed the pilin sortase, which mediates pilus fiber formation. In addition, nearly every sequenced Gram-positive bacterial species encodes a Class A sortase, also called the housekeeping sortase. Sortases are transpeptidases that recognize and cleave the threonine-glycine bond within an LPXTG (or LPXTG-like) motif found within a C-terminal cell wall sorting signal (CWSS) of some secreted proteins, leading to the covalent linkage of proteins to the cell wall (by Class A housekeeping sortases) or to one another (by Class C pilin sortases) [55]. The paradigmatic Gram-positive pilus system for which we have the most information is in *C. diphtheriae* (see review by Ref. [56]). Studies in *C. diphtheriae* and *S. pyogenes* have demonstrated that sortase-mediated attachment of pilin subunits to one another during pilus biogenesis proceeds via isopeptide bond formation between the threonine and glycine of the cleaved LPXTG (or LPXTG-like) motif in one pilus subunit and a conserved lysine present within the neighboring pilin subunit [46, 57–59]. Sequential transpeptidation reactions result in a pilus fiber consisting of a linear array of pilin molecules, the bulk of which is composed of the major pilin subunit, as shown by immunogold-EM studies [46]. These pili range from 0.3–3 μm wide and can be as long as 10 μm [60]. The major pilin subunit is essential for pilus fiber formation [46, 61, 62]. In part, pilus length is determined by availability of the major pilus subunit, since overexpression leads to longer pili [46, 61, 63]. In comparison, minor subunits are generally dispensable for pilus formation and are typically incorporated into the pilus fiber in characteristic spatial locations along the pilus fiber: at the base, at the tip, or in patches along the length of the pilus as shown by immunogold-EM (Figure 1). The molecular details required for minor pilus subunit incorporation were first assessed in detail for *C. diphtheriae*. In this system, fiber incorporation of the minor tip-associated pilus subunit SpaC required the lysine of the major subunit's YPKN pilin motif, whereas incorporation of the SpaB minor subunit instead utilizes a glutamic acid within the E-box

conserved element in the major SpaA subunit [46, 58]. The presence of pilin motifs and E-boxes in many other Gram-positive pilin sequences suggests that there may be conserved elements for minor subunit incorporation; indeed the YPKN motif is also required for BcpA pilus polymerization in *Bacillus cereus*[64]. However, not all Gram-positive pilus systems studied thus far strictly adhere to these motif requirements. Pilus polymerization in *S. pyogenes* requires a non-canonical QVPTG motif within the CWSS of the major pilin subunit, as well as a lysine residue that is not associated with a canonical YPKN-type pilin motif. A similar non-canonical VPPTG motif within the *S. pyogenes* minor Cpa subunit is required, along with the conserved lysine of the major subunit for Cpa incorporation into the pilus [65, 66]. Further study of the sequence elements and mechanism(s) for minor pilin subunit incorporation, the basis for pilin sortase specificity, and the functional consequences of the localization pattern of subunit incorporation on individual pili will greatly increase our overall understanding of pilus biogenesis in Gram-positive bacteria.

Pilus structure of SA pili

Structural insights into the assembly of SA pili first emerged from crystallization of the minor pilin adhesive subunit from *Streptococcus agalactiae* and major pilin subunit from *S. pyogenes*, revealing a two-domain organization in each consisting of a modified Ig fold, similar to the Ig-like fold for CU pilin subunits [57, 67] (Figure 3a). An intermolecular isopeptide bond between the major pilin subunits of *S. pyogenes* formed between the invariant lysine of the pilin motif of one molecule with the threonine of the LPXTG in the next pilin molecule. Furthermore, two potentially self-generated intramolecular lysine-asparagine bonds were revealed within each subunit (Figure 3a). These covalent linkages, along with intermolecular bonding, provide a basis for the structural integrity necessary to maintain such long thin pili [57]. Based on sequence comparison, Kang *et al.* predicted the presence of intramolecular bonds in other Gram-positive pilin proteins [57]. Indeed, intramolecular amide bonds were recently demonstrated within the *B. cereus* BcpA major pilus subunit, one of which contributes to pilus fiber formation [68], as well as within the major SpaA pilin subunit of *C. diphtheriae*[59]. SpaA pilins also contain an additional intramolecular disulfide bond (Figure 3b), which is surprising since other pilin proteins examined lack cysteines and it was thought that Gram-positive bacteria lack disulfide bond-forming machinery [59, 69].

