Title
New Paradigms of Pilus Assembly Mechanisms in Gram-Positive Actinobacteria

Journal
Trends in Microbiology, 28(12)

ISSN
0966-842X

Authors
Ramirez, Nicholas A
Das, Asis
Ton-That, Hung

Publication Date
2020-12-01

DOI
10.1016/j.tim.2020.05.008

Peer reviewed

Trends in Microbiology

CellPress REVIEWS

Review

New Paradigms of Pilus Assembly Mechanisms in Gram-Positive Actinobacteria

Nicholas A. Ramirez,[1] Asis Das,[2] and Hung Ton-That[1,3,*]

Adhesive pili in Gram-positive bacteria represent a variety of extracellular multiprotein polymers that mediate bacterial colonization of specific host tissues and associated pathogenesis. Pili are assembled in two distinct but coupled steps, an orderly crosslinking of pilin monomers and subsequent anchoring of the polymer to peptidoglycan, catalyzed by two transpeptidase enzymes – the pilus-specific sortase and the housekeeping sortase. Here, we review this biphasic assembly mechanism based on studies of two prototypical models, the heterotrimeric pili in *Corynebacterium diphtheriae* and the heterodimeric pili in *Actinomyces oris*, highlighting some newly emerged basic paradigms. The disparate mechanisms of protein ligation mediated by the pilus-specific sortase and the spatial positioning of adhesive pili on the cell surface modulated by the housekeeping sortase are among the notable highlights.

Highlights

Covalently linked pili are assembled on the cell surface of many Gram-positive bacteria via a biphasic mechanism whereby pilus polymerization is catalyzed by the pilus-specific sortase followed by cell wall anchoring of pili by the housekeeping sortase.

Pilus-mediated adhesion depends on pilus length, which is modulated by the housekeeping sortase via unique structural features.

Some Gram-positive surface proteins with the LPXTG motif may hijack a pilus assembly machine via molecular mimicry to be displayed at the pilus tip.

Pilus-specific sortase enzymes provide a bioconjugation tool via the formation of an isopeptide bond that is mechanically stable and less susceptible to proteolytic cleavage.

Covalently-Linked Pili of Gram-Positive Bacteria

Fiber-like appendages called 'pili' or 'fimbriae' are microscopic structures present on the cell surface of both Gram-negative and Gram-positive bacteria. They are involved in a wide range of cellular activities, including adherence, motility, conjugation, and virulence [1–3]. Among these, the only pilus form known to date in which individual subunits are covalently bonded is the pili that are assembled by the action of sortase enzymes conserved in Gram-positive bacteria [4], but not in Gram-negative bacteria that produce pili in which the monomer subunits are joined via protein–protein interaction. The various sortases found thus far are broadly grouped into six classes (SrtA–SrtF), based on sequence alignment and substrate preference [5,6]; of these, only members of the class C and class A/E sortases are shown to catalyze the two distinct steps of Gram-positive pilus assembly: pilus polymerization and anchoring of pili to the cell wall, respectively (Box 1) [7].

Historically, the connection between sortase and pilus polymerization was somewhat serendipitous, based on two types of genetic observations. First, it was recognized that the protein sequences of different fimbrial subunits in the actinobacterium *Actinomyces naeslundii* harbor the same C-terminal cell wall sorting signal (CWSS) as the classically defined cell surface protein, protein A of *Staphylococcus aureus* [8,9] (Box 1). Second, many sets of genes coding for sortase enzymes and surface proteins with the LPXTG motif are clustered in the same operons in the actinobacterium *Corynebacterium diphtheriae* [10]. Indeed, immunoelectron microscopic analysis using antibodies against some of these surface proteins revealed the presence of distinct classes of pili on the surface of *C. diphtheriae* [10]. Since the first demonstration of the essential function of specific sortases in pilus assembly in *C. diphtheriae* [3,10,11], the past decade has seen extensive investigations of pilus assembly in many other Gram-positive bacteria, including *Bacillus cereus*, *Enterococcus faecalis*, and streptococci [12–18]. Because both *Actinomyces* and *C. diphtheriae* continue to serve as excellent models in the studies of the genetic, biochemical, and structural mechanisms of Gram-positive pilus assembly, we focus our present review on these two actinobacterial species only to highlight the recent advances in the field. For a more comprehensive

[1]Molecular Biology Institute, University of California, Los Angeles, CA, USA
[2]Department of Medicine, Neag Comprehensive Cancer Center, University of Connecticut Health Center, Farmington, CT, USA
[3]Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, CA, USA

*Correspondence: htonthat@dentistry.ucla.edu (H. Ton-That).

Box 1. Overview of Classes A, C, and E Sortases

Staphylococcus aureus SrtA is the prototype class A sortase [61], which recognizes an LPXTG motif, preceding a hydrophobic domain and a positively charged tail that together constitute a 'cell wall sorting signal' located at the C-terminus of a sortase substrate [62]. As a transpeptidase, SrtA catalyzes cell wall anchoring of surface proteins harboring the tripartite sorting signal by first hydrolyzing the peptide bond between threonine and glycine and then covalently joining the cleaved threonine residue to the pentaglycine peptide of lipid II in the cell wall [63]. Structurally, the class A sortase harbors a unique β-barrel fold, with the catalytic site containing the sole cysteine residue [64], which is absolutely required for sortase activity [65].

Class C sortases, or pilus-specific sortases, are found in bacterial species that produce covalently linked pili [5]. They are structurally similar to class A sortases with the eight-stranded β-barrel fold encapsulating the active site [66]. Unique to class C sortases is a flexible N-terminal hydrophobic 'lid' that covers the catalytic pocket and has been proposed to play a role in substrate recognition and sortase stability [6,67–70].

Present in various Gram-positive bacterial species and abundant in actinobacteria [5,71], class E sortase enzymes recognize a distinct LAXTG sorting motif [5]. Compared with sortases of classes A and C, structures of class E sortases have been less well studied, with only two available sortase structures from *Streptomyces coelicolor* and *A. oris* [60,72]. While both harbor the conserved eight-stranded β-barrel fold without the aforementioned lid, they contain a conserved tyrosine residue within the β3/β4 sheet that appears to be involved in the recognition of the LAXTG sorting motif [60,72].

description of pilus assembly in Gram-positive bacteria, we refer the reader to several excellent publications elsewhere [19–21].

