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Sortase enzymes in Gram-positive bacteria

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Summary

In Gram-positive bacteria proteins are displayed on the cell surface using sortase enzymes. These cysteine transpeptidases join proteins bearing an appropriate sorting signal to strategically positioned amino groups on the cell surface. Working alone, or in concert with other enzymes, sortases either attach proteins to the cross-bridge peptide of the cell wall or they link proteins together to form pili. Because surface proteins play a fundamental role in microbial physiology and are frequently virulence factors, sortase enzymes have been intensely studied since their discovery a little more than a decade ago. Based on their primary sequences and functions sortases can be partitioned into distinct families called class A to F enzymes. Most bacteria elaborate their surfaces using more than one type of sortase that function non-redundantly by recognizing unique sorting signals within their protein substrates. Here we review what is known about the functions of these enzymes and the molecular basis of catalysis. Particular emphasis is placed on ‘pilin’ specific class C sortases that construct structurally complex pili. Exciting new data have revealed that these enzymes are amazingly promiscuous in the substrates that they can employ and that there is a startling degree of diversity in their mechanism of action. We also review recent data that suggest that sortases are targeted to specific sites on the cell surface where they work with other sortases and accessory factors to properly function.

Introduction

Sortase enzymes decorate the surfaces of Gram-positive bacteria with a diverse array of proteins that enable each microbe to effectively interact with its environment. They either ‘sort’ proteins to the cell surface by covalently joining them to the cell wall, or polymerize proteins to construct pili, multi-subunit hair-like fibres that extend from the cell surface to promote bacterial adhesion (Scott and Barnett, 2006; Scott and Zahner, 2006; Mandlik *et al.*, 2008a; Proft and Baker, 2009; Clancy *et al.*, 2010; Kline *et al.*, 2010; Hendrickx *et al.*, 2011). Sortases have attracted great interest as potential drug targets as many of the surface proteins they display are virulence factors (Suree *et al.*, 2007; Maresso and Schneewind, 2008). They have also been developed into powerful molecular biology reagents to site-specifically attach proteins to a variety of biomolecules (Tsukiji and Nagamune, 2009; Proft, 2010; Popp *et al.*, 2011). All sortase enzymes characterized to date function as cysteine transpeptidases that join proteins containing a cell wall sorting signal (CWSS) to an amino group located on the cell surface (Marraffini and Schneewind, 2006; Clancy *et al.*, 2010).

Although they are not essential for bacterial viability when cells are grown in rich media, sortases can be important virulence factors as they display surface proteins that mediate bacterial adhesion to host tissues, host cell entry, evasion and suppression of the immune response and acquisition of essential nutrients. The sorting reaction catalysed by the sortase A protein from *Staphylococcus aureus* (Sa-SrtA) is best understood and begins when a full-length precursor protein containing an amino terminal leader peptide is exported from the cytoplasm through the secretory pathway (Fig. 1A). The C-terminal CWSS is then processed by Sa-SrtA. The CWSS consists of a LPXTG motif (where *X* denotes any amino acid), followed by a segment of hydrophobic amino acids, and a tail composed primarily of positively charged residues. The C-terminal charged tail presumably retards export, positioning the protein for processing by the extracellular membrane associated Sa-SrtA enzyme. A highly conserved active site cysteine residue in Sa-SrtA then nucleophilically attacks the backbone carbonyl carbon of the threonine residue in the LPXTG motif, breaking the threonine and glycine peptide bond and creating a sortase-protein complex in which the components are linked via a thioacyl bond. The protein is then transferred by Sa-SrtA to the cell wall precursor lipid II, when the amino group in this molecule nucleophilically attacks the thioacyl linkage to create an isopeptide linked protein-lipid II product. Transglycosylation and transpeptidation reactions that synthesize the cell wall then incorporate this product into the peptidoglycan, where it is covalently linked to the cross-bridge peptide. Other sortases catalyse a similar transpeptidation reaction, but join remarkably different LPXTG motifs and amino groups.

Since the discovery of Sa-SrtA a little more than decade ago by Schneewind and colleagues (Mazmanian *et al.*, 1999), over 800 genes encoding related proteins have been identified in ~260 distinct bacterial species (Finn *et al.*, 2010). The vast majority of sortases are found in Gram-positive bacteria that contain a conventional cell wall (they are absent in Mollicutes) (Pallen *et al.*, 2001). Most bacterial species contain multiple sortase enzymes that have been named in an *ad hoc* manner (e.g. SrtA, SrtB, SrtC, etc.). To provide a framework in which to discuss their functions, we first grouped sortases from Gram-positive bacteria into families based upon their primary sequences (Fig. 2). Approximately 60% of all sortase proteins can be partitioned into six distinct families of enzymes that share related amino acid sequences. These include class A to D enzymes that have been described previously (Comfort and Clubb, 2004; Dramsi *et al.*, 2006), and class E and F enzymes that are defined here. Experimental and bioinformatics analyses indicate members of each group recognize distinct CWSSs in which the LPXTG sequence is varied (hereafter called sorting signal motifs). Class A enzymes are present in Firmicutes and have been studied extensively. They appear to perform a housekeeping role in the cell as members of this group are capable of anchoring a large number of functionally distinct proteins to the cell wall. Class B enzymes are also present in Firmicutes and can have distinct functions. Some members of this group attach haem-receptors to the peptidoglycan, while others assemble pili. Class C enzymes are broadly distributed in Gram-positive bacteria and function as pilin polymerases that construct pili. Class D enzymes predominate in Bacilli and in *Bacillus anthracis* this type of enzyme anchors proteins to the cell wall that facilitate sporulation. Actinobacteria contain class E and F enzymes whose functions are largely unknown. In *Corynebacterium diphtheriae* a class E enzyme appears to perform a housekeeping function similar to class A enzymes (Ton-That and Schneewind, 2003), while class F enzymes have yet to be studied. Sortases are also present in a few Gram-negative and archaeobacterial species, but the functions of these enzymes are unknown (Pallen *et al.*, 2001; 2003; Comfort and Clubb, 2004). In this Microreview we discuss what is known about the functions and mechanism of transpeptidation of different types of sortase enzymes.

Sortases that attach proteins to the cell wall (class A, B, D and E enzymes)

Housekeeping class A enzymes

Nearly all bacteria in the Firmicutes subfamily contain a single class A sortase whose primary sequence is most closely related to the prototypical Sa-SrtA enzyme. Like Sa-SrtA, members of this group are believed to attach proteins to the cross-bridge peptide of the cell wall, presumably by first linking it to lipid II. Extensive experimental work on class A enzymes from different bacteria has revealed that each enzyme anchors a large number of functionally distinct proteins to the cell wall (Marraffini *et al.*, 2006). This has led to the notion that they perform a housekeeping function in the cell, and is consistent with the finding that genes encoding class A enzymes are not genomically clustered with genes encoding potential protein substrates that contain a CWSS (Pallen *et al.*, 2001; Comfort and Clubb, 2004). The busiest enzyme in this group appears to be the sortase A from *Listeria monocytogenes*, which based on a genome sequence analysis is predicted to display an astounding 43 distinct proteins (Boekhorst *et al.*, 2005). Most surface proteins attached by class A enzymes contain a canonical LPXTG motif within their CWSS and have diverse functions that can promote bacterial adhesion, nutrient acquisition, host cell invasion, and immune evasion. Class A enzymes have attracted significant interest as potential drug targets because a number of clinically important pathogens use these sortases to display virulence factors and they are attenuated in their virulence if their *srtA* gene is eliminated (*S. aureus*, *L. monocytogenes*, *Streptococcus pyogenes* and *Streptococcus pneumoniae* among others) (Suree *et al.*, 2007; Maresso and Schneewind, 2008).

Class B enzymes have diverse functions

Class B enzymes are widely distributed in Firmicutes and have primary sequences that are most closely related to the sortase B enzyme from *S. aureus* (Sa-SrtB) (Mazmanian *et al.*, 2002). Although they share significant primary sequence homology, members of this group can have radically distinct functions. In some pathogenic microbes class B sortases attach haemoproteins to the cell wall (Mazmanian *et al.*, 2002; 2003; Maresso and Schneewind, 2006; Maresso *et al.*, 2006). In contrast, recent studies have shown that the class B enzymes in *S. pyogenes* assemble pili (Kang *et al.*, 2011). This fundamental difference highlights the limits of assigning function strictly based on primary sequence homology and emphasizes the need for experimental work to decipher sortase function. Here we discuss class B enzymes that anchor haemoproteins, while pilin-specific class B sortases are discussed in the section reviewing functionally homologous class C enzymes.