The C-terminal pilin domain revealed in the X-ray crystal structure of the *S. agalactiae* minor pilin subunit mediates binding to host cells and the N-terminal domain is hypothesized to confer structural attributes, analogous to the two-domain makeup of the CU pilus tip adhesins [67]. Indeed, minor subunits in *C. diphtheriae* and *S. pneumoniae* are implicated in adhesion to host cells [70, 71]. In contrast to the well-characterized CU pilus adhesins, a molecular receptor has been identified for only one SA pilus: the *S. pyogenes* major pilin protein Spy0128 specifically interacts with the soluble scavenger receptor gp340 [72]. No receptors have been identified for SA pilus minor pilin subunits, although many contain MSCRAMM motifs predicted to mediate attachment to extracellular matrix molecules.

Sortase requirements for pilus growth

Pilus loci can encode 1–3 pilin sortases. In organisms bearing only one pilus sortase, the pilus-associated sortase is essential for fiber formation and incorporation of all minor subunits [46, 61, 62, 73]. Studies in organisms with pilus loci encoding more than one sortase have shown both functional specificity and redundancy among the enzymes. For instance, in a pilus locus in *S. agalactiae* (Group B Streptococcus, GBS) which contains two pilin sortases, each sortase was required for the incorporation of a single specific minor subunit [63]. *S. pneumoniae* encodes three pilus sortase enzymes (called SrtB-D or SrtC1–

3), although there are conflicting reports as to the specific contribution of each pilin sortase to fiber formation. While deletion of any single pilin sortase does not abrogate pilus formation [74, 75], the absence of both SrtB/SrtC-1 and SrtC/SrtC-2 eliminates piliation and indicates that SrtD/SrtC02 is not sufficient for pilus polymerization [74]. Pneumococcal SrtB/SrtC-1 plays a specific role for incorporation of at least one minor pilin (RrgC) into the pilus fiber [74, 75]. SrtC/SrtC-2 is not sufficient for heterotrimeric pilus assembly, since SrtC/SrtC-2 alone cannot incorporate one of the minor subunits, RrgC, into the pilus [74].

Fully polymerized fibers are attached to the cell wall by the housekeeping sortase in *C. diphtheriae*, with deletion of this gene resulting in the secretion of pilus fibers into the culture supernatant [76]. However, deletion of the housekeeping SrtA in *S. pneumoniae* did not result in an increase in shed pilus fibers, suggesting that a role for the housekeeping sortase in pilus attachment to the cell wall may not be universal among Gram-positive bacteria [75]. An accessory pilin protein in *C. diphtheriae*, SpaB, is also important for efficient anchoring of the pilus fiber to the cell wall, leading to the proposal that SpaB can serve as the pilus terminator when it is attached to the cell wall by the housekeeping sortase [77]. Similarly, the housekeeping sortase of *S. agalactiae* appears to act in concert with a specific minor subunit, GBS150, for cell wall anchoring of the pilus [78]. Pilus length termination by minor pilin subunits in Gram-positive bacteria may be analogous to PapH termination of pilus growth of Pap CU pili, since deletion of either *spaB* or *papH* results in longer pili presumably due to a termination defect [21, 77]. Consistent with a role in pilus termination, *C. diphtheriae* SpaB was observed at the base of the pilus fiber, but was also observed along the length of the pilus fiber (Figure 1). Thus, it will be important to specifically determine the mechanistic basis underlying differential locations and roles for SpaB in pilus biogenesis, the range of mechanisms by which pilus termination and cell wall attachment can occur in other Gram-positive pilus systems, and the different roles for sortases and minor pilins in pilin polymerization and function.