Corynebacterium diphtheriae Pili Offer a New Paradigm for Protein Ligation

Assembly of the SpaA Pilus

The causative agent of human diphtheria *C. diphtheriae* is among the earliest bacterial species where pili were identified [22,23]. As in many Gram-positive bacteria [4], the *C. diphtheriae* genes coding for distinct pilin subunits and dedicated pilus-specific sortases, which are all class C sortases, are organized into three operons [10]. Together, they encode three distinct pilus types specified by their major subunits: SpaA-type, SpaD-type, and SpaH-type pili [10,24,25]. Pili constitute one of the major virulence factors in *C. diphtheriae*: A mutant devoid of all pilins is highly attenuated in virulence in a mouse model of diphtheritic toxemia [26]. Each type of *C. diphtheriae* pili is heterotrimeric, meaning that each pilus type is made of three distinct pilin subunits; however, it is important to note further that the various molecules of the same pilus type (SpaA, SpaD, or SpaH type) vary in their length. Of these, the most highly studied is the SpaA pilus, which is composed of the shaft pilin SpaA, the tip adhesin SpaC, and the base pilin SpaB anchored to the cell wall; assembly of this pilus requires the cognate sortase SrtA, a class C sortase [10] (Box 1). While all three pilins contain the CWSS, only SpaA has a recognizable pilin motif with a conserved lysine residue that serves as a nucleophile essential for sortase-mediated crosslinking of pilin monomers. In this reaction that can occur repeatedly, the pilus-specific sortase SrtA catalyzes hydrolysis of the LPXTG motif in a pilin subunit and links the cleaved threonine residue to the lysine residue within the pilin motif of another pilin adjoining on the bacterial membrane (Figure 1). Because SpaC resides at the pilus tip (the tip first rule), the first transpeptidation reaction must occur between SpaC and SpaA, linking the threonine residue of the SpaC LPXTG motif to the reactive lysine residue within the pilin motif of SpaA (Figure 1). Subsequently, pilus elongation ensues whereby SpaA pilins are added to the growing chain of pilus polymers. Ultimately, pilus polymerization is terminated with addition of SpaB (the base pilin), which is then anchored to peptidoglycan by the housekeeping sortase SrtF (class E sortase), whose gene is not genetically linked (or in proximity) to any of the three pilus gene clusters [27] (Figure 1). This biphasic mechanism of pilus assembly appears to be universally applicable to other Gram-positive bacterial pili studied to date [12,28–30].

Genetic and biochemical studies together have provided groundbreaking evidence to support the various steps of the aforementioned model (Figure 1). Importantly, X-ray crystallographic

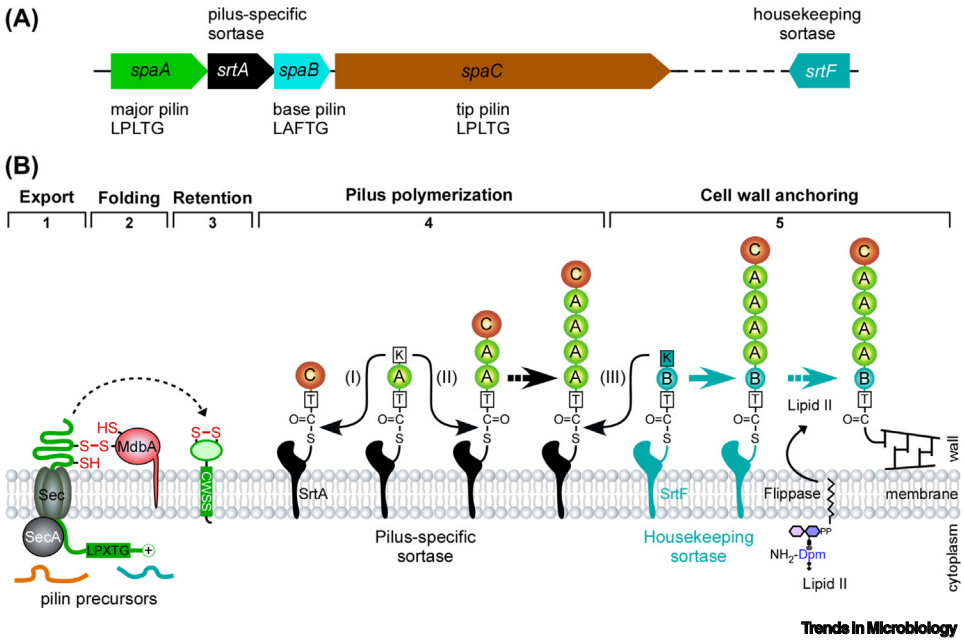

Figure 1. Assembly of the Heterotrimeric SpaA Pili in *Corynebacterium diphtheriae*. (A) Shown is the *spaA* pilus gene cluster in strain NCTC13129, encoding the pilus shaft SpaA, pilus base SpaB, and pilus tip SpaC and the pilus-specific sortase SrtA. The SpaABC pilins contain a cell wall sorting signal with the LPLTG or LAFTG motif. The housekeeping sortase gene *srtF* is located elsewhere in the bacterial chromosome. (B) A biphasic assembly mechanism of Gram-positive pili is depicted here with the SpaA-type pili. Pilin precursors are secreted in an unfolded state across the cytoplasmic membrane through the Sec translocon (Step 1). The membrane-bound thiol-disulfide oxidoreductase MdbA mediates disulfide bond formation and folding of pilin subunits (Step 2) prior to their insertion into the membrane (Step 3). The pilus-specific sortase SrtA catalyzed pilus polymerization into pilus fibers through successive lysine-transpeptidase reactions (Step 4). Incorporation of SpaB to the base of the pilus signals cell wall anchoring of the pilus by housekeeping sortase SrtF (Step 5). The order of the described transpeptidation reactions is marked by roman numerals (I–III). Reproduced, with permission, from [7].

studies of the SpaA pilin led to the discovery of an intramolecular disulfide bond and the role of a disulfide bridge-forming machine, termed 'MdbA,' which is critically involved in mediating the post-translocational folding of the SpaA precursor pilin prior to its polymerization by pilus-specific sortase SrtA in the exoplasmic environment [26]. As expected from the model, deletion of *srtA* completely abolishes SpaA pilus polymerization [10], as does the genetic replacement of the nucleophilic lysine residue of the pilin motif (lysine-to-alanine substitution) that prevents pilus crosslinking [11]. The complete loss of SpaA pilus assembly *in vivo* in the absence of SrtA also demonstrates the substrate specificity of the pilus-specific sortase enzyme SrtA, as other pilus-specific sortases expressed *in vivo* cannot substitute for SrtA function. When *srtF* or *spaB* is absent, however, abundant amounts of the generated pilus polymers are secreted into the culture medium, supporting the model that the pilus polymerization phase precedes the cell wall anchoring phase and that SpaB incorporation acts as a pilus termination switch in this biphasic mode of pilus assembly [27,31].