In *S. aureus* and *B. anthracis* a single class B enzyme anchors the IsdC haemoprotein to the cell wall (Mazmanian *et al.*, 2002; Maresso *et al.*, 2006). In both microbes, IsdC is thought to facilitate haem capture from human haemoglobin by relaying haem from upstream haem-receptors to a membrane transporter complex that imports haem into the cell (Maresso and Schneewind, 2006; Maresso *et al.*, 2006). In contrast to Sa-SrtA, Sa-SrtB and its substrate IsdC are only expressed in iron deplete conditions (Mazmanian *et al.*, 2002). Class B enzymes recognize an unusual NP(Q/K)(T/S)(N/G/S)(D/A) sorting signal that differs markedly from the canonical LPXTG motif recognized by class A enzymes. Interestingly, the class A and B enzymes in *S. aureus* may attach their substrates to different sites within the cell wall. Sa-SrtA attaches its substrates to surface exposed sites that are heavily cross-linked. As demonstrated by protease sensitivity and immunoblot experiments, Sa-SrtB attaches IsdC to a buried site within the peptidoglycan that is not heavily cross-linked (Mazmanian *et al.*, 2003; Marraffini and Schneewind, 2005). It has been hypothesized that the positional difference of the attached proteins results from Sa-SrtB's ability to attach IsdC to preassembled peptidoglycan instead of lipid II, but this has not been demonstrated experimentally.

Gene knockout studies in *S. aureus* and *L. monocytogenes* indicate that their class B enzymes are needed to establish persistent infections, but that they are less important for virulence than their class A enzymes (Mazmanian *et al.*, 2002; Jonsson *et al.*, 2003; Newton *et al.*, 2005). However, recent findings suggest that the Sa-SrtB enzyme could play a more prominent role in pathogenesis as *S. aureus* has been shown to have enhanced specificity for human haemoglobin such that murine models of infection may underestimate the pathogenic importance of the Sa-SrtB anchored IsdC protein and other iron-regulated surface determinant proteins that participate in the capture of haem-iron from human haemoglobin during infections (Pishchany *et al.*, 2010). Sortases belonging to class B are also present in *Clostridium perfringens*, *Clostridium difficile* and *Enterococcus faecalis*. However, their functions remain unknown and the genes encoding these enzymes are not clustered with genes encoding putative sortase substrates.

Class D, E and F enzymes

Much less is known about members of the class D, E and F sortases. Class D enzymes are present in bacilli and have thus far only been characterized in *B. anthracis* (Marraffini and Schneewind, 2006; 2007). This microbe encodes a single class D enzyme (called Ba-SrtC) that attaches two proteins to the cell wall, BasH and BasI. Deletion of this enzyme reduces the efficiency of spore formation under oxygen limiting conditions, but is otherwise dispensable for pathogenesis in mice (Marraffini and Schneewind, 2006). Remarkably, the class D enzyme attaches each of its substrates to a distinct structure; it attaches BasI to the diaminopimelic acid moiety of the peptidoglycan of predivisional cells, while it attaches BasH exclusively to the forespore. The *srtC* and *basI* genes are coexpressed from the same operon before formation of the septum that divides the mother cell from the forespore enabling conventional anchoring of the BasI protein to the cell wall (Marraffini and Schneewind, 2006; 2007). In contrast, the *basH* gene is located elsewhere in the genome and is primarily expressed in the forespore after septum formation. BasH is then presumably attached to the fore-spore by class D enzymes that are transferred to the forespore from the mother cell's membrane. Interestingly, *B. anthracis* contains class A and D sortase enzymes that recognize closely related sorting signals; both BasH and BasI contain a LPNTA sorting signal that differs only slightly from the canonical LP[A/N/K]TG sorting signal present in proteins anchored by *B. anthracis* SrtA. Nevertheless, the enzymes function non-redundantly suggesting that they may have evolved a high degree of specificity for their respective sorting signals (Marraffini and Schneewind, 2006). Class D enzymes in other bacilli may perform similar functions as genes encoding these enzymes are frequently clustered with genes encoding proteins with a LPNTA sorting signal motif, which in some instances share primary sequence homology with BasI and/or BasH. However, it remains unclear why certain species including *B. cereus* and *Bacillus halodurans* contain a second class D sortase gene that is not genomically clustered with potential protein substrate genes.

Instead of a class A enzyme, some high G + C bacterial species may use a class E enzyme as their housekeeping sortase (Ton-That and Schneewind, 2003). This idea is based on the finding that genes encoding class A and E enzymes are never found in the same organism, and similar to class A enzymes, the genes encoding class E enzymes are not positioned adjacent to genes encoding potential protein substrates (Comfort and Clubb, 2004).

Comparative genome analyses suggest that class E enzymes recognize an LAXTG sorting signal, instead of the canonical LPXTG motif processed by class A enzymes (Comfort and Clubb, 2004). This unique specificity has been demonstrated for the class E enzyme in *C. diphtheriae* (named Cd-SrtF), which is the only class E enzyme that has been characterized experimentally (Chang *et al.*, 2011). Interestingly, Cd-SrtF also attaches assembled pili to the cell wall, a function shared by some class A enzymes (discussed below) (Budzik *et al.*, 2007; Swaminathan *et al.*, 2007; Nobbs *et al.*, 2008). In *Streptomyces coelicolor*, class E

enzymes display chaplin surface proteins containing an LAXTG sorting signal, which presumably mediate aerial hyphae formation. (Claessen *et al.*, 2003; Elliot *et al.*, 2003; Capstick *et al.*, 2007). Class F enzymes are present in *S. coelicolor* and other Actinobacteria, but their functions have yet to be explored (a total of 57 enzymes cluster into this family and are present in 20 bacterial species). It should be noted that in a previous study class D and E sortases were collectively grouped into a single family called class D sortases (Dramsi *et al.*, 2005). However, based on our analyses class D and E enzymes share only limited primary sequence homology with one another and are predicted to process distinct sorting signals. Moreover, unlike class D enzymes, genes encoding class E enzymes are not genomically clustered near their substrates.

Sortases that assemble pili: class C enzymes

Gram-positive bacteria use class C enzymes to build pili that promote microbial adhesion and biofilm formation. The pili extend 0.2–3.0 μm from the cell surface and are assembled in a two-stage process. First, one or more class C enzymes form the long thin shaft of the pilus by linking together pilin subunits via isopeptide bonds. The base of the pilus is then anchored to the cell wall by a housekeeping sortase or, in some cases, the class C enzyme itself. Although the general features of this process appear to be conserved, there is great species-specific variation in pilus structure and the mechanism of biogenesis. For example, depending upon the organism both the number and type of sortase enzymes involved varies, and in some cases, accessory factors appear to be needed (Fig. 3). Pili in Gram-positive bacteria are constructed from either two or three types of pilin subunits. In two-component pili the shaft of the pilus is formed by multiple copies of a major pilin subunit, while the tip of the pilus contains a single copy of a minor ‘tip’ pilin subunit that typically functions as an adhesin. Three-component pili are similar, but they also contain a minor ‘basal’ pilin subunit that is covalently attached to the cell wall. Several transmission electron microscopy (EM) and immuno-gold labelling studies have led to the conclusion that the minor ‘basal’ pilin subunits are also interspersed throughout the shaft of the pilus, presumably because the sortase enzymes are promiscuous in the substrates they recognize. However, this issue is controversial as more recent high-resolution EM imaging of *S. pneumoniae* pili indicates that only the major pilin subunit is present in the shaft (discussed below). Interestingly, some pilin subunits within the pilus contain intra-protein isopeptide bonds that form spontaneously. These bonds presumably stabilize the structure of the pilus, but they have not been shown to be required for pilus biogenesis (Kang *et al.*, 2007; Kang and Baker, 2009; Budzik *et al.*, 2009a). Because of space restraints, we only summarize what is known about the functions of sortases in the best characterized pilus systems and refer the reader to several excellent reviews on this topic for more detailed information (Ton-That *et al.*, 2004a; Scott and Zahner, 2006; Telford *et al.*, 2006; Mandlik *et al.*, 2008a; Proft and Baker, 2009; Kline *et al.*, 2010; Hendrickx *et al.*, 2011).