Localized pilus assembly

Like their Gram-negative counterparts, pilus subunits in Gram-positive bacteria are targeted to the membrane for Sec-mediated translocation by the presence of a Sec signal sequence. Recent studies in a variety of Gram-positive bacteria show that protein translocation can be coordinated at distinct sites on the cell surface. In *Bacillus subtilis*, SecY and SecA proteins of the general secretory pathway are colocalized at multiple discrete sites, forming a punctate helical pattern along the cell membrane of this rod-shaped bacterium [79]. The ATPase of the Sec machinery, SecA, in the Gram-positive cocci *S. pyogenes*, *Streptococcus mutans*, and *Enterococcus faecalis* is reported to localize to distinct domains leading to the proposal that protein secretion may be spatially restricted [80–82]. This localized secretion domain has been termed the ExPortal in *S. pyogenes*[51, 81]. However, a separate study reported homogenous SecA localization throughout the *S. pyogenes* cell membrane, leading to an alternative model for localization in which domains within the N-terminus of secreted proteins themselves, and not the location of the Sec machinery, are proposed to direct the localization of secreted proteins in Gram-positive cocci [83]. A YSIRK domain within the N-terminal secretion signal differentially influenced the sites of several sortase substrates on the cell surface in both *S. pyogenes* and *Staphylococcus aureus*, although it is unclear is if this is a consequence of localized secretion, sortase function, or due to another factor [83, 84].

After translocation across the cell membrane, pilus subunits must efficiently interact with their cognate sortase(s) for fiber assembly and cell wall attachment. This process may be facilitated by spatially coordinating secretion and sortase processing, whereby SecA and sortases are co-localize at a single site, as has been observed in *S. mutans* and *E. faecalis*[80, 82]. Consistent with this notion, the housekeeping sortase of *S. pyogenes* localizes to

distinct membrane foci that are most often found near the division septum, sites of nascent cell wall synthesis and thus the predicted sites for sortase-mediated attachment of pilus fibers to lipid II precursors [85]. The pilus-associated sortase of *E. faecalis* also localizes to distinct foci found predominantly at the division plane, and the pilus subunits accumulate in foci in the absence of the pilus sortase suggesting they are secreted and processed at focal membrane domains [82]. Similarly, pilin subunits in *C. diphtheriae* can be detected in clusters on the cell surface [70, 86]. In *S. pneumoniae*, pili viewed by immunofluorescence are topologically localized to symmetric foci at the equatorial plane, supporting the hypothesis that pili are assembled and attached to the nascent cell wall in a spatially restricted manner [74]. Interestingly, the focal deposition of pneumococcal pili required the presence of either the SrtB/SrtC-1 or the SrtD/SrtC-3 pilin sortase, but not the SrtC/SrtC-1 pilin sortase, leading to the speculation that SrtB/SrtC-1 and/or SrtD/SrtC-3 may also be found focally localized at sites of pilus biogenesis. Together these reports suggest a localized pilus assembly complex whereby pilin subunits are secreted at focal membrane domains where both pilin and housekeeping sortases are also spatially restricted. Clustering of related pathways in this way may enable newly secreted pilin proteins to quickly come into contact with their cognate sortase for efficient pilus polymerization prior to cell wall attachment and subsequent distribution around the cell surface. A positively charged cytoplasmic amino acid motif has recently been shown to be important for focal localization of the pilin sortase of *E. faecalis*[82]. Mislocalization of the pilin sortase by mutation of the positively charged motif resulted in a decrease in piliation, supporting the idea that localized sortase action enhances efficient pilus production. It remains to be seen how localized pilus secretion and assembly platforms extends to other Gram-positive organisms, especially the rod-shaped bacilli; how pilus systems utilizing multiple pilin sortases organize their assembly machinery; whether a hierarchy of sortase interaction occurs; and what molecular and cellular factors are involved on mediating localization.