Recently, *in vitro* reconstitution of a Gram-positive pilus assembly system was described for the first time. The system uses recombinant sortase enzyme and pilin substrate proteins and has provided yet another foundational support for the biphasic model described above. This remarkable success in biochemical reconstitution was facilitated by structural genetic studies of the pilus-specific sortase SrtA, which uncovered novel structural features of the enzyme, on the one hand, and, on the other hand, also led to the engineering of a robust protein-polymerizing

machine. As outlined in Box 1, the pilus-specific sortase SrtA harbors a structural lid that appears to occlude the enzyme's catalytic pocket. Mutations of this lid could unmask the active site, thereby amplifying the net rounds of SpaA polymerization by this mutant enzyme (^{Cd}SrtA^{2M}) to a level that was never observed with the wild-type enzyme under comparable conditions [32].

The *in vitro* pilus assembly reaction contained the recombinant SrtA sortase truncated for its membrane localization domain and a SpaA protein devoid of its hydrophobic domain and charged tail [32], generating substantial amounts of pilus polymers within 24 h that were easily detected by SDS-PAGE and Coomassie staining. Importantly, electron microscopic sampling revealed SpaA polymers with many >1-μm-long fibers, while mass spectrometry authenticated the isopeptide linkage connecting individual subunits. Polymerization was abolished by a catalytic site mutation (C222A) and a pilin motif mutation (K190A); furthermore, the formation of an acyl-enzyme intermediate between SpaA and sortase, as the model predicted, was also observed. Remarkably, when SpaB or SrtF protein was added to the reaction, pilus polymerization was terminated, another prediction of the model [32]. The fact that SpaA polymers are formed without the presence of the tip pilin SpaC confirms the previous genetic observation that SpaC is dispensable for pilus assembly [10]. In essence, this test tube version of the reaction recapitulates much of the pilus assembly process that is observed in *C. diphtheriae* cells.

Protein Ligation with Pilus-Specific Sortase

The ability of a sortase to ligate proteins or peptides has significant implications in protein engineering, cell biology, and biomedicine. Indeed, prior to the work with a pilus-specific sortase described above, Mao and coworkers creatively used the most active recombinant sortase enzyme studied to date [i.e., *S. aureus* SrtA (^{Sa}SrtA)] in protein ligation [33]. In this study, the recombinant staphylococcal SrtA is capable of joining one substrate protein that is C-terminally tagged with the LPXTG peptide with another substrate N-terminally tagged with the G_n peptide (n = 1–5). Ploegh and colleagues further advanced this method for protein labeling in living cells [34]. Dubbed 'sortagging,' this method appears to be a promising new engineering tool, and it has been further optimized for site specificity and the ability to covalently link peptides to a variety of nonpeptide substrates, including folate, amino-terminated or glycine-tagged polyethylene glycol, and beads [33,35]. Sortagging was also used to create peptide nucleic acid cell-penetrating peptide conjugants, thus providing an exciting new tool for designing highly specific cell-permeable drug therapies [36]. Importantly, the high affinity of sortase for the LPXTG motif has yielded yet another protein capture method in which LPXTG motif–containing peptides can be efficiently and specifically captured from complex cell lysates [35,37]. The ability of sortase to mediate the stable anchoring of proteins to surfaces for microarray-based protein activity assays has also been explored, as staphylococcal SrtA has been used to covalently attach LPXTG-containing proteins to planar surfaces, such as glass coverslips, following treatment of the surface with aminosilane and oligoglycine [37]. Sortase protein–labeling technology is not limited to protein extract applications, as staphylococcal SrtA was used to efficiently ligate a modified pentaglycine probe to the surface of the transmembrane protein CD40L in live HEK293T cells [34]. Finally, Tanaka and colleagues successfully used sortagging as a method of protein-specific fluorescent labeling by conjugated glycine-containing biotin, enhanced GFP, and Alexa Fluor probes to the transmembrane protein osteoclast differentiation factor in HEK293T cells, without inducing any toxic phenotype to the cell culture [38].

The protein ligation reaction catalyzed by *S. aureus* SrtA is limited to the N-terminal to C-terminal protein joining involving two defined substrates. By comparison, a variety of pilus-specific sortase enzymes identified to date offer the unique advantage as a bioconjugation tool via the formation of an isopeptide bond that is mechanically stable and less susceptible to proteolytic cleavage

[39,40]. Recently, McConnell and colleagues generated a recombinant *C. diphtheriae* sortase enzyme termed '^{Cd}SrtA^{3M}' that is more reactive than ^{Cd}SrtA^{2M} described above. When a substrate protein containing the pilin motif was incubated with GFP harboring a C-terminal LPLTG motif in the presence of ^{Cd}SrtA^{3M}, the mutant enzyme catalyzed the covalent joining of the two recombinant proteins [41]. Furthermore, these authors demonstrated that both sortases ^{Sa}SrtA and ^{Cd}SrtA^{3M} can be used in sequential transpeptidation reactions to modify a protein of interest at distinct sites and with high specificity. Using a small ubiquitin-like modifier (SUMO) engineered to contain an N-terminal pentaglycine peptide and a C-terminal pilin motif, ^{Cd}SrtA^{3M} was first used to catalyze the addition of a fluorescein isothiocyanate (FITC) tag harboring the LPLTG motif to the lysine residue of the pilin motif. ^{Sa}SrtA was then used to conjugate Alexa Fluor 546 harboring the LPATG motif to SUMO via pentaglycine [41]. Because of the high degree of specificity for the ε-amine nucleophile within the pilin motif, protein ligation using pilus-specific sortase enzyme may provide selective labeling [41].