The paradigm: the three-component spaA pilus from *C. diphtheriae*

The prototypical SpaA pilus in *C. diphtheriae* is assembled in a two-stage process (Figs 1B and 3B). First, a class C sortase (called Cd-SrtA) joins the pilin subunits (SpaA, SpaB and SpaC) to construct the pilus shaft (Ton-That and Schneewind, 2003). This structure is then attached to the cell wall by a housekeeping class E sortase (called Cd-SrtF) (Swaminathan *et al.*, 2007). The shaft of the pilus is primarily formed by the main pilin subunit SpaA, and is decorated with two minor pilins that are present at lower abundance, SpaB and SpaC. The SpaC pilin is located at the tip of the pilus, while SpaB is primarily located at its base, but also infrequently interspersed throughout the shaft (Ton-That and Schneewind, 2003; Mandlik *et al.*, 2008b). In the working model of the assembly process put forth by Ton-That and colleagues, assembly begins with the export of the tip protein SpaC, which contains an LPLTG sorting signal that is cleaved between the threonine and glycine residues by the class

C enzyme (Guttilla *et al.*, 2009). This forms a sortase–SpaC complex wherein a thioacyl bond connects the sortase active site cysteine residue to the threonine carbonyl carbon in SpaC. Similarly, another copy of this class C enzyme recognizes a LPLTG sorting signal in the main pilin subunit, SpaA, forming a thioacyl sortase–SpaA intermediate. These acyl intermediates are then thought to react with one another when the free amino group originating from a lysine residue present in SpaA (Lys190) attacks the acyl bond in the sortase–SpaC intermediate. Lys190 is located in a ‘pilin’ motif (residues WXXXVX-VYPK) that is frequently conserved in other main pilin subunits (Ton-That and Schneewind, 2003). Its nucleophilic attack on the acyl bond results in dissolution of the sortase–SpaC intermediate and the creation of a sortase–SpaA–SpaC complex in which the SpaA and SpaC components are covalently linked to each other by an isopeptide bond (the carbonyl carbon in the threonine residue in the sorting signal motif of SpaC is joined to the ϵ -amino group of Lys190 in SpaA) (Ton-That and Schneewind, 2003). The nascent pilus is elongated through repetition of this transpeptidation reaction, with additional SpaA pilin subunits presumably entering the assembly reaction pre-loaded onto a sortase in the form of an acyl intermediate. Evidence that pilin subunits enter the reaction as sortase attached acyl intermediates is indirect. It is based upon the observation that only small amounts of polymerized pili are released into the solvent in wild-type cells, presumably because during the assembly process the nascent pilus is always covalently linked to a membrane associated sortase via a thioacyl bond. Furthermore, biochemical data demonstrate that *C. diphtheriae* class C enzymes form stable complexes with pilin subunits (Guttilla *et al.*, 2009). However, it is also conceivable that SpaA proteins that have yet to be processed by sortase might serve as substrates in the reaction instead of SpaA proteins that are acyl linked to sortase. In this alternative model, the side-chain of Lys190 within a membrane associated SpaA protein containing an intact CWSS would nucleophilically attack the acyl bond that joins the pilin polymer to the sortase. As a result, the growing pilus would become temporally associated with the membrane via the C-terminus of the recently added SpaA subunit. The polymer would then be transferred to a class C sortase when the enzyme cleaved the threonine residue in the LPLTG motif of the terminal membrane associated SpaA protein. As in the aforementioned ‘preloaded’ model that assumes that SpaA enters the assembly reaction as a sortase–SpaA acyl intermediate, a repetition of this process would grow the pilus from its base.

The class C enzyme also attaches the minor pilin SpaB to the SpaA pilus. SpaB does not contain a pilin motif, but alanine substitution of Lys139 within this protein abolishes its cross-linking to the SpaA pilus, suggesting this lysine is linked to the threonine residue within the sorting signal motif of SpaA (Mandlik *et al.*, 2008b). The minor pilin SpaC lacks a lysine residue that can be used by the class C enzyme as a nucleophile, explaining why it is only found at the tip of the pilus. Fascinatingly, incorporation of SpaB can act as a molecular switch to promote pilus anchoring to the cell wall (Mandlik *et al.*, 2008b). In accordance with the model put forth by Ton-That and colleagues, this occurs when the class E sortase (called Cd-SrtF) cleaves the LAFTG sorting signal to form a Cd-SrtF–SpaB acyl intermediate (Guttilla *et al.*, 2009). In a reaction that is presumably catalysed by the class C enzyme, Lys139 within the SpaB component of the Cd-SrtF–SpaB acyl intermediate attacks the acyl bond that joins the terminal SpaA subunit of the pilus polymer to the catalytic cysteine residue of the class C enzyme. This transfers the pilus fibre to Cd-SrtF and terminates the polymerization reaction because Cd-SrtF is incapable of using lysine residues located within the pilin subunits as a nucleophile, but instead transfers the pilus to the free amino group located within lipid II (Swaminathan *et al.*, 2007). Cd-SrtF can then recognize the LAFTG sorting signal within SpaB and attach the completed pilus to the free amino group within lipid II. In the final assembly step, cell wall biogenesis reactions then incorporate the lipid II linked pilus into the murein sacculus.

Pilus assembly appears to be a stochastic process, as both the number and length of pili is determined by the relative abundance of the major and minor pilin subunits (Swierczynski and Ton-That, 2006; Mandlik *et al.*, 2008b; Quigley *et al.*, 2009). For example, while it has not been shown in the SpaA pilus, in the related *C. diphtheriae* SpaH pilus, an increase in the amount of SpaH, the major pilin component, results in longer pilus fibres. As the SpaH pilus also contains a minor basal pilin that can be incorporated by its class C sortase that can terminate the assembly reaction (described below), increased amounts of the major pilin subunit presumably enable it to out-compete the minor basal pilin for access to the sortase thereby causing longer pili to be produced. Furthermore, the prevalence of the minor tip pilin used to construct the *S. pyogenes* T3 pilus (further discussed below) has been shown to influence the number of pili that are displayed suggesting that it competes with the major pilin subunit in this system to gain access to the sortase (Swierczynski and Ton-That, 2006; Quigley *et al.*, 2009). Although they play a key structural role, the minor pilin subunits in many pili are not required for pilus biogenesis. For example, in the SpaA pilus, neither SpaB nor SpaC are required for SpaA polymerization, albeit anchoring of the pilus to the cell wall is inefficient when SpaB is absent (Ton-That and Schneewind, 2003; Mandlik *et al.*, 2008b). SpaA, along with major pilin subunits from many other pilin systems, contains a highly conserved E-box motif, which when mutated prevents SpaA pilus formation (Ton-That *et al.*, 2004b). This motif does not appear to be directly involved in the sortase catalysed transpeptidation reaction, but a glutamic acid residue within it catalyses the formation of an intra-protein isopeptide bond that stabilizes the pilin subunit (Kang *et al.*, 2009).

The class C enzyme that assembles the SpaA pilus is amazingly promiscuous, as it can recognize two distinct sorting signals (LPLTG in SpaA and SpaC, and LAFTG in SpaB) and it can employ lysine residues that originate from different proteins (either Lys190 within the pilin motif of SpaA or Lys139 in SpaB) (Fig. 3B). In addition, in the absence of the housekeeping sortase Cd-SrtF, the class C enzyme attaches the pilus to the cell wall, albeit at a reduced rate (Swaminathan *et al.*, 2007). This indicates that it is able to use diaminopimelic acid as a nucleophile when it is located in the cross-bridge peptide of lipid II and/or in preassembled peptidoglycan. This ability may function as a fail-safe mechanism enabling pilus attachment if the housekeeping sortase is not available. Interestingly, when the SpaB sorting signal motif (LAFTG) is altered to the SpaA sorting signal moiety (LPLTG), the class C sortase was more successful in attaching the pilus to the cell wall in the absence of the class E sortase (Chang *et al.*, 2011). This suggests that the LAFTG sorting signal motif of SpaB is preferentially recognized by the class E enzyme, while the LPLTG motif in SpaA and SpaC is a better substrate for the class C enzyme. Very recently, the class C and E enzymes have been shown to work conjunctively to attach SpaB-SpaC heterodimers to the cell wall (Chang *et al.*, 2011). Mechanistically, this process is related to the pilus assembly reaction, with the class C enzyme linking SpaB via its K139 residue to the CWSS of SpaC. The class E enzyme then specifically recognizes the LAFTG sorting signal in SpaB to attach the heterodimer to the cell wall. Assembly of a heterodimer consisting of two disparate pilin adhesins may be a novel strategy for corynebacteria to form a lasting intimate zone of adhesion with their host cells.

