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Anchoring surface proteins to the bacterial cell wall by sortase enzymes: How it started and what we know now

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Abstract

In Gram-positive bacteria, the peptidoglycan serves as a placeholder for surface display of a unique class of monomeric and polymeric proteins, or pili – the precursors of which harbor a cell wall sorting signal with LPXTG motif that is recognized by a conserved transpeptidase enzyme called sortase. Since this original discovery over two decades ago, extensive genetic, biochemical and structural studies have illuminated the basic mechanisms of sortase-mediated cell wall anchoring of surface proteins and pili. We now know how LPXTG-containing surface proteins are folded post-translocationally, how sortase enzymes recognize substrates, and how a remnant of the cell wall sorting signal modulates intramembrane signaling. In this review, we will highlight new findings from a few model experimental paradigms and present future prospects for the field.

Keywords

Gram-positive bacteria; sortase; transpeptidation; peptidoglycan; pilus assembly; cell wall sorting signal; LPXTG; pilin motif

INTRODUCTION

During the month of May in 1972, Sjöquist and colleagues sent several manuscripts to *European Journal of Biochemistry*, reporting their application of the cell wall hydrolases lysostaphin and lysozyme to release protein A (SpA) from the cell envelope of the Gram-

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None

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positive pathogen *Staphylococcus aureus* [1,2]. Biochemical characterizations of isolated cell wall and released SpA led them to conclude that SpA is part of the *S. aureus* cell wall and that this surface protein is covalently linked to peptidoglycan [2]. This remarkable insight fueled, a few decades later, the discoveries of the C-terminal cell wall sorting signal (CWSS) essential for surface proteins including SpA to be cross-linked to peptidoglycan [3–5] and also the transpeptidase enzyme termed sortase catalyzing this covalent attachment [6,7].

Sortase turned out to be ubiquitously present in Gram-positive bacteria, often in multiple copies in bacterial genomes [8,9]. The presence of multiple sortases in a single organism has raised some intriguing questions. For example, do various sortases perform redundant or specific different functions? Are there families of sortases that are structurally and enzymatically conserved? Are sortase substrates varied? What determines substrate specificity? Indeed, sortases are broadly divided into six classes, class A to F, based on sequence homology and substrate preference [10]. Of these, the most well characterized is the class A sortase with the archetype *S. aureus* sortase SrtA, which mediates the covalent attachment of surface proteins to the cell wall mentioned above [10]. Interestingly, class C sortases, the only enzyme family known to date with protein polymerizing activity [11], build covalently linked protein polymers or pili, which are then anchored to the cell wall by a sortase of either class A or class E [12,13]. While the class C sortases are designated ‘pilus-specific’, the class A/E sortases are distinguished by the ‘housekeeping’ designation. The remaining classes of sortase are thought to mediate cell wall anchoring of specific surface proteins typically encoded by genes in the same genetic locus as sortase [14]. Here, we briefly review what we know about sortase-mediated cell wall anchoring of surface proteins including protein polymers, such as pili (or fimbriae), and some of the current outstanding questions in this field. For in-depth reviews on this topic, readers are referred to recent publications elsewhere [15,16].

BLAST FROM THE PAST

The Cell Wall Sorting Signal (CWSS) –

A fundamental question posed by the evidence for cell wall linkage of a surface protein is whether such a protein harbors a specific signature that enables a cellular export machinery to link the substrate protein to the cell wall. Answer to this question came from the groundbreaking study of Schneewind and colleagues, who reported the discovery of the CWSS – a tripartite domain consisted of an LPXTG motif, a hydrophobic domain, and a charged tail – located near the C-terminus of their favorite model protein SpA of *S. aureus* [5]. Indeed, genetic deletions of the CWSS, or the hydrophobic domain or charged tail alone resulted in extracellular secretion of SpA, while omission of the LPXTG motif alone caused protein mislocalization, i.e. SpA was detectable in all cellular compartments – extracellular milieu, cell wall, membrane and cytoplasm. Intriguingly, the positively charged tail was considered as a retention signal since serine substitution of the positively charged arginine residues following the hydrophobic domain caused complete abolition of cell wall sorting [17]. To date, it remains unclear how these positively charged residues hold the protein precursor in place, though it is tempting to speculate that these residues may be required for

interaction with a membrane retention factor. Remarkably, when a fusion protein, comprised of the SpA signal peptide followed by the *Escherichia coli* periplasmic alkaline phosphatase (PhoA) and the SpA CWSS, is expressed in *S. aureus*, the fusion protein is targeted to cell wall in the same manner as SpA [5]. Hence, the CWSS is proven to be necessary and sufficient for cell wall anchoring of surface proteins. Important among these CWSS containing proteins are the microbial surface components that recognize the adhesive matrix molecules, called MSCRAMMs and defined by the presence of two adjacent IgG-like folded subdomains, shown to be necessary for bacterial colonization and infection [18].