There are no reports examining the localization of CU pilus secretion or assembly sites, although conflicting reports exist regarding whether Sec components display any subcellular organization in Gram-negative organisms [87–89]. It will be interesting to know whether other Gram-negative pilus assembly apparatuses, such as that of the CU pili, localize to discrete sites, and if so, how localized biogenesis occurs in these cells, and how this translates into pilus display around the cell surface. The approaches used and mechanisms unveiled in the study of SA pilus localization may be useful in guiding future investigation of localization in Gram-negative pilus systems.

Concluding remarks

In the past several years, highly resolved molecular and structural details of the well-studied Gram-negative CU pilus system have been reported, important not only for their insights into our understanding of pilus biogenesis, but as a detailed model of protein secretion and translocation across biological membranes. From a practical standpoint, understanding the fine structural details of the CU pilus biogenesis pathway has allowed for the creation of rationally designed compounds which inhibit pilus formation by interfering with chaperone-subunit-usher interactions [90], inhibit biofilm formation *in vitro*, and attenuate virulence *in vivo*[7], paving the way for future virulence factor-specific therapeutics. In contrast, SA pili of Gram-positive bacteria have only recently been appreciated, during which time we have seen rapid advancement in our understanding of these ranging from genetics to biochemistry to pathogenesis. The search for housekeeping sortase-inhibitors as potential anti-infective therapies is an active area of research and several candidate inhibitors have been identified [8]. A deeper understanding of pilin sortase structure and function relationships will be important for extending inhibitor searches to pilin sortases. While bacterial pili are thought to function in adherence, the biogenesis pathways of these extracellular fibers are

specialized to circumvent inherent physiological differences between Gram-negative and Grampositive bacteria, such as the presence of an oxidizing periplasmic space to a thick cell wall, respectively. It will be of interest to determine which aspects of pilus biogenesis are conserved across pilus types and organisms (Box 1). Consideration of increasingly refined details of well-established CUr pilus systems along with new paradigms emerging from the world of Gram-positive pili will facilitate our understanding of conserved and dedicated aspects of bacterial pilus biogenesis.

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Box 1

Outstanding questions CU assembled pili

CU assembled pili

- How does the pilin/chaperone complex dissociate at the usher?
- How does the usher facilitate productive DSE reactions and extrusion of polymerized pili to the surface of the bacterium?
- What are the molecular details governing the specificity between the different pilin subunits during DSE?
- How is the pilus fiber anchored to the cell?
- Do Gram-negative bacteria employ spatially localized secretion and assembly strategies for efficient pilus polymerization?

SA pili

- How does the putative chaperone protein act to facilitate pilus polymerization?
- Do Gram-positive bacteria utilize a dual platform system at the membrane to facilitate secretion, substrate recognition, and assembly?
- What mediates the incorporation and location of minor subunits in the pilus fiber?
- How do Gram-positive bacteria spatially and temporally coordinate secretion with sorting?
- What are the molecular receptors for the pilus adhesins?

General questions

- How are multiple pilus operons regulated to efficiently display one particular pilus type at the appropriate time?
- What determines the specificity for assembly in organisms with multiple, distinct pili?