Two-component pili

Pili in *B. cereus*, *Actinomyces* spp., and the FCT-1 *S. pyogenes* pilus systems are less complex and contain only two types of pilin subunits (Mora *et al.*, 2005; Budzik *et al.*, 2007; Mishra *et al.*, 2007). The pilus in *B. cereus* is prototypical and consists of a major pilin protein BcpA that forms the shaft, and a minor tip pilin, BcpB (Fig. 3A). These proteins are linked together by a single class C enzyme (called Bc-SrtD) (Budzik *et al.*, 2009b). It cleaves related sorting signals within BcpA (LPVTG) and BcpB (IPNTG), and catalyses a transpeptidation that joins the threonine residues in each signal to the side-chain of Lys162

in BcpA (located within a pilin motif) (Budzik *et al.*, 2008a). In a striking contrast to the three-component pili, two-component pili do not contain a minor basal pilin subunit. Assembly is terminated by a class A enzyme (called Bc-SrtA), which transfers the pilus fibre to the cell wall by joining the sorting signal within the basal BcpA subunit to the diaminopimelic acid moiety within lipid II and/or the cell wall (Budzik *et al.*, 2008b). Sortase specificity for the sorting signal appears to regulate the assembly process, as the class A enzyme is unable to process the IPNTG signal within BcpB, facilitating usage of this tip pilin by the class C enzyme. Interestingly, in the absence of the housekeeping enzyme pili are not covalently attached to the cell wall, indicating that, unlike the SpaA pilus in *C. diphtheria*, in *B. cereus* the class C enzyme is unable to attach the pilus to the cell wall and is reliant on the housekeeping sortase to perform this task (Budzik *et al.*, 2007).

Assembling pili using two class C enzymes

Gram-positive bacteria frequently use two class C sortase enzymes to assemble pili from three distinct pilin subunits. *C. diphtheriae* expresses SpaD- and SpaH-type pili in addition to the SpaA-type pili (Gaspar and Ton-That, 2006; Swierczynski and Ton-That, 2006). These separate pilus types each use their own specific sortases, and no cross-reactivity has been observed in spite of the similarities between their respective sorting signals. The SpaD pilus is assembled by two class C enzymes (called Cd-SrtB and Cd-SrtC), and consists of a main pilin, SpaD, a minor tip pilin, SpaF, and a basal pilin, SpaE, which is also interspersed throughout the shaft (Fig. 3C). The class C sortases appear to have partially redundant functions as each can polymerize SpaD and attach the SpaF tip pilin. However, only Cd-SrtB can incorporate SpaE presumably by joining the lysine within SpaE to the threonine residue within the CWSS of SpaD (Gaspar and Ton-That, 2006). This suggests that both enzymes are able to use the lysine residue within the SpaD pilin motif as a nucleophile and that each can recognize the LPMTG and LPKTG sorting signals within SpaD and SpaF respectively. The assembly process is completed when the terminal SpaE subunit in the fibre is transferred to the housekeeping class E enzyme, which then incorporates the fibre into the cell wall (Swaminathan *et al.*, 2007). This mechanism again highlights the substrate promiscuity of class C enzymes, as Cd-SrtB presumably uses distinct lysine residues located within the SpaD and SpaE proteins as nucleophiles, and it also recognizes distinct sorting signals within all three pilin subunits to form acyl intermediates. The SpaH pilus appears to be assembled using a similar mechanism, as it is also constructed from three pilin subunits (SpaG, SpaH and SpaI) by two class C enzymes (Cd-SrtD and Cd-SrtE) (Swierczynski and Ton-That, 2006).

Pilus assembly in *Streptococcus agalactiae* follows a similar pattern as the SpaD pilus in *C. diphtheria*, but is slightly more complicated (Fig. 3D). Its P1-2a pilus is formed from a major pilin, GBS59, which contains a pilin motif, and two minor pilins, GBS67 and GBS150 (Dramsi *et al.*, 2006; Rosini *et al.*, 2006). Two class C enzymes, Sag-SrtC3 and Sag-SrtC4, function redundantly to polymerize GBS59. Interestingly, recent work suggests that each class C enzyme predominantly incorporates a specific minor pilin into the pilus, with significantly reduced ability to incorporate the other minor pilin. Sag-SrtC4 incorporates the minor pilin GBS67 found at the pilus tip, and Sag-SrtC3 specifically incorporates GBS150, which is found within the shaft and at its base (Rosini *et al.*, 2006). Low-resolution EM images also reveal that GBS67 is incorporated throughout the pilus shaft; however, this could be artefactual (discussed below). There is conflicting data regarding the role of the class C enzymes in this system, as Dramsi *et al.* have proposed that they function redundantly to incorporate both minor pilins (Dramsi *et al.*, 2006). The class C enzymes in this system are unable to attach the pilus to the cell wall, which instead requires the class A housekeeping sortase that recognizes the LPKTG sorting signal present within GBS150 (Nobbs *et al.*, 2008). *S. agalactiae* also expresses an additional three-component

pilus system, PI-1, whose subunits share primary sequence homology with the components of the aforementioned PI-2a pilus. This pilus is also assembled by two class C enzymes (Sag-SrtC1 and Sag-SrtC2) that operate redundantly to polymerize the main pilin, suggesting that it is constructed in a similar manner as the PI-2a pilus (Dramsi *et al.*, 2006; Rosini *et al.*, 2006).

Streptococcus pneumoniae pili are even more complicated, as the pilus encoded from the *rlrA* pathogenicity islet is formed by three pilin proteins (RrgA, RrgB and RrgC) and three sortases (Spn-SrtC-1, Spn-SrtC-2 and Spn-SrtC-3) (Hava *et al.*, 2003; LeMieux *et al.*, 2006). Transmission EM images have shown that the shaft of the pilus is primarily composed of RrgB, and that the minor pilins RrgA and RrgC are located at the tip and base of the pilus respectively (Hilleringmann *et al.*, 2009). RrgA may also be integrated throughout the pilus (LeMieux *et al.*, 2006; Falker *et al.*, 2008) and can form heterodimers with RrgC similar to what is seen in *C. diphtheriae* (LeMieux *et al.*, 2008). Spn-SrtC-1 recognizes each of the individual subunits and polymerizes mature pilus structures *in vivo*, and can readily polymerize RrgB subunits *in vitro* (Falker *et al.*, 2008; Manzano *et al.*, 2008). The role of Spn-SrtC-2 is less clear, with some research groups reporting an ability to polymerize the major pilin subunit, RrgB, along with the ancillary RrgA (Falker *et al.*, 2008), while others report no RrgB polymerization function by Spn-SrtC-2 alone (Manzano *et al.*, 2008). Spn-SrtC-3 has been implicated in pilus localization on the cellular surface, suggesting a direct role in anchoring the constructed pilus to the cell wall (Falker *et al.*, 2008). As with Spn-SrtC-2, the ability of Spn-SrtC-3 to polymerize specific pilus subunits has not yet been fully elucidated. Unlike pili in *C. diphtheriae*, *B. cereus* and *S. agalactiae*, the housekeeping sortase is not required for anchoring the *S. pneumoniae* pili to the cellular surface (LeMieux *et al.*, 2008). This may be due to the inability of the Spn-SrtA enzyme to recognize the sorting signals on each of the *S. pneumoniae* pilin subunits, and because all three SrtC enzymes have demonstrated an ability to attach pilin subunits to the peptidoglycan (Neiers *et al.*, 2009).