Actinomyces Fimbriae: A Paradigm of Tissue Tropism, Hijacking of Pilus Machinery, and Spatial Positioning of Pili

Heterodimeric Fimbriae of *Actinomyces oris*

Actinomyces are one of the most dominant and earliest colonizing genera of microbes present in the human oral cavity, with *A. oris* (formerly called *Actinomyces naeslundii*) detected in children as young as 1 year old [42,43]. *A. oris* is a major contributor to dental plaque through its ability to coaggregate with other microbial species and thus a key to the genesis of complex biofilms on the surface of teeth and the mucosal epithelia [44–46]. This intrinsic adherence property of *A. oris* is largely attributed to the presence of two distinct fimbrial types: type 1 and type 2 fimbriae. *A. oris* has served as a pioneering model of tissue tropism mediated by Gram-positive pili, as type 1 fimbriae mediate bacterial adherence to the salivary proline-rich proteins normally coating the tooth enamel [47], whereas type 2 fimbriae promote bacterial binding to receptor polysaccharides present on the surface of oral streptococci and various host cells [48–51]. Unlike *C. diphtheriae* and many other Gram-positive bacteria, *A. oris* fimbriae are heterodimeric, containing a tip component and another entity forming the pilus shaft. In the case of type 1 fimbriae, FimP forms the pilus shaft with FimQ located at the tip, and their assembly requires the pilus-specific sortase SrtC1, whose genes are all tightly linked together on the *Actinomyces* genome [52]. The specificity of *Actinomyces* sortases appears to be strict, as the pilus-specific sortase SrtC2 is selectively required for formation of type 2 fimbriae only, which consist of the shaft FimA and tip FimB [53]. Since there are only two components in each fimbria, the last subunit of the shaft pilins should be the pilus base. This has raised an intriguing question of how pilus polymerization in *Actinomyces* or in any other two-component pilus systems, such as *B. cereus* [12] and *Streptococcus suis* [54], is terminated.

Using the type 2 fimbriae of *A. oris* as a prototype, the model of pilus assembly in *Actinomyces* is illustrated in Figure 2 [55]. Similar to what is described above for *C. diphtheriae*, pilin precursors translated in the cytoplasm are transported across the cytoplasmic membrane by the Sec translocon, and post-translocational protein folding is mediated by the disulfide bond machine MdbA, permitting membrane insertion of the pilin precursors [56]. When both tip and shaft pilins are available on the membrane, pilus polymerization is catalyzed by the pilus-specific sortase SrtC2 [57]. Finally, cell wall anchoring of the type 2 fimbriae is mediated by the housekeeping sortase SrtA, a class E sortase [58]. Surprisingly, unlike all known sortases studied to date, *A. oris srtA* is an essential gene. Genetic and biochemical studies to reveal the basis of *srtA* essentiality (Box 2) have serendipitously uncovered the molecular basis of regulated pilus polymerization and spatial positioning of pilus adhesins in *Actinomyces* that could not have been envisioned before (see below).

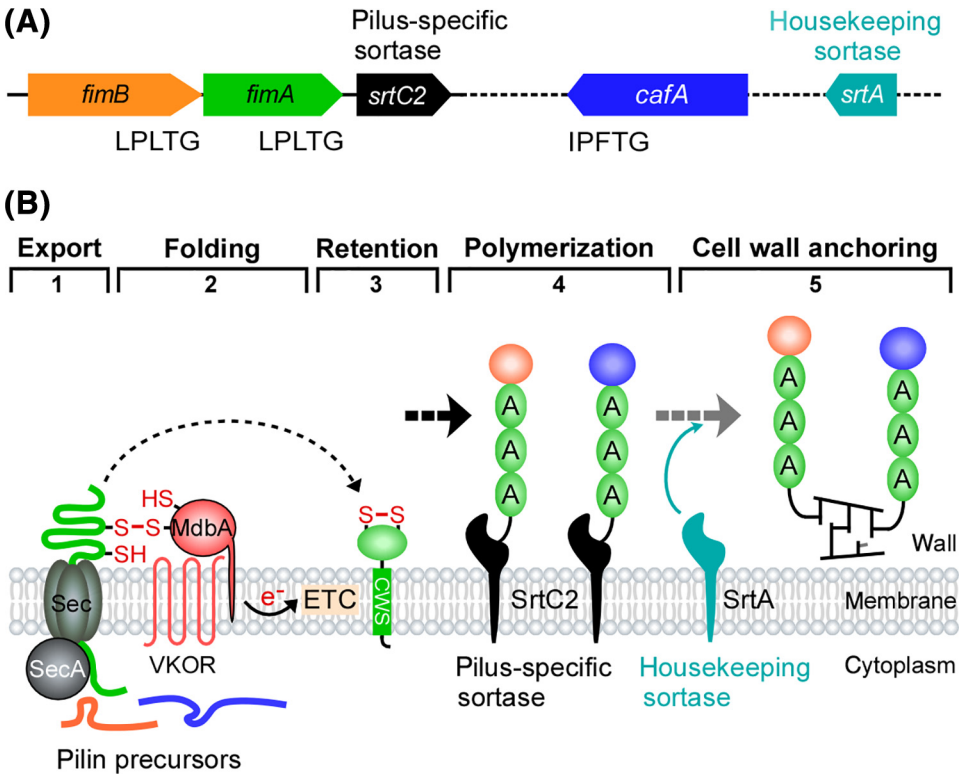

Figure 2. Assembly of the Heterodimeric Type 2 Fimbriae in *Actinomyces oris*. (A) The type 2 fimbriae are encoded by the three-gene operon. Genes encoding the coaggregation factor CafA and the housekeeping sortase SrtA are located elsewhere. (B) Similar to the assembly mechanism of the SpaA pili, assembly of the type 2 fimbriae begins with translocation (Step 1) and post-translocational folding of the shaft pilin FimA and tip pilin FimB mediated by the disulfide bond–forming machine MdbA/VKOR (vitamin K epoxide reductase) coupled to the electron transport chain (ETC) (Step 2). Membrane insertion (Step 3) of these pilins permits pilus polymerization catalyzed by the pilus-specific sortase SrtC2 (Step 4), followed by cell wall anchoring of the pilus polymer catalyzed by the housekeeping sortase SrtA. Reproduced, with permission, from [55].