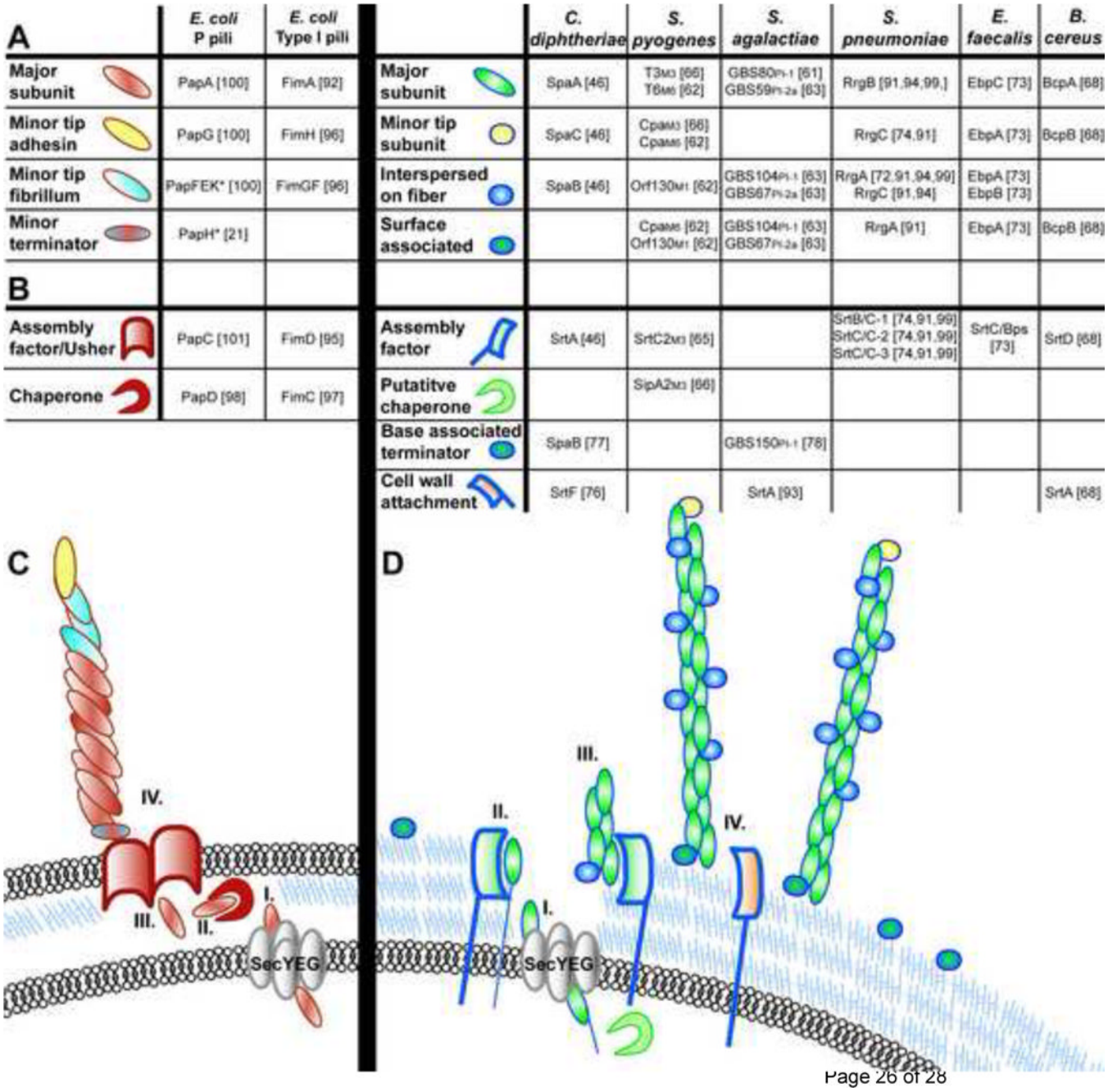

Figure 1. CU and SA pilus and fiber assembly components

(a)–(b) *E. coli* prototype CU assembled pili. **(a)** Pilus subunit location within wild-type pilus fibers are depicted, as determined by immunoelectron microscopy. "*" indicates subunits whose location was deduced from biochemical and/or structural studies and not by immunoelectron microscopy. Empty boxes indicate no available data for that pilus biogenesis component. **(b)** Schematic of pilus biogenesis machinery for CU assembled pili. *E. coli* CU pilin subunits are translocated via the Sec machinery through the inner membrane (i), after which they associate with dedicated chaperone proteins in the periplasm (ii) which prevent subunit misfolding and facilitate delivery of the subunits to the outer membrane usher protein (iii) through which subunits are secreted and which serves as a platform for