Accessory factors and class B enzymes are involved in pilus biogenesis

In nearly all pilus systems studied to date, the minor pilins are dispensable for polymerization of the main pilin subunit that forms the shaft of the pilus (*C. diphtheriae*, *B. cereus*, *S. pneumoniae*, *S. pyogenes* and *S. agalactiae*). Interestingly, a two-component pilus in *Streptococcus suis* appears to be the exception to this rule. Similar to the *B. cereus* paradigm, it contains a minor pilin that is located at the tip of the pilus (Sgp2) and a major pilin that forms the shaft (Sgp1). When Sgp2 is deleted, there is a significant decrease in the amount of polymerized Sgp1, a phenotype that is similar to knocking out the class C enzyme (Ss-SrtG) (Okura *et al.*, 2011). This suggests that the minor pilin Sgp2 functions as a chaperone for Sgp1, or that its presence, through an unknown mechanism, is required to initiate the polymerization of Sgp1. In a surprising example of convergent evolution, a class B enzyme, not a class C enzyme, assembles pili in *S. pyogenes* (Kang *et al.*, 2011). The FCT-3 pilus in *S. pyogenes* serotype M3 is constructed by a single class B sortase, Sp-SrtC2, which polymerizes the major pilin subunit Tee3/FctA and attaches the minor tip pilin Cpa (Fig. 3E) (Quigley *et al.*, 2009). This system also contains a minor pilin FctB that contains a LPLAG sorting signal. Its location and sortase dependent incorporation into the pilus has not been extensively explored. However, based on amino acid sequence homology, it is likely located at the base of the pilus (Smith *et al.*, 2010). Interestingly, assembly of the FCT-3 pilus also requires the SipA2/LepA protein that shares sequence homology with signal peptidases, but is presumably inactive as it is missing key active sites residues (Zahner and Scott, 2008). It has been proposed that SipA2/LepA functions as a chaperone that associates with Tee3/FctA, reminiscent of pilus assembly in Gram-negative bacteria. Sp-SrtC2 highlights the diversity of sortases, as the side-chain amino group of K173 within

the main pilin Tee3/FctA that it joins to other subunits is not located within a pilin motif. In addition, Cpa and Tee3/FctA contain very distinct signals, VPPTG and QVPTG, respectively, which are nevertheless recognized by Sp-SrtC2. The use of peptidase-like proteins as chaperones may be common to many pilus systems as a SipA homologue is also required for the assembly of the PI-2 pilus in *S. pneumoniae* (Bagnoli *et al.*, 2008) and fimbrial gene clusters in *Actinomyces naeslundii* also contain genes encoding peptidase-like proteins (Mishra *et al.*, 2007).

Class C sortase substrate recognition

Across the multiple species and pilin systems studied to date, the pilin polymerizing sortases demonstrate a unique variance in their ability to recognize a variety of sorting signals and amino groups. For example, cell wall attachment of pili in *S. agalactiae* relies on the housekeeping sortase as the class C sortase is unable to do this, while in *S. pneumoniae* the housekeeping sortase is unnecessary for cell wall attachment (LeMieux *et al.*, 2008; Nobbs *et al.*, 2008). The pilin polymerizing sortases also have little ability to function outside of their specific system. For example, Cd-SrtA is able to polymerize SpaA, but not SpaH, even though both major pilin subunits have identical LPLTG motifs (Swierczynski and Ton-That, 2006). There are obviously other factors beyond the CWSS that control how sortases function and recognize specific substrates. This is likely the case for other types of sortases as domain switching experiments performed by Barnett and co-workers have shown that both the class A and pilin polymerase class B enzymes in *S. pyogenes* recognize features in their protein substrates in addition to their respective sorting signals (Barnett *et al.*, 2004). The recent crystal structure of FctB, the anchoring pilin subunit from *S. pyogenes*, has provided insight into how minor pilins are recognized and attached to the cell wall. FctB contains an Ig-like domain with striking resemblance to the N-terminus of the previously solved *S. pyogenes* major pilin, with nearly identical placement of the intermolecular isopeptide bond-forming lysine (Kang *et al.*, 2007; Linke *et al.*, 2010). This helps explain how a single sortase can recognize both lysines on the major and anchoring pilin subunits. Unique to FctB, however, is a long proline-rich C-terminal tail forming a polyproline-II (PPII) helix before the start of the sorting signal. In the major pilin, the sorting signal begins immediately after the globular domain, with little separation (Linke *et al.*, 2010). Closer examination of other pilin sequences revealed that most anchoring pilin subunits have a proline-rich tail separating the globular domain from the sorting signal (Linke *et al.*, 2010). This difference may allow the housekeeping sortase to preferentially utilize the anchoring pilin subunits, while the pilin polymerizing sortase prefers the sorting signal more proximal to the globular domain (see discussion on SrtC structure below).

Some class C sortases also promiscuously integrate pilin subunits. In many species, the basal pilin is also intermittently integrated into the pilus shaft rather than terminating polymerization and resulting in cell wall anchoring (Fig. 3). For example, in *C. diphtheriae*, SpaB is generally necessary for cell wall anchoring, but it is not sufficient, as in some cases it can be incorporated into the pilus by being attached to a SpaA molecule (Fig. 3B). This process occurs less frequently than SpaA incorporation, as immuno-gold microscopy experiments indicate that only small amounts of SpaB are interspersed throughout the pilus shaft (Ton-That and Schneewind, 2003). This presumably occurs because Cd-SrtA occasionally recognizes the LAFTG sorting signal of SpaB and attaches it to the pilin motif lysine within SpaA. It is unclear how frequently the basal pilin is incorporated in the pilus shaft; however, it has been reported in other pilin systems, including SpaD from *C. diphtheriae* (Gaspar and Ton-That, 2006), *S. agalactiae* (Rosini *et al.*, 2006) and *S. pneumoniae* (LeMieux *et al.*, 2006; Falker *et al.*, 2008). In some cases, the minor tip pilin has also been seen interspersed throughout the pilus shaft. However, this issue is controversial, as more recent higher resolution EM studies of a pilus from *S. pneumoniae*

have shown that its minor tip and basal pilin subunits are only present at the termini (LeMieux *et al.*, 2008; Hilleringmann *et al.*, 2009). For the case of the minor tip pilin, its presence within the shaft of the pilus in low-resolution EM images could be artefactual and caused by the bundling of pili of differing lengths. The presence of basal minor pilin in these EM images presumably is not artificial, as bundling would not explain its positioning distal to the cell wall. Instead, the presence of the basal minor pilin subunits within the shaft presumably occurs because the sortase enzymes that assemble them are promiscuous in the substrates that they recognize.

Molecular basis of catalysis – why so slow?

Given their key roles in displaying virulence factors and assembling pili, considerable effort has been put forth to understand the catalytic mechanism of transpeptidation. This process is best understood for Sa-SrtA, as the transpeptidation reaction it catalyses can be replicated *in vitro* using peptide substrates (Marraffini *et al.*, 2006; Clancy *et al.*, 2010). Structural and biochemical experiments have shown that Sa-SrtA adopts an eight-stranded β -barrel structure that houses three essential active site residues: His120, Cys184 and Arg197 (Fig. 4). Transpeptidation proceeds through a ping-pong mechanism that is initiated when the LPXTG sorting signal binds to a large groove adjacent to the active site (Huang *et al.*, 2003; Frankel *et al.*, 2005). An NMR structure of Sa-SrtA covalently bound to an analogue of the sorting signal revealed that binding occurs through an induced-fit mechanism (Suree *et al.*, 2009). A large active loop, called the $\beta 6/\beta 7$ loop, undergoes a disordered–ordered transition so as to partially encapsulate the leucine residue within the sorting signal. The active site thiol of Cys184 then nucleophilically attacks the carbonyl carbon of the substrate threonine residue, forming a transient tetrahedral oxyanion intermediate that may be stabilized by Arg197 (Huang *et al.*, 2003; Frankel *et al.*, 2005). This intermediate rearranges into a more stable thioacyl enzyme-substrate complex after breakage of the threonine-glycine peptide bond. The terminal amine group within the pentaglycine branch of lipid II then nucleophilically attacks the carbonyl carbon of the threonine, initially generating a second tetrahedral intermediate that subsequently rearranges into the final product. How lipid II is recognized remains a mystery, but based on NMR data, binding of the sorting signal may induce a structural change that unmasks a second groove on the enzyme that interacts with this substrate (Suree *et al.*, 2009). The side-chain of His120 is positioned within this groove, and may function as a general acid to protonate the substrate leaving group of the first tetrahedral intermediate, and/or as a general base that deprotonates the amine group of the lipid II nucleophile (Frankel *et al.*, 2005; 2007). Other sortases likely use a similar mechanism to catalyse transpeptidation as the active site arginine, cysteine and histidine residues are conserved.