Coaggregation Factor CafA Illustrates Pilus Hijacking in Gram-Positive Bacteria

The discovery that CafA is the coaggregation factor in *A. oris* has several significant implications, one that provides a paradigm of a surface protein hijacking the pilus assembly machine for pilus display and another a concept of spatial positioning of pilus adhesins for biological functions (see below). As mentioned above, type 2 fimbriae are essential for *A. oris* interactions, or coaggregation, with other oral bacteria, especially oral streptococci, as deletion of *fimA* abrogates coaggregation with *Streptococcus oralis* [57]. Surprisingly, *A. oris* coaggregation could not be blocked by polyclonal antibodies against FimA, nor was the process affected by a deletion

Box 2. Molecular Basis of *srtA* Essentiality in *A. oris*

Unlike all other sortases studied to date, *A. oris srtA* is an essential gene, as deletion of *srtA* has proved to be lethal [58]. A genetic suppressor screen – by Tn5 transposon mutagenesis – subsequently revealed that *srtA* essentiality is linked to the toxic accrual of a normally cell wall–anchored glycoprotein GspA, an SrtA substrate harboring a cell wall sorting signal with the LAXTG motif. In the absence of *srtA*, glycosylated GspA accumulates in the cytoplasmic membrane, causing lethal 'glyco-stress' accompanied by expansion of the cell envelope and cell growth arrest [58]. An *lcp* mutant devoid of the glycosyltransferase LcpA and unable to glycosylate GspA [73] is one of 13 identified suppressor mutants, so is a *gspA* mutant lacking the cell wall sorting signal that permits membrane insertion prior to cell wall anchoring [58]. This illustrates the power of forward genetic analysis and the continued utility of isolating genetic suppressors in unveiling the intricacies of microbial genetic mechanisms.

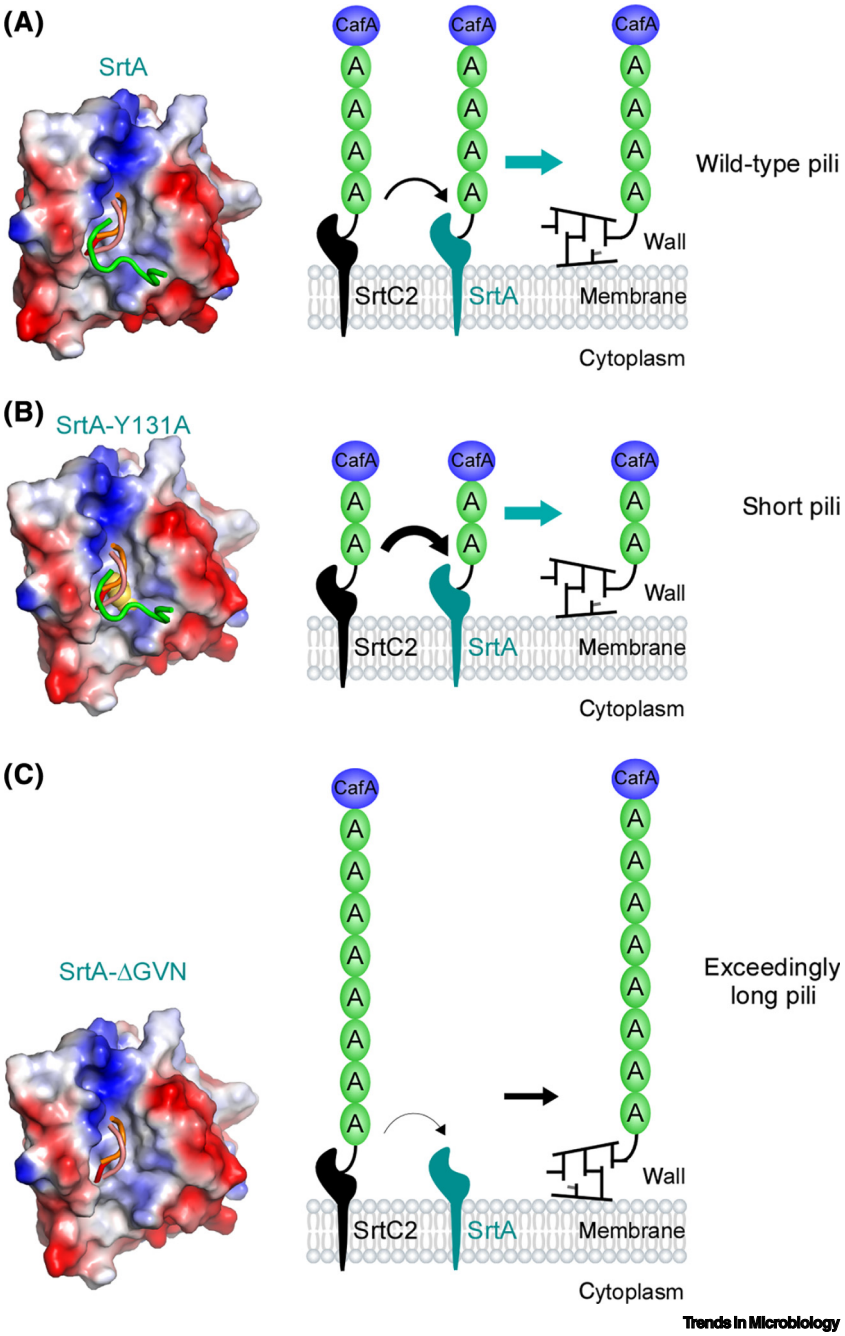

Figure 3. A Model of Pilus Length Modulated by Sortase Enzymes in *Actinomyces oris*. X-ray crystallization revealed the structural features of the housekeeping sortase SrtA: the tyrosine residue Y131, and the tripeptide loop GVN. (A) In the wild-type MG1, balanced activities of the pilus-specific sortase SrtC2 and the housekeeping sortase SrtA produce type 2 fimbriae with typical length. (B) Alanine substitution of Y131 enhances SrtA sortase activity for the LPLTG motif of FimA, interfering with pilus polymerization, resulting in premature cell wall anchoring of short pili. (C) Mutation or deletion of the GVN loop reduces SrtA preference for the LPLTG motif of FimA, leading to continuous polymerization by SrtC2 and generating exceedingly long fimbriae that can be anchored to the cell wall by this pilus-specific sortase enzyme. Reproduced, with permission, from [60].

of *fimB*, the gene that encodes the type 2 fimbrial tip pilin FimB [59]. Thus, a hunt was on for discovering the responsible adhesive principle that defied molecular genetic identification strategies used so far. One potential scenario was that the FimA shaft contains some other protein to mediate coaggregation. This prompted a systematic elimination of each individual LPXTG-containing surface protein encoded in the *Actinomyces* genome. Indeed, among the 14-candidate surface protein–encoding genes successfully deleted, one displayed a clear-cut coaggregation defect on its own [59]. The implicated gene product, thus named CafA, was subsequently proved to be the long-sought-after coaggregation factor by biochemical experiments: Antibodies against CafA captured type 2 fimbriae and blocked bacterial coaggregation. Electron microscopic analyses revealed that CafA localizes at the pilus tip, forming a distinct pilus structure with shaft pilin FimA. Intriguingly, the CWSS sequence of CafA is strikingly similar to that of FimB, leading to the hypothesis that some Gram-positive surface proteins may hijack a pilus assembly machine via molecular mimicry to be displayed at the pilus tip [59]. As significant as this may be for advances in oral bacterial biology and therapeutic intervention, the broader implication of whether the general mechanism of pilus hijacking is more widespread remains to be investigated.