Sortase-mediated cell wall anchoring of surface proteins: Insights from studies of staphylococcal SrtA –

Biochemical revelation that SpA was covalently linked to peptidoglycan via its threonine residue within the LPXTG motif as the terminal end led to a logical proposal that the LPXTG motif is proteolytically processed [19,20]. Indeed, a "*biochemical/genetic tour de force*" of roughly 4,000 radiolabeling/immunoprecipitation experiments ultimately uncovered the gene that coded for the responsible enzyme, which proved to be a transpeptidase in nature [6]. Termed sortase, SrtA, this transpeptidase was shown to cleave the LPXTG motif between the threonine (T) and glycine (G) residues and link the cleaved threonine residue to the penta-glycine crossbridge of lipid II, a precursor of the cell wall [7,21]. Alongside, the first three-dimensional structure of sortase determined by nuclear magnetic resonance (NMR) revealed an eight-stranded ß-barrel structure of *S. aureus* SrtA [22], which holds the catalytic pocket comprised of the essential catalytic residues C184 and H120 [23]. This unique ß-barrel fold is a common feature in all sortase enzymes structurally characterized to date [24]. Thus, the *S. aureus* SpA experimental model has led to the elucidation of the sortase-mediated cell wall anchoring pathway of surface proteins in Gram-positive bacteria, which involves several discernible steps [25]. Briefly, following translocation from the cytoplasm to the cytoplasmic membrane by the Sec secretory machine and trans-membrane insertion via the CWSS, SpA is recognized by sortase SrtA and cleaved at the LPXTG motif between T and G, forming an acyl enzyme intermediate. This intermediate is resolved by sortase-mediated catalysis of linking the stem peptide of lipid II precursor to the cleaved threonine residue; lastly, SpA-linked lipid II is incorporated into the cell wall, hence displaying the matured SpA molecule on the bacterial surface (Fig. 1).

Sortase-mediated pilus polymerization and cell wall anchoring of pili (or fimbriae): a tale of two sortases –

As mentioned earlier, only class C sortases are capable of polymerizing individual subunit proteins into covalently-linked pilus polymers [10,26]. Described first in *Corynebacterium diphtheriae* and *Actinomyces oris*, the class C sortase enzymes in these organisms assemble heterotrimeric and heterodimeric polymers, respectively [11,27]. As an archetype example of a heterotrimeric pilus, the prototypical SpaA pilus of *C. diphtheriae* is made of the pilus shaft SpaA presenting SpaC at the tip and connected to the cell wall via SpaB at the pilus base [11]. Like *S. aureus* SpA, all three corynebacterial pilins (SpaABC) harbor an N-terminal signal peptide for protein translocation and a C-terminal CWSS. Intriguingly, the CWSS of SpaB contains an LAXTG motif [12] predicted to be a substrate of class E sortase [14]. Unlike SpA, SpaB, and SpaC, SpaA possesses a 'pilin' motif with a reactive lysine

residue that serves as a nucleophile for the transpeptidation reaction linking individual pilin subunits to pilus polymers. According to the current model of pilus assembly [10], the Sec machinery directs pilin precursors to the pilusosome, a dedicated pilus assembly machine, where *C. diphtheriae* SrtA (^{Cd}SrtA), a class C sortase, catalyzes the first transpeptidation reaction that links the SpaC LPXTG motif via threonine to the lysine residue within the SpaA's pilin motif. Subsequent transpeptidation reactions employ the SpaC-SpaA dimer and joins SpaA's LPXTG motif to the pilin lysine residue of adjacent SpaA subunit and carries out this reaction with additional SpaA to extend the pilus shaft structure. Ultimately, the pilus polymerization is terminated with the entry of SpaB to the pilus base [28], which is then anchored to the cell wall by ^{Cd}SrtF, a class E sortase we term the house keeping sortase [12] (Fig. 2A).