Atomic structures of representative class A, B and C enzymes have revealed interesting structural variations that could contribute to function (Fig. 4) (Ilangovan *et al.*, 2001; Zhang *et al.*, 2004; Zong *et al.*, 2004a,b; Manzano *et al.*, 2008; 2009; Neiers *et al.*, 2009; Race *et al.*, 2009; Suree *et al.*, 2009; Weiner *et al.*, 2010; Cozzi *et al.*, 2011; Kang *et al.*, 2011; Lu *et al.*, 2011; Persson, 2011). Perhaps not surprisingly, class C enzymes that assemble pili exhibit some of the largest structural differences with Sa-SrtA. Crystal structures have revealed that these enzymes contain a novel polypeptide segment called a ‘lid’ that contacts the active site and masks the presumed binding site for the sorting signal (Manzano *et al.*, 2008; Neiers *et al.*, 2009; Cozzi *et al.*, 2011; Lu *et al.*, 2011; Persson, 2011). *S. agalactiae* SrtC1 proteins containing mutations in the lid display similar *in vitro* activity compared with the wild-type enzyme (Cozzi *et al.*, 2011), and recent studies of *S. pneumoniae* SrtC-1 suggest that its lid is unstable in the apo-enzyme (Manzano *et al.*, 2009). This has led to the hypothesis that the lid has a regulatory function, restricting sorting signal access to the active site until other factors or substrates involved in pilus biogenesis dislodge it (Cozzi *et al.*,

2011). Interestingly, not all class C enzymes contain a lid, suggesting that other regulatory mechanisms may be at work for these sortases. This issue is only beginning to be explored, but studies thus far have shown that enzymes lacking a lid can require additional factors for proper pilus assembly. For example, the *S. suis* SrtC enzyme does not contain a lid and the assembly reaction it mediates requires the minor pilin subunit Spg2 to initiate pilus formation (Lu *et al.*, 2011; Okura *et al.*, 2011). The pilin polymerase class B enzyme from *S. pyogenes* also doesn't contain a lid and requires the SipA2/LepA protein to construct the pilus (Zahner and Scott, 2008; Kang *et al.*, 2011).

Class B sortases also exhibit unique structural variations that may enable them to recognize unusual sorting signals (Zhang *et al.*, 2004; Zong *et al.*, 2004b; Kang *et al.*, 2011). In the structures of these enzymes the short $\beta 6/\beta 7$ loop present in class A and C sortases is replaced with a large extended α -helical segment. In Sa-SrtB, this segment contains residues important for recognizing its novel NPQTN sorting signal, as domain swapping experiments that replaced the $\beta 6/\beta 7$ loop in Sa-SrtA with the corresponding extended loop in Sa-SrtB changed the specificity profile towards that of Sa-SrtB (Bentley *et al.*, 2007). Interestingly, the recently determined structure of the pilin polymerase class B enzyme from *S. pyogenes* also contains an extended α -helical segment, which is presumably involved in recognizing an unusual EVPTG sorting signal (Kang *et al.*, 2011). Structural studies of several class A enzymes have also revealed interesting variations that may be important for function. In addition to Sa-SrtA, the structures of the class A enzymes from *B. anthracis* and *S. pyogenes* have been determined (Race *et al.*, 2009; Weiner *et al.*, 2010). Surprisingly, both enzymes appear to contain a preformed binding pocket for the sorting signal, as the $\beta 6/\beta 7$ loop in these enzymes is structurally ordered in the absence of their substrate. Thus, unlike Sa-SrtA, these enzymes may recognize the LPXTG sorting signal through a lock-and-key mechanism. Interestingly, the *B. anthracis* class A enzyme also contains an N-terminal α -helical appendage that contacts the imidazole ring of the active site histidine residue (Weiner *et al.*, 2010). It may participate in the recognition of lipid II, or could have a regulatory function similar to the lid structures found in class C enzymes. Clearly, much work needs to be done to understand how these structural differences impact sortase function.

A common feature shared by sortase enzymes studied to date is that they have low enzymatic activity *in vitro*. For the case of Sa-SrtA, McCafferty and colleagues have proposed that key active site residues in this enzyme are improperly ionized (Frankel *et al.*, 2005). To be active, the Cys184 and His120 side-chains presumably need to be in their ionized thiolate and imidazolium forms respectively. However, based on pKa measurements, only ~ 0.1% of the Sa-SrtA exists in this state; in the remainder of the enzymes the cysteine and histidine residues are uncharged (Connolly *et al.*, 2003; Frankel *et al.*, 2005). The available structural data suggest that other sortases may have a similar problem that reduces their activity, because in all structures determined to date the active site histidine and cysteine residues are positioned too far apart from one another to form a thiolate-imidazolium ion pair, and are thus presumably uncharged. This has recently been substantiated for the *B. anthracis* SrtA enzyme, as pKa measurements indicate that its active site histidine is predominantly uncharged at physiological pH (Weiner *et al.*, 2010). On the cell surface, sortase enzymes may need to associate with other factors to be fully functional. These factors may facilitate catalysis by properly guiding the substrates into the enzyme active site and/or they could alter the electrostatic environment such that the active site cysteine was converted to its nucleophilic thiolate form. This notion is compatible with biochemical data that have shown that some sortases require the Spa2/LepA protein to be fully functional (Zahner and Scott, 2008; Okura *et al.*, 2011).

Colocalizing protein secretion, anchoring and cell wall synthesis machinery may increase the efficiency of protein display

Several recent findings suggest that efficiency and fidelity of protein anchoring is increased by coordinating this process with protein secretion and cell wall synthesis. The secretion translocon in Gram-positive bacteria is localized to discrete sites. In rod-shaped *Bacillus subtilis*, protein secretion appears to be restricted to spiral-like clusters along the longitudinal axis of the cell (Campo *et al.*, 2004), while in streptococcal cells single or multiple distinct secretion foci have been detected adjacent to the newly forming septum (Rosch and Caparon, 2004; Carlsson *et al.*, 2006). Immuno-gold electron micrographs indicate that sortases colocalize with the SecA component of the translocation machinery at these foci, suggesting that protein secretion and anchoring occur at the same site in streptococci and enterococci (Hu *et al.*, 2008; Kline *et al.*, 2009). The efficiency of the sorting reaction may also be increased by localizing it to sites where the cell wall is being synthesized and where the lipid II substrate is presumably most abundant. This has been observed in *S. pyogenes* as its class A enzyme predominately localizes to the septum, with its presence peaking during septation and then decreasing after cell division (Raz and Fischetti, 2008). Similarly, in dividing *S. aureus* cells proteins attached to the cell wall by Sa-SrtA are mainly deposited at two to four foci close to a layer of newly synthesized cell wall. Cell growth and expansion then generate a more diffuse distribution of the proteins in a ring-like pattern (DeDent *et al.*, 2007; 2008). Interestingly, in *S. aureus* some proteins may also be anchored to a second site that is on the cell pole distal to the septum. The final destination of Sa-SrtA attached proteins seems to be determined by their signal peptides, with proteins harbouring a conventional signal sequence at their N-termini being directed to peripheral sites, while proteins containing a signal peptide with an YSIRK/GS motif are retained at sites close to the septum where their attachment is presumably coupled to cell wall synthesis (DeDent *et al.*, 2008). Sequence elements within sortases may also function to properly direct them to specific sites on the cell surface, as deletion of the positively charged residues at the C-terminus of the class C enzyme from *E. faecalis* resulted in decreased focal localization of the enzyme and disrupted pilus biogenesis (Kline *et al.*, 2009). Interestingly, deletion of *srtA* or *srtC* genes involved in pilin biogenesis also caused their respective substrates to accumulate at a single focus, suggesting that the CWSS or another sequence element may act to retain the substrates at the secretion locus before them being processed by a sortase. In *C. diphtheria*, the major and minor pilins that form the SpaA pilus colocalize with their cognate sortase, and led to the proposal by Ton-That and colleagues that pilus biogenesis in Gram-positive bacteria occurs within highly organized 'pilosome' assembly centres (Guttilla *et al.*, 2009). Formation of sortase oligomers could further function to increase the efficiency of the sorting reaction. Sa-SrtA dimers detected by gel electrophoresis under native conditions and size exclusion chromatography were reported to display increased activity compared with monomeric proteins (Lu *et al.*, 2007). Sortase dimerization could be disrupted by amino acid substitutions in the putative interaction surface (Zhu *et al.*, 2008). However, it remains to be seen whether sortases also interact on the cell surface and whether formation of such oligomers facilitates catalysis *in vivo*.