Spatial Positioning of Pilus Adhesins

Over a decade ago, it was speculated that Gram-positive adhesins appended at the pilus tip mediate the initial bacterial encounter with host cells due to the extended nature of pili [3]. Because pilus lengths vary greatly within individual pilus types and among various types of pili, it is important to know whether pilus-mediated adhesion processes depend on pilus length and whether and how pilus length is controlled in Gram-positive bacteria. A breakthrough in this problem came from the observation that *A. oris* mutants lacking the housekeeping sortase *srtA* produced exceedingly long pili, as might be expected, but surprisingly, they failed to adhere to oral streptococci [58]. This was puzzling, since the coaggregation factor CafA was still abundantly detected at the tips of these long pili [60]. Through a series of probing experiments, Chang and colleagues demonstrated that as the pilus length was shortened by inducing expression of *srtA*, coaggregation could be restored, supporting the notion that the enzymatic activity of the housekeeping sortase SrtA is a key determinant of pilus length modulation. This new insight leads to the important question of whether SrtA activity is subject to regulation functionally or genetically. X-ray crystallization revealed that SrtA harbors two structural elements: a conserved tyrosine residue Y131 and a GVN tripeptide loop that may be of regulatory significance. Indeed, alanine substitution of Y131 residue resulted in the production of shorter pili and defective coaggregation by *A. oris*, whereas mutations of the GVN loop led to assembly of extremely long pili and no coaggregation by the mutant bacteria [60], the phenotype similar to *srtA* depletion [58].

These results led to a mechanistic model that Y131 mutations alter the preference of the class E sortase SrtA, which normally recognizes the LAXTG motif, toward the LPXTG motif present in the FimA pilin subunits. As a result, pilus polymerization is terminated by SrtA-catalyzed cell wall anchoring of the FimA polymer, leading to the display of short pili on the cell surface. Conversely, in the case of the GVN motif, its mutations diminished SrtA's preference for the LAXTG motif, making SrtA's capacity limited for cell wall anchoring. As a consequence, pilus polymerization continues unperturbed, leading to extremely long pili (Figure 3). Consistent with this model, the deletion of *gspA*, which codes for one of the most abundant SrtA substrates with the LAXTG motif, resulted in normal assembly of FimA pili in the GVN mutation background and enabled positive coaggregation by the mutant strain. Together, these structural genetic findings provide compelling grounds to posit that the housekeeping sortase functions as a molecular ruler for pilus polymerization and, as such, a positive effector of bacterial coaggregation and virulence.

Concluding Remarks

Collective efforts during the last decade dissected the molecular assembly mechanisms of Gram-positive pili and probed their roles in bacterial pathogenesis and their use in the development of vaccines. While pilus vaccines have yet to emerge in the clinical arena, we now have made great strides in the basic biology and have a clearer view of pilus biogenesis in Gram-positive bacteria. A common feature in these monoderms is the biphasic mode of pilus assembly by distinct steps of enzymatic catalysis involving two sortases whereby pilus polymerization catalyzed by pilus-specific sortase is followed by cell wall anchoring of pili promoted by the housekeeping sortase. Regardless of the sortase enzymes involved, the basic principle of these transpeptidation reactions in the polymerization phase is the same: the enzymatic cleavage of a substrate and covalent linkage of the cleaved substrate to a nucleophilic acceptor. This transpeptidation reaction generates an isopeptide bond that is mechanically strong and can resist a potential unfolding force up to 690 pN [39]. This unique property of isopeptide bonding, via lysine and threonine residues, is protease resistant and offers a versatile tool in protein engineering and bioconjugation [41].

Where do we go from here? In spite of differences with their Gram-negative counterparts in the manner of assembly, the heteromeric pili of Gram-positive bacteria play significant roles in bacterial physiology and virulence as those of Gram-negative bacteria. Today, however, many fundamental questions regarding this are waiting to be addressed (see Outstanding Questions). Given the importance of these questions and the genetic and biochemical versatility of *C. diphtheriae* and *A. oris* as model organisms and their importance in significant human conditions, we believe these two systems will continue as fertile and attractive experimental models of pilus biogenesis in Gram-positive bacteria for some time to come.

Outstanding Questions

In the actinobacteria *C. diphtheriae* and *A. oris*, the membrane-bound disulfide bond–forming machine MdbA promotes post-translocational folding of pilins. Given that no major protein-folding machines are linked to pilus assembly in Firmicutes, how do these organisms solve the protein-folding problem in pilus assembly?

In a heterodimeric pilus system such as *A. oris*, the last shaft pilin acts as a pilus base and stop signal, and the housekeeping sortase appears to control pilus polymerization and hence pilus length. How does the housekeeping sortase indiscriminately recognize this base pilin from the rest? This raises an intriguing possibility that pilus polymerization and termination may depend on the stoichiometric availability of pilin substrates and sortase enzymes at the pilusosome. If so, does this require additional factors?

How are the tip pilins FimB and CafA in *A. oris* involved in pilus assembly? What mechanisms govern how a tip pilin is spatiotemporally incorporated only at the tips of pili?

How do surface proteins with the LPXTG motif such as CafA in *A. oris* hijack the sortase machine to be incorporated into the pilus tip?

Pili are found in the culture medium, especially in late log phase and stationary phase. What are the roles of secreted pili, or are they products of cell wall turnover?

Acknowledgements

We would like to dedicate this review to our late former mentor and colleague Dr Olaf Schneewind, who discovered the cell wall anchoring pathway of Gram-positive bacterial surface proteins. His amazing insights and warm friendship will be sorely missed. We thank our laboratory members for critical review of the manuscript and for discussion. Work in the Ton-That laboratory related to Gram-positive pili has been supported by the National Institute of Dental and Craniofacial Research (NIDCR)/National Institutes of Health (NIH) under award numbers DE025015 and DE017382 (H. T-T.).