In theory, the first transpeptidation reaction can occur between SpaA pilins, leading to formation of a pilus comprised of only two components – SpaA multimer and the pilus base SpaB. Alternatively, the last SpaA subunit can function as base in place of SpaB, resulting a heterodimeric polymer consisting of SpaC and SpaA multimer. While these pilus forms have not been documented in *C. diphtheriae*, albeit the presence of reported various SpaB and SpaC multimers [29], the *A. oris* fimbriae has set a precedent for heterodimeric pili [10]. The type 2 fimbriae of *A. oris* are built by the fimbrial shaft FimA and the fimbrial tip FimB or CafA [30,31]. Fimbrial polymers are synthesized by the class C sortase, ^{Ao}SrtC2, and they are anchored to the cell wall by ^{Ao}SrtA, a class E sortase, via the last subunit FimA as base [32] (Fig. 2B).

SORTASES & BEYOND: WHAT WE KNOW NOW

Post-translocational folding of LPXTG-containing surface proteins –

Since LPXTG-containing surface proteins possess an N-terminal signal peptide for Sec-mediated membrane translocation, they are transported across the membrane in an unfolded state. Post-translocational folding of exported proteins in Gram-positive bacteria is thought in general to be mediated by some foldases, such as PrsA and peptidylprolyl cis/trans-isomerases, and thiol-disulfide oxidoreductases [33]. In the high GC-content Gram-positive bacteria (Actinobacteria) *C. diphtheriae* and *A. oris*, the membrane-bound thiol-disulfide oxidoreductase MdbA constitutes the major folding pathway of non- and LPXTG-containing exported proteins, which contain more than two cysteine residues, including ^{Cd}SpaA and ^{Ao}FimA [34,35]. MdbA mediates oxidative protein folding by catalyzing formation of disulfide bonds in Cys-containing substrates emerged from the Sec translocon [36]. Given the fact that most exported proteins from Actinobacteria contain paired cysteines, whereas those from aerobic Firmicutes largely exclude cysteine residues [37], it remains unclear how post-translocational folding of cysteine-less surface proteins in Actinobacteria, like *C. diphtheriae* SpaB, is mediated.

Remarkable substrate specificity of sortases and their co-evolution –

One of the most remarkable features of sortases is their specificity for substrates in spite of common or similar sorting signals in the substrates recognized by the sortases. Multiple sortases that are present in an organism work on their designated substrates with exquisite

specificity. For instance, in addition to producing the prototype SrtA, which processes LPXTG-containing substrates [38], *S. aureus* expresses a second sortase enzyme named SrtB; this sortase recognizes the only known substrate, IsdC, a heme-scavenging protein that harbors an NPQTN motif in place of the LPXTG motif [39]. Like ^{Sa}SrtA, ^{Sa}SrtB harbors an eight-stranded β-barrel [40], with the β6/β7 loop potentially presenting a substrate recognition site since swapping the corresponding loop in ^{Sa}SrtA with this loop produces a ^{Sa}SrtA hybrid protein that actively acylates NPQTN-containing substrates, though it fails to catalyze the transpeptidation step of the sorting reaction [41]. It is noteworthy that in *Streptococcus pyogenes* SrtA recognizes the LPETG, LPETA and LPKLG motifs; swapping the β7/β8 loop of ^{Sp}SrtA with that of ^{Sa}SrtA makes the hybrid ^{Sp}SrtA enzyme no longer reactive with LPETA-containing substrates but hyperactive towards the LPETG motif, the preferred substrate of ^{Sa}SrtA [42]. It is likely that both β6/β7 and β7/β8 loops create a molecular surface that confers substrate specificity of sortase enzymes.