ordered pilus assembly (iv). **(c)–(d)** Gram-positive SA pili. **(c)** Table of pilus subunits for Gram-positive SA pili. Subscript for *S. pyogenes* indicates M1, M3 or M6 serotype and for *S. agalactiae* indicates the PI-1 or PI-2a pilus island. Slash marks (/) indicate differing names for the same protein in the literature. **(d)** Schematic of Gram-positive SA pili. Gram-positive pilin subunits are targeted for translocation across the cell membrane by the general secretion machinery via an N-terminal signal sequence (i). Presumably, subunits are then retained in the cell membrane by a transmembrane domain (ii) prior to LPXTG recognition and cleavage by sortase enzymes leading to ordered pilus fiber assembly (iii) and attachment to the cell wall (iv).

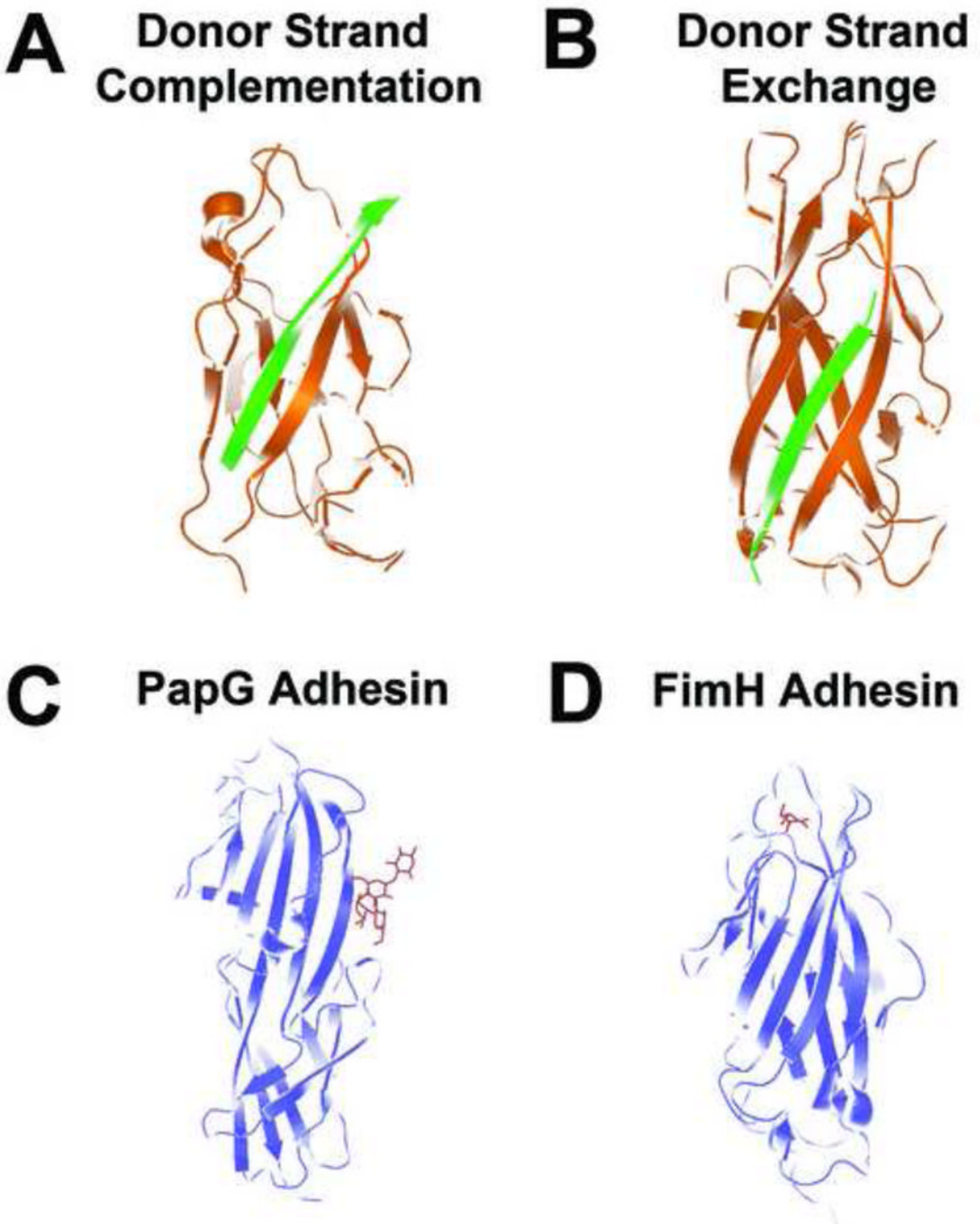

Figure 2. CU pilin subunit structure

(a) Donor strand complementation. The interactive subunit assembly site created by the incomplete immunoglobulin fold of the PapE (orange) pilin subunit is capped by the G1 strand of the PapD chaperone (green), providing the steric information necessary to complement the Ig-like fold of the subunit and stabilize the subunit prior to pilus assembly (PDB code 1NOL). **(b)** Donor strand exchange. PapE (orange) pilin structure consists of an incomplete Ig fold, which is completed within the pilus structure by the N-terminal extension of the neighboring pilin subunit, PapK (green), to form a canonical Ig domain in the polymerized pilus fiber (PDB code 1N12). **(c)** PapG ligand-binding domain (blue) bound

to GbO4 (red), which consists of the PapG ligand tetrasaccharide GalNAc beta 1–3Gal alpha 1–4Gal beta 1–4Glc linked to ceramide (PDB code 1J8R). **(d)** FimH ligand-binding domain interacting with its D-mannose ligand (red) (PDB code 1KLF).