References

1. Kline, K.A. *et al.* (2010) A tale of two pili: assembly and function of pili in bacteria. *Trends Microbiol.* 18, 224–232
2. Thanassi, D.G. *et al.* (2012) Surface organelles assembled by secretion systems of Gram-negative bacteria: diversity in structure and function. *FEMS Microbiol. Rev.* 36, 1046–1082
3. Mandlik, A. *et al.* (2008) Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. *Trends Microbiol.* 16, 33–40
4. Ton-That, H. and Schneewind, O. (2004) Assembly of pili in Gram-positive bacteria. *Trends Microbiol.* 12, 228–234
5. Spirig, T. *et al.* (2011) Sortase enzymes in Gram-positive bacteria. *Mol. Microbiol.* 82, 1044–1059
6. Dramsi, S. *et al.* (2005) Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. *Res. Microbiol.* 156, 289–297
7. Siegel, S.D. *et al.* (2016) Biogenesis of the Gram-positive bacterial cell envelope. *Curr. Opin. Microbiol.* 34, 31–37
8. Yeung, M.K. *et al.* (1998) Identification of a gene involved in assembly of *Actinomyces naeslundii* T14V type 2 fimbriae. *Infect. Immun.* 66, 1482–1491
9. Yeung, M.K. and Ragsdale, P.A. (1997) Synthesis and function of *Actinomyces naeslundii* T14V type 1 fimbriae require the expression of additional fimbria-associated genes. *Infect. Immun.* 65, 2629–2639
10. Ton-That, H. and Schneewind, O. (2003) Assembly of pili on the surface of *Corynebacterium diphtheriae*. *Mol. Microbiol.* 50, 1429–1438
11. Ton-That, H. *et al.* (2004) Sortases and pilin elements involved in pilus assembly of *Corynebacterium diphtheriae*. *Mol. Microbiol.* 53, 251–261
12. Budzik, J.M. *et al.* (2007) Assembly of pili on the surface of *Bacillus cereus* vegetative cells. *Mol. Microbiol.* 66, 495–510
13. Nallapareddy, S.R. *et al.* (2006) Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *J. Clin. Invest.* 116, 2799–2807
14. Lauer, P. *et al.* (2005) Genome analysis reveals pili in group B *Streptococcus*. *Science* 309, 105
15. Dramsi, S. *et al.* (2006) Assembly and role of pili in group B streptococci. *Mol. Microbiol.* 60, 1401–1413
16. Manetti, A.G. *et al.* (2007) *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. *Mol. Microbiol.* 64, 968–983
17. Falker, S. *et al.* (2008) Sortase-mediated assembly and surface topology of adhesive pneumococcal pili. *Mol. Microbiol.* 70, 595–607
18. Turroni, F. *et al.* (2013) Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium-host interactions. *Proc. Natl. Acad. Sci. U. S. A.* 110, 11151–11156
19. Telford, J.L. *et al.* (2006) Pili in Gram-positive pathogens. *Nat. Rev. Microbiol.* 4, 509–519
20. Danne, C. and Dramsi, S. (2012) Pili of gram-positive bacteria: roles in host colonization. *Res. Microbiol.* 163, 645–658
21. Hendrickx, A.P. *et al.* (2011) Architects at the bacterial surface – sortases and the assembly of pili with isopeptide bonds. *Nat. Rev. Microbiol.* 9, 166–176