In *A. oris*, the housekeeping sortase SrtA (class E sortase) was identified, along with two class C sortases, SrtC1 and SrtC2 [43]. Intriguingly, *A. oris* SrtA (^{Ao}SrtA) harbors two structural elements that are conserved in class E sortase enzymes [44], the Y131 residue and the GVN motif, each of which was shown to be involved in substrate recognition by mutational analysis [32]. Since ^{Ao}SrtA recognizes its preferred LAXTG-containing substrates, it was suggested that a bias in the reactivity of SrtA towards the LPXTG motif (present in pilus proteins and recognized by class C sortases) is ascribed to Y131A mutation, leading to abrupt termination of pilus polymerization; as a result, short pili are formed. By comparison, an ^{Ao}SrtA mutant strain with mutations in the GVN motif produces long pili. This is rationalized by the relatively high affinity of the ^{Ao}SrtA mutant for LAXTG-containing surface proteins and low affinity for the pilus proteins, resulting in prolonged pilus polymerization that is catalyzed by class C sortase enzymes (Fig. 2C). Much more genetic, biochemical and structural work is still needed to fully dissect the nature of co-evolution of sortases and their substrates and the regulation of their enzymatic activities in physiological context.

Significantly, unlike the class A and class E sortases, the class C sortase enzymes distinguish themselves by possessing a molecular lid encapsulating the active site presumably involved in substrate recognition and catalysis [45]. Recent studies have shown that mutations of this lid in the *C. diphtheriae* pilus-specific sortase SrtA greatly enhance its transpeptidase activity in vitro [46–48]. This enhanced and novel enzymatic activity permits the development of a powerful new protein-ligation technology, a limited version of which first emerged exploiting the *S. aureus* SrtA [49,50].

Mighty remnants- an intramembrane sensory circuit built on the CWSS leftover –

It has long been thought that once the amino-terminal surface protein part is cleaved off at the LPXTG motif of CWSS, the hydrophobic domain and the charged tail segment that is left in the cytoplasmic membrane (see Fig. 1) is ultimately degraded [38]. It is refreshing to alter that logical notion by noting that the remnants left in the membrane serve a fateful biological regulatory purpose as recently demonstrated in a common pathogen in the human oral cavity. Herzberg and colleagues reported that the cleaved C-terminal peptide from the

CWSS (termed C-pep) acts as a ligand for a histidine sensor kinase that is part of a two-component signal transduction system (TCS) that controls biofilm formation by *Streptococcus gordonii* [51]. This exciting work revealed that the cleaved membrane-bound C-peps of the cell wall-anchored adhesins SspA and SspB bind to and inhibit the intramembrane sensor histidine kinase SGO_1180. This interaction in turn inhibits activation of the cognate response regulator SGO_1181, which otherwise up-regulates transcription of the *scaCBA* locus that codes for the lipoprotein adhesin ScaA. Because ScaA is critical for *S. gordonii* biofilms' biomass, upregulation of *scaA* promotes biofilm expansion. Considering that SGO_1180 is conserved among streptococci, this C-pep mediated homeostatic mechanism is likely to be conserved in Gram-positive bacteria.

CONCLUDING REMARKS

The past decade has seen a notable transition in the field of sortases – a departure from the longstanding investigations into sortase mechanisms to the application of sortases for protein ligation and labeling, utilizing and adapting mechanistic studies to bioconjugation and protein engineering technology. Nonetheless, new findings and paradigms regarding sortase-mediated surface assembly continue to emerge, raising intriguing new questions. Noteworthy is the finding that the remnant of the CWSS after processing by sortase serves as a signaling molecule of an intramembrane sensory circuit that controls biofilm formation by an oral pathogen which appears to be common in streptococci [51]. It leaves wide open the obvious question whether some of the many cleaved CWSS peptides of non-streptococcal Gram-positive bacteria serve the same path, and which ones are subjected to degradation or involved in a cellular process that has yet to be discovered. In Gram-positive bacteria with multiple sortases, such as *C. diphtheriae* and *A. oris*, coordination between pilus-specific sortase and housekeeping sortase enzymes regulates pilus length and cell wall anchoring of surface proteins, which are critical for cell-cell interaction and pathogenesis [32]. Since all sortases studied to date are structurally conserved, it is likely that additional factors may be involved in this coordination. As such and beyond, both *C. diphtheriae* and *A. oris* continue as excellent experimental models with their unique attributes and niches in the human oral cavity for sortase-mediated cell surface assembly in Gram-positive bacteria.

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HIGHLIGHTS

- Sortase covalently links surface proteins and pili with the CWSS to peptidoglycan.
- Unfolded precursors of surface proteins and pili are folded non- or oxidatively.
- Sortase enzymes contain structural elements that determine substrate specificity.
- The CWSS remnant serves as a ligand for an intramembrane sensory circuit.