Conclusion

Members of the sortase enzyme superfamily decorate the surfaces of Gram-positive bacteria with proteins that play key roles in microbial physiology and pathogenesis. Genome sequencing efforts have identified nearly a thousand sortase homologues whose functions are only beginning to be discovered. All sortases characterized to date are cysteine transpeptidases that either covalently join proteins to the cell wall peptidoglycan, or link proteins together to construct pili. Based on their primary sequences around ~60% of sortase

homologues in Gram-positive bacteria can be clustered into six families, called class A to F enzymes. Class A and C sortases have been studied extensively. Most class A enzymes appear to have a housekeeping role in the cell as members of this group attach a large number of functionally distinct proteins to the cell wall, while all class C enzymes studied to date assemble pili. The functions of other types of sortases are less clear as only a few representative members have been investigated, and in some instances related enzymes within a given class have been shown to have distinct functions. In the future, it will therefore be important to move beyond classification schemes that are based solely on overall primary sequence homology by identifying specific enzyme features that confer function. In particular, structure-function studies will need to be performed to identify enzyme determinants that control substrate specificity. This will bring to light how sortases selectively route different proteins to the cell surface by recognizing their unique sorting signals, and reveal how they can function to either attach proteins to the cell wall or assemble pili. It will also be critical to learn the molecular basis through which sortases work with other enzymes and accessory factors on the cell surface, which will require obtaining a better understanding of the targeting and retention mechanisms of these proteins as well as the generation of robust *in vitro* systems to study pilus assembly. Many surprises likely await as hundreds of sortase homologues can't be classified into the aforementioned subfamilies suggesting that they will be found to perform novel functions on the cell surface.

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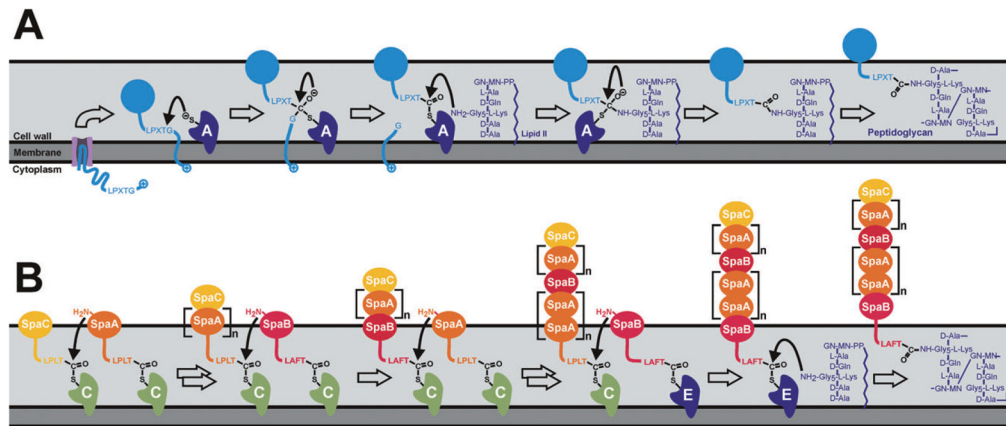


Fig. 1. Mechanisms of sortase mediated attachment of surface proteins and pilus assembly at the bacterial cell wall.

A. The *S. aureus* housekeeping sortase A anchors surface proteins to the peptidoglycan. The precursor protein containing an amino terminal leader peptide is secreted across the membrane through the Sec pathway. The exported protein (light blue) is processed by the sortase enzyme (dark blue, labelled 'A'), which recognizes the LPXTG sequence and cleaves the surface proteins between the threonine and glycine residues of the motif. The enzyme then recognizes the pentaglycine cross-bridge peptide of lipid II as the second substrate. Subsequent formation of a peptide bond between the carbonyl of the threonine and the free amino group of the cross-bridge peptide results in covalent attachment of the protein to lipid II. The surface protein is then fully incorporated into the cross-linked peptidoglycan via the transglycosylation and transpeptidation reactions during the bacterial cell wall synthesis. The sphere coloured light blue represents the folded form of the cell surface displayed protein.

B. Pilin-specific and housekeeping sortases assemble the SpaA pilus in *C. diphtheria*. The formation of complexes between the pilus-specific sortase C (light green) and the tip protein SpaC (light orange) initiates pilus assembly. The class C enzyme also recognizes the main pilin subunit SpaA (orange) forming SrtC-SpaA complexes. Nucleophilic attack by the free amino group originating from a lysine residue present in SpaA results in dissolution of the sortase-SpaC intermediate and the formation of a sortase-SpaA-SpaC complex. Repetition of this transpeptidation reaction results in pilus elongation. The class C sortase also incorporates the minor pilin SpaB (red) into the growing shaft by an analogous mechanism. Termination of pilus biogenesis is presumably initiated when the pilin polymer is transferred to the class E type housekeeping sortase (dark blue), which subsequently catalyses the nucleophilic attack by the amino group within lipid II. In the final assembly step the lipid II linked pilus is incorporated in the murein sacculus via normal cell wall biosynthesis.

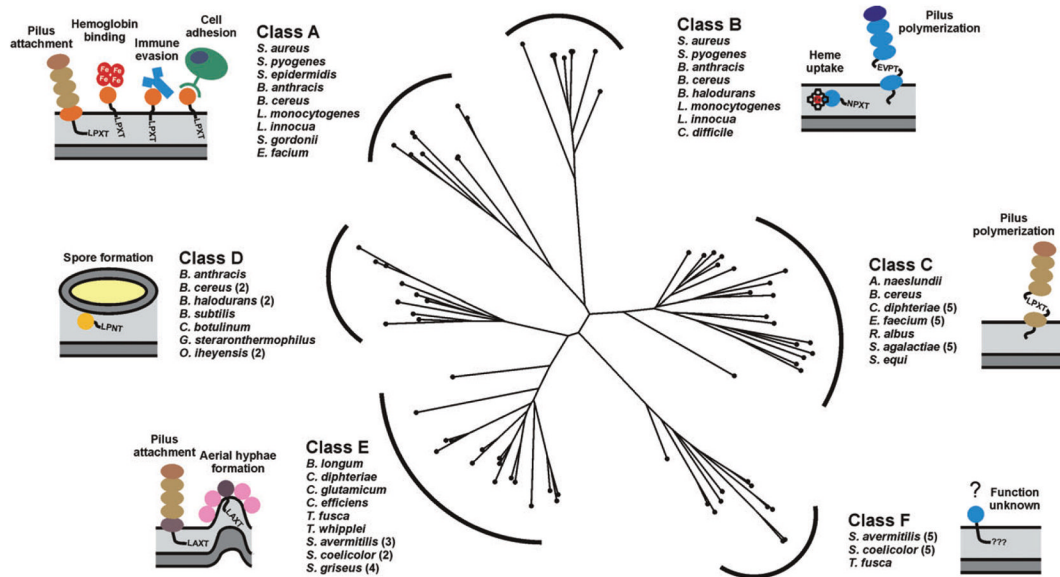


Fig. 2.