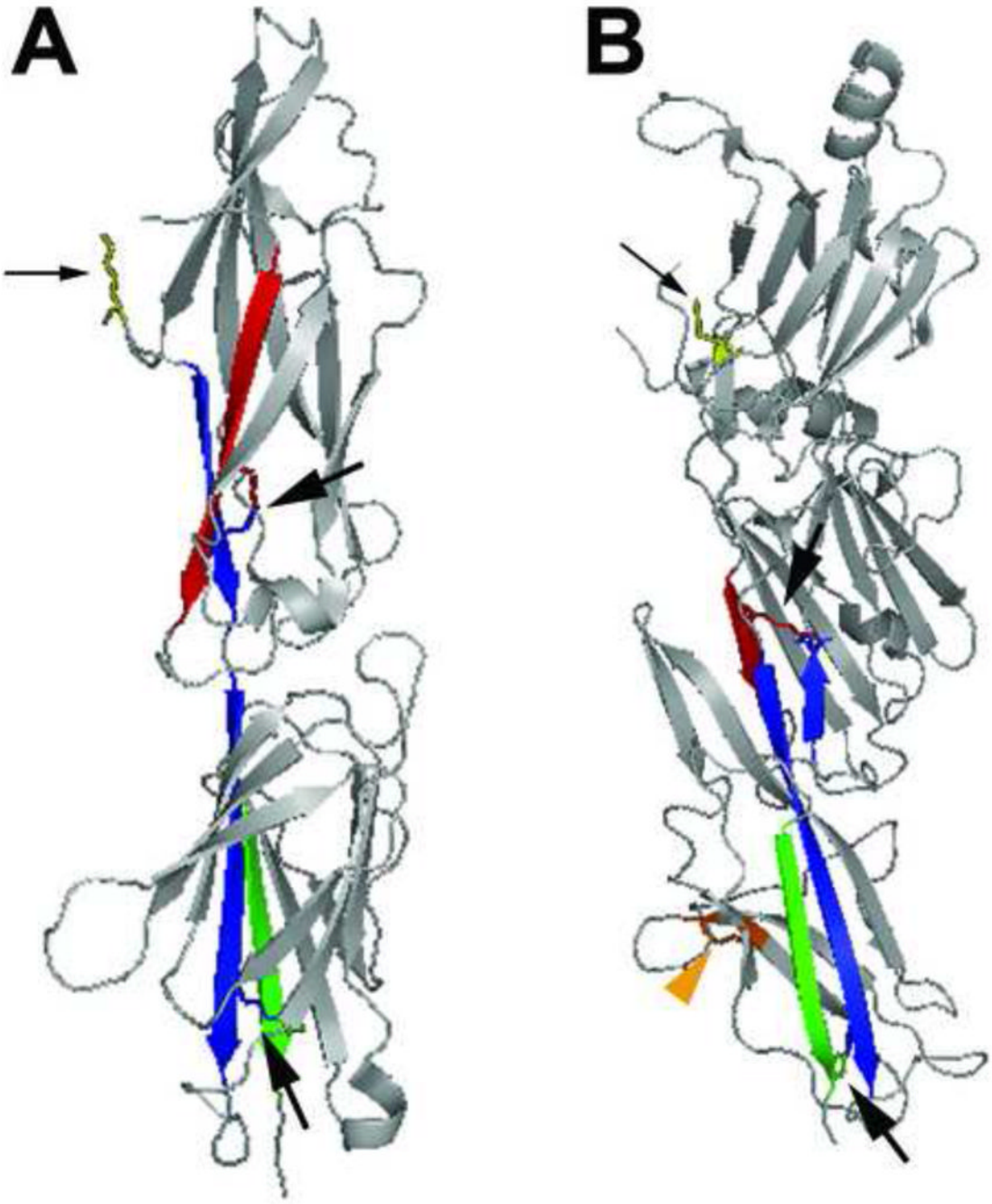

Figure 3. SA pilin subunit structure

(a) The 32.5 kD two-domain *S. pyogenes* major pilin subunit structure in ribbon diagram (PDB code 3B2m). The N- and C-terminal β sheets of the molecule are colored red and green, respectively. The interacting β sheet for each amide bond is colored blue. Stick diagrams indicate Lys-Asn intramolecular bonds within each domain of the subunit (indicated by large black arrows) and the conserved lysine (yellow, small black arrow) that becomes covalently linked to the threonine of cleaved LPXTG-like motif in the neighboring subunit. **(b)** The 47 kD three-domain *C. diphtheriae* SpaA major pilin subunit structure in ribbon diagram (PDB code 3HR6). The N- and C-terminal β sheets that participate in

intramolecular amide bonds are colored red and green (in the middle and C-terminal domain, respectively); the interacting β sheet for each amide bond is colored blue. Stick diagrams indicate Lys-Asn intramolecular bonds within each domain of the subunit (large black arrows), the conserved lysine of the YPKN pilin motif (yellow, small black arrow) that becomes covalently linked to the threonine of cleaved LPXTG motif in the neighboring subunit, and the disulfide bond in the C-terminal domain (orange, orange arrowhead).

Table 1

Pilus system attributes for CU and SA pili

	CU assembled Pili	**SA pili**
Fiber initiation	Tip adhesins with highest binding affinity for the usher are incorporated first	Major subunit sufficient to initiate fiber polymerization by the pilin sortase
Fiber organization	Tip adhesin Tip fibrillum Major subunit backbone	Minor subunit tip in some SA pili Minor subunits interspersed Major subunit backbone
Fiber organization	Non-covalent interactions via DSE	Covalent inter- and intra-subunit peptide bonds
Fiber termination	Incorporation of minor pilin subunit that cannot undergo DSE	Incorporation of minor pilin subunit that is attached to the cell wall by the housekeeping sortase
Receptor interactions	Ligand interactive sites mapped to tip adhesin	Minor subunits mediate adhesion, molecular basis undetermined
Assembly platform	Usher–mediated, location not examined	Secretion and sortase enzymes spatially restricted
Pilus fiber display	Peritrichous	Focal assembly; peritrichous display and/or absent from septum after cell wall attachment