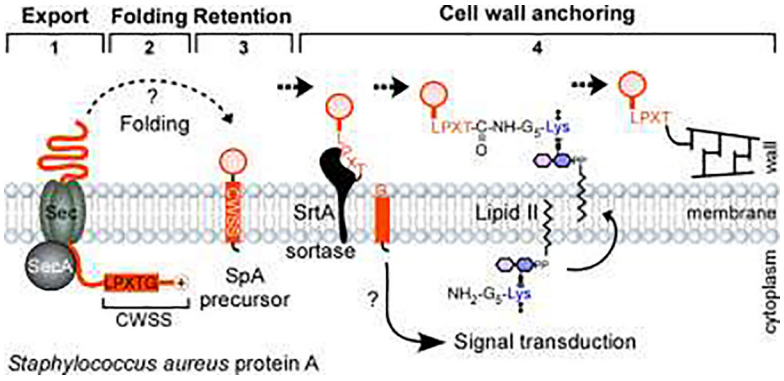

Figure 1: Sortase-mediated cell wall anchoring of surface proteins in Gram-positive bacteria.

Gram-positive bacteria employ sortase enzymes to attach surface proteins on the bacterial cell wall. Using S. aureus protein A (SpA) as a model system, cell wall anchoring of surface proteins can be divided into 4 distinct steps. After synthesized in the cytoplasm, the unfolded precursor of SpA is translocated across the membrane by the Sec translocon (Export) prior to embedding to the cytoplasmic membrane through the CWSS domain (Retention). Note that how SpA retains its native state (Folding) is unclear. During cell wall anchoring of SpA, the housekeeping sortase SrtA (class A) captures SpA on the membrane and catalyzes the sorting transpeptidation, i.e. cleavage of the LPXTG motif between threonine and glycine and coupled linkage of the threonine residue to the pentaglycyl stem peptide of lipid II, anchoring surface proteins to the bacterial cell wall. It is noteworthy that in *S. gordonii*, the cleaved CWSS terminal peptide that is left over and retained as a trans-membrane remnant acts as a signal molecule in a physiologically important and homeostatically controlled signal transduction circuit [51]. It is not clear if a similar mechanism would be observed in *S. aureus*.