22. Rogers, E.A. *et al.* (2011) Adhesion by pathogenic corynebacteria. *Adv. Exp. Med. Biol.* 715, 91–103
23. Yanagawa, R. and Honda, E. (1976) Presence of pili in species of human and animal parasites and pathogens of the genus *Corynebacterium*. *Infect. Immun.* 13, 1293–1295
24. Gaspar, A.H. and Ton-That, H. (2006) Assembly of distinct pilus structures on the surface of *Corynebacterium diphtheriae*. *J. Bacteriol.* 188, 1526–1533
25. Swierczynski, A. and Ton-That, H. (2006) Type III pilus of corynebacteria: pilus length is determined by the level of its major pilin subunit. *J. Bacteriol.* 188, 6318–6325
26. Reardon-Robinson, M.E. *et al.* (2015) A thiol-disulfide oxidoreductase of the Gram-positive pathogen *Corynebacterium diphtheriae* is essential for viability, pilus assembly, toxin production and virulence. *Mol. Microbiol.* 98, 1037–1050
27. Swaminathan, A. *et al.* (2007) Housekeeping sortase facilitates the cell wall anchoring of pilus polymers in *Corynebacterium diphtheriae*. *Mol. Microbiol.* 66, 961–974
28. Nobbs, A.H. *et al.* (2008) Sortase A utilizes an ancillary protein anchor for efficient cell wall anchoring of pili in *Streptococcus agalactiae*. *Infect. Immun.* 76, 3550–3560
29. Nielsen, H.V. *et al.* (2013) Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *J. Bacteriol.* 195, 4484–4495
30. Sillanpaa, J. *et al.* (2013) Contribution of individual Ebp pilus subunits of *Enterococcus faecalis* OG1RF to pilus biogenesis, biofilm formation and urinary tract infection. *PLoS One* 8, e68813
31. Mandlik, A. *et al.* (2008) The molecular switch that activates the cell wall anchoring step of pilus assembly in gram-positive bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 105, 14147–14152
32. Chang, C. *et al.* (2018) *In vitro* reconstitution of sortase-catalyzed pilus polymerization reveals structural elements involved in pilin cross-linking. *Proc. Natl. Acad. Sci. U. S. A.* 115, E5477–E5486
33. Mao, H. *et al.* (2004) Sortase-mediated protein ligation: a new method for protein engineering. *J. Am. Chem. Soc.* 126, 2670–2671
34. Popp, M.W. *et al.* (2007) Sortagging: a versatile method for protein labeling. *Nat. Chem. Biol.* 3, 707–708
35. Parthasarathy, R. *et al.* (2007) Sortase A as a novel molecular 'stapler' for sequence-specific protein conjugation. *Bioconjug. Chem.* 18, 469–476
36. Pritz, S. *et al.* (2007) Synthesis of biologically active peptide nucleic acid-peptide conjugates by sortase-mediated ligation. *J. Org. Chem.* 72, 3909–3912
37. Chan, L. *et al.* (2007) Covalent attachment of proteins to solid supports and surfaces via sortase-mediated ligation. *PLoS One* 2, e1164
38. Tanaka, T. *et al.* (2008) Site-specific protein modification on living cells catalyzed by sortase. *Chembiochem* 9, 802–807
39. Echelman, D.J. *et al.* (2016) CnaA domains in bacterial pili are efficient dissipaters of large mechanical shocks. *Proc. Natl. Acad. Sci. U. S. A.* 113, 2490–2495
40. Mishra, A. *et al.* (2011) Two autonomous structural modules in the fimbrial shaft adhesin FimA mediate *Actinomyces* interactions with streptococci and host cells during oral biofilm development. *Mol. Microbiol.* 81, 1205–1220
41. McConnell, S.A. *et al.* (2018) Protein labeling via a specific lysine-isopeptide bond using the pilin polymerizing sortase from *Corynebacterium diphtheriae*. *J. Am. Chem. Soc.* 140, 8420–8423
42. Papaioannou, W. *et al.* (2009) The microbiota on different oral surfaces in healthy children. *Oral Microbiol. Immunol.* 24, 183–189
43. Sarkonen, N. *et al.* (2000) Oral colonization with *Actinomyces* species in infants by two years of age. *J. Dent. Res.* 79, 864–867
44. Cisar, J.O. *et al.* (1979) Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. *Infect. Immun.* 24, 742–752
45. Skopek, R.J. *et al.* (1993) Dental plaque development on defined streptococcal surfaces. *Oral Microbiol. Immunol.* 8, 16–23
46. Zijnge, V. *et al.* (2010) Oral biofilm architecture on natural teeth. *PLoS One* 5, e9321
47. Gibbons, R.J. *et al.* (1988) Adsorbed salivary proline-rich protein 1 and statherin: receptors for type 1 fimbriae of *Actinomyces viscosus* T14V-J1 on apatitic surfaces. *Infect. Immun.* 56, 2990–2993
48. McIntire, F.C. *et al.* (1978) Mechanism of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34. *Infect. Immun.* 21, 978–988
49. Stromberg, N. and Karlsson, K.A. (1990) Characterization of the binding of *Actinomyces naeslundii* (ATCC 12104) and *Actinomyces viscosus* (ATCC 19246) to glycosphingolipids, using a solid-phase overlay approach. *J. Biol. Chem.* 265, 11251–11258
50. Ruhl, S. *et al.* (2000) Identification of polymorphonuclear leukocyte and HL-60 cell receptors for adhesins of *Streptococcus gordonii* and *Actinomyces naeslundii*. *Infect. Immun.* 68, 6346–6354
51. Ruhl, S. *et al.* (1996) Recognition of immunoglobulin A1 by oral *Actinomyces* and streptococcal lectins. *Infect. Immun.* 64, 5421–5424
52. Mishra, A. *et al.* (2007) Sortase-catalyzed assembly of distinct heteromeric fimbriae in *Actinomyces naeslundii*. *J. Bacteriol.* 189, 3156–3165
53. Wu, C. *et al.* (2012) Structural determinants of *Actinomyces* sortase SrtC2 required for membrane localization and assembly of type 2 fimbriae for interbacterial coaggregation and oral biofilm formation. *J. Bacteriol.* 194, 2531–2539
54. Okura, M. *et al.* (2011) The minor pilin subunit Sgp2 is necessary for assembly of the pilus encoded by the srtG cluster of *Streptococcus suis*. *J. Bacteriol.* 193, 822–831
55. Sanchez, B.C. *et al.* (2017) Electron transport chain is biochemically linked to pilus assembly required for polymicrobial interactions and biofilm formation in the Gram-positive actinobacterium *Actinomyces oris*. *mBio* 8, e00399-17
56. Reardon-Robinson, M.E. *et al.* (2015) A disulfide bond-forming machine is linked to the sortase-mediated pilus assembly pathway in the Gram-positive bacterium *Actinomyces oris*. *J. Biol. Chem.* 290, 21393–21405
57. Mishra, A. *et al.* (2010) The *Actinomyces oris* type 2 fimbrial shaft FimA mediates co-aggregation with oral streptococci, adherence to red blood cells and biofilm development. *Mol. Microbiol.* 77, 841–854
58. Wu, C. *et al.* (2014) Lethality of sortase depletion in *Actinomyces oris* caused by excessive membrane accumulation of a surface glycoprotein. *Mol. Microbiol.* 94, 1227–1241
59. Reardon-Robinson, M.E. *et al.* (2014) Pilus hijacking by a bacterial coaggregation factor critical for oral biofilm development. *Proc. Natl. Acad. Sci. U. S. A.* 111, 3835–3840
60. Chang, C. *et al.* (2019) Cell-to-cell interaction requires optimal positioning of a pilus tip adhesin modulated by gram-positive transpeptidase enzymes. *Proc. Natl. Acad. Sci. U. S. A.* 116, 18041–18049
61. Mazmanian, S.K. *et al.* (1999) *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. *Science* 285, 760–763
62. Navarre, W.W. and Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol. Mol. Biol. Rev.* 63, 174–229
63. Ton-That, H. *et al.* (1999) Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proc. Natl. Acad. Sci. U. S. A.* 96, 12424–12429
64. Ilangovan, U. *et al.* (2001) Structure of sortase, the transpeptidase that anchors proteins to the cell wall of *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U. S. A.* 98, 6056–6061
65. Ton-That, H. *et al.* (2002) Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*: cysteine 184 and histidine 120 of sortase form a thiolate-imidazolium ion pair for catalysis. *J. Biol. Chem.* 277, 7447–7452
66. Khare, B. and S, V.L.N. (2017) Pilus biogenesis of Gram-positive bacteria: roles of sortases and implications for assembly. *Protein Sci.* 26, 1458–1473
67. Khare, B. *et al.* (2011) The crystal structure analysis of group B *Streptococcus* sortase C1: a model for the 'lid' movement upon substrate binding. *J. Mol. Biol.* 414, 563–577
68. Khare, B. *et al.* (2011) Structural differences between the *Streptococcus agalactiae* housekeeping and pilus-specific sortases: SrtA and SrtC1. *PLoS One* 6, e22995

69. Persson, K. (2011) Structure of the sortase AcSrtC-1 from *Actinomyces oris*. *Acta Crystallogr. D Biol. Crystallogr.* 67, 212–217
70. Manzano, C. *et al.* (2009) Sortase activity is controlled by a flexible lid in the pilus biogenesis mechanism of Gram-positive pathogens. *Biochemistry* 48, 10549–10557
71. Comfort, D. and Clubb, R.T. (2004) A comparative genome analysis identifies distinct sorting pathways in Gram-positive bacteria. *Infect. Immun.* 72, 2710–2722
72. Kattke, M.D. *et al.* (2016) Crystal structure of the *Streptomyces coelicolor* sortase E1 transpeptidase provides insight into the binding mode of the novel class E sorting signal. *PLoS One* 11, e0167763
73. Siegel, S.D. *et al.* (2019) Structure and mechanism of LcpA, a phosphotransferase that mediates glycosylation of a Gram-positive bacterial cell wall-anchored protein. *mBio* 10, e01580-18