Phylogenetic tree showing the relationships among the six classes of sortases from Gram-positive bacteria. A multiple sequence alignment based on pairwise constraints of a selected set of 73 sortase proteins was generated using the program COBALT and a phylogenetic tree constructed using the neighbour joining method (Papadopoulos and Agarwala, 2007). The analysed sortases can be partitioned into six distinct subfamilies based on their primary sequences. It should be noted that the class D and E enzymes described here are collectively referred to as a class D enzymes by Biene and colleagues (Dramsai *et al.*, 2005). Class D and E enzymes have also previously been referred to as subfamily-4 and -5 enzymes (Comfort and Clubb, 2004). The bacterial species associated with the enzyme classes A–F are listed and schematic representations of the main biological function of their corresponding sortase substrates are illustrated. The bacterial species and accession numbers of the amino acid sequences used for the alignment are as followed: *Actionomyces naeslundii* (AAC13546), *Bacillus anthracis* (NP_847260; NP_843215; NP_846988), *Bacillus cereus* (NP_834511; NP_830752; NP_830495; NP_834250; NP_832268), *Bacillus halodurans* (NP_244463; NP_244878; NP_244160), *Bacillus subtilis* (NP_388801), *Bifidobacterium longum* (NP_695779), *Clostridium botulinum* (YP_001254630), *Clostridium difficile* (YP_001089230), *Corynebacterium diphtheriae* (NP_940343; NP_940532; NP_940531; NP_938627; NP_940575; NP_938624), *Corynebacterium efficiens* (NP_739396), *Corynebacterium glutamicum* (NP_602126), *Enterococcus faecium* (ZP_05713110; ZP_05714716; ZP_05713995; ZP_05713369; ZP_00603528; ZP_05713288), *Geobacillus* sp. (YP_003672707), *Listeria innocua* (NP_470268; NP_471617), *Listeria monocytogenes* (NP_464454; NP_465705), *Oceanobacillus iheyensis* (NP_691253; NP_694114), *Ruminococcus albus* (ZP_06717336), *Streptomyces avermitilis* (NP_826393; NP_827770; NP_826840; NP_828474; NP_821266; NP_825513; NP_825514; NP_826383; NP_825510), *Streptomyces coelicolor* (NP_628037; NP_628038; NP_627928; NP_631498; NP_626722; NP_625233; NP_627070), *Streptomyces griseus* (YP_001825232; YP_001825235; YP_001826193; YP_001825236), *Staphylococcus aureus* (NP_375640; NP_374252), *Staphylococcus epidermidis* (NP_765631), *Streptococcus agalactiae* (NP_687667; NP_687668; NP_687670; NP_688403; NP_688404), *Streptococcus equi* (YP_002746265), *Streptococcus gordonii* (YP_001450517), *Streptococcus pyogenes* (NP_802272; NP_801365), *Thermobifida fusca* (YP_290439; YP_288977), *Tropheryma whipplei* (NP_787692).

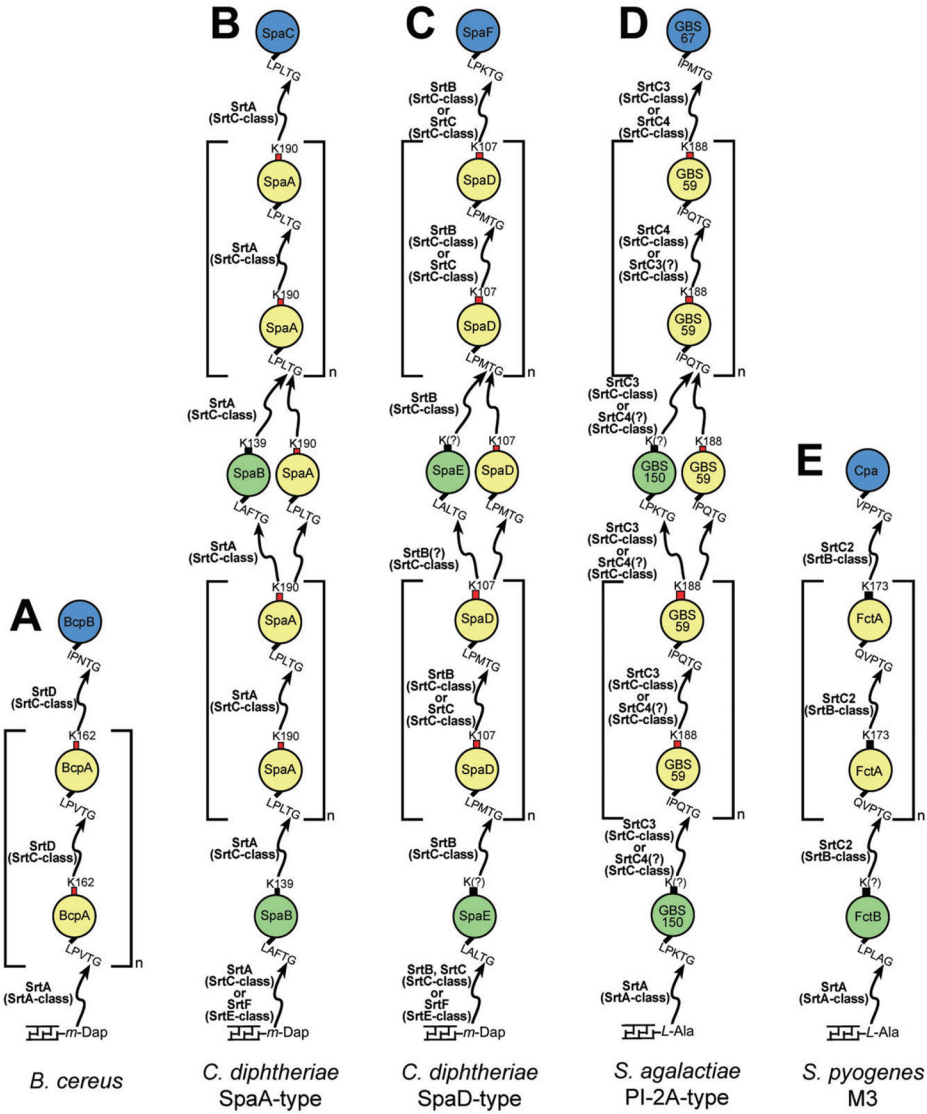


Fig. 3. The role of sortase enzymes in pilus assembly in various Gram-positive bacteria. Gram-positive bacteria are diverse in their assembly of pili with respect to the number of pilin subunits, number of sortases, class of sortases, as well as the type of sorting signals, lysine residues and cell wall acceptors recognized. Red boxes indicate lysine residues within a WXXXV.XVYPK pilin motif, while black boxes indicate the absence of an obvious pilin motif. Lack of biochemical data demonstrating which lysine residue is involved in pilus polymerization is indicated with (?).

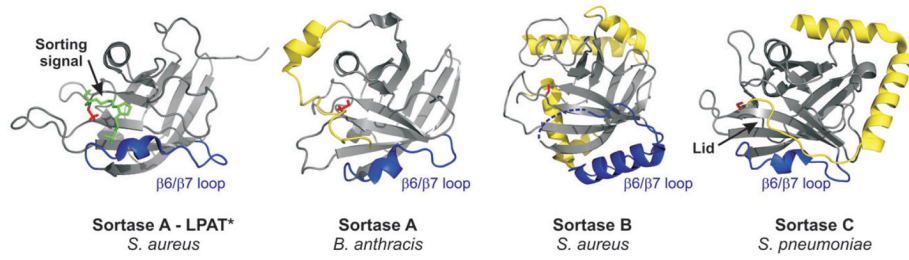


Fig. 4. Sortase structure overview. Representative structures of sortase enzymes from different classes are shown. Class A: SrtA from *S. aureus* in complex with the sorting signal analogue LPAT* (green) (PDB code 2KID) and SrtA from *B. anthracis* (PDB code 2KW8). Class B: SrtB from *S. aureus* (PDB code 1NG5). Class C: SrtC1 from *S. pneumoniae* (PDB code 3G66). The $\beta 6/\beta 7$ loops are coloured in blue. Unique structural features are highlighted in yellow: the N-terminal extension in *B. anthracis* SrtA, the additional helices in SrtB from *S. aureus* and the flexible lid in *S. pneumoniae* SrtC1. The catalytic cysteine residues are highlighted in red and shown in a stick representation.