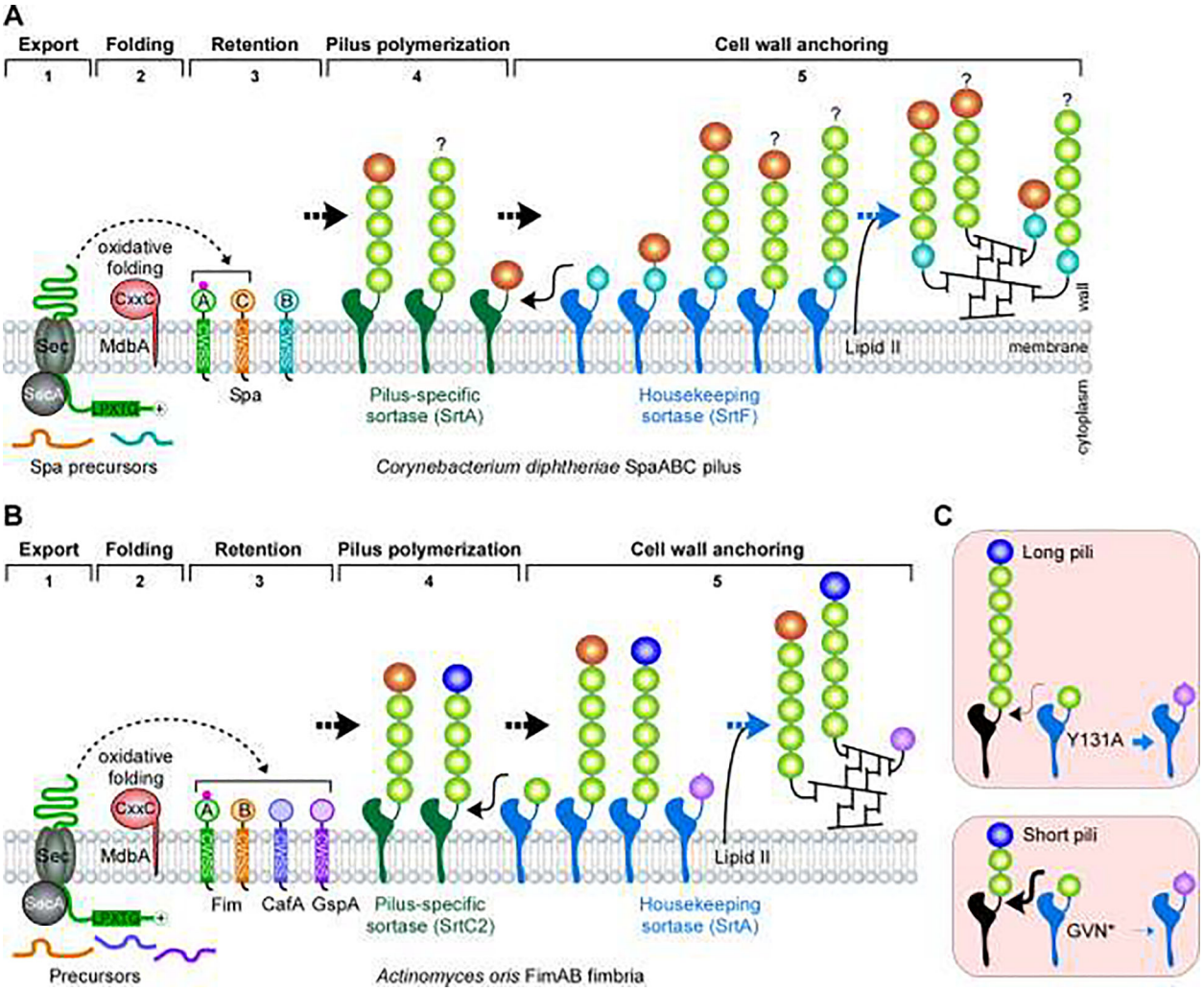

Figure 2: Sortase-mediated pilus assembly in Gram-positive bacteria.

(A) Presented is an assembly model for a heterotrimeric pilus based on the *C. diphtheriae* SpaABC pilus. Pilus assembly can be divided into 5 distinct steps, beginning with the synthesis of Spa precursors in the cytoplasm, followed by membrane translocation of unfolded protein precursors (Export) and oxidative folding (Folding) of precursor proteins with Cys pairs (SpaA and SpaC) by the thiol-disulfide oxidoreductase MdbA prior to embedding to the cytoplasmic membrane through the CWSS domain (Retention). Note that how Cys-less SpaB retains its native state is unknown. Membrane-bound pilus-specific sortase (SrtA), a class C sortase, polymerizes individual pilin monomers, via the pilin motif (pink) in the major shaft SpaA (green), into pilus polymers (Pilus polymerization). Polymerization is terminated with the entry of SpaB to the pilus base catalyzed by the housekeeping sortase SrtF (class E sortase), which mediates cell wall anchoring of the heterotrimeric polymers (Cell wall anchoring). Furthermore, it is possible that tip-less pili or pili with or without SpaB (question marks) may potentially be assembled; however, these pilus forms have not been documented although a SpaC-SpaC dimer was reported. **(B)** Presented is an assembly model for a heterodimeric pilus based on the *A. oris* FimAB fimbria. The Export, Folding, and Retention steps of fimbrial proteins and surface proteins,

like GspA (purple), are similar as those described for the *C. diphtheriae* SpaABC pilus in (A). Pilus-specific sortase SrtC2 catalyzes pilus polymerization, linking FimA subunits into polymers containing the tip pilin FimB or the coaggregation factor CafA. The housekeeping sortase SrtA (class E sortase) terminates pilus polymerization, inserting the last FimA subunit into the pilus polymer. The heterodimeric polymers are then anchored to the cell wall. **(C)** *A. oris* SrtA contains two conserved structural elements found in class E sortases, the Tyr^{131} residue and the GVN motif. It was proposed that Y131A mutation shifts SrtA preference for the LAXTG-containing substrates, for example GspA, hence impeding pilus termination. As a result, SrtC2 continues to polymerize pilins, leading to formation of long pili. In contrast, mutations of the GVN motif (GVN*) alters SrtA preference for the LPXTG-substrates, such as FimA, leading to early termination of pilus polymerization that generates short pili.