Architects at the bacterial surface — sortases and the assembly of pili with isopeptide bonds

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Abstract | The cell wall envelope of Gram-positive bacteria can be thought of as a surface organelle for the assembly of macromolecular structures that enable the unique lifestyle of each microorganism. Sortases — enzymes that cleave the sorting signals of secreted proteins to form isopeptide (amide) bonds between the secreted proteins and peptidoglycan or polypeptides — function as the principal architects of the bacterial surface. Acting alone or with other sortase enzymes, sortase construction leads to the anchoring of surface proteins at specific sites in the envelope or to the assembly of pili, which are fibrous structures formed from many protein subunits. The catalysis of intermolecular isopeptide bonds between pilin subunits is intertwined with the assembly of intramolecular isopeptide bonds within pilin subunits. Together, these isopeptide bonds endow these sortase products with adhesive properties and resistance to host proteases.

Electron microscopy was essential for the study of microbial surfaces, as only this technology could reveal the intricate structures of cell wall peptidoglycan, S-layers (crystalline surface layers), pili and surface proteins[1–4]. Pili in Gram-positive bacteria were first detected in 1968, when Yanagawa and colleagues[5,6] reported fibrous structures on the surface of *Corynebacterium renale* and *Corynebacterium diphtheriae*. The discovery that sortase enzymes are responsible for the surface display and cell wall anchoring of surface proteins carrying the sortase recognition motif LPXTG, and that these enzymes catalyse pilus polymerization, was an important insight into the cell biology of Gram-positive bacteria[7–9]. Sortases are transpeptidase enzymes that covalently connect their substrates to a target molecule[7,10–12]. Structures of multiple sortases from different bacteria have been solved and display comparable overall compositions[13–21]; the backbone is similar among isoforms and consists of an uneven eight-stranded β-barrel with connecting random-coil loops. All sortases contain a TLXTC motif in their active site as well as multiple conserved residues that are required for transpeptidation activity.

Genome sequencing of Gram-positive bacteria has uncovered thousands of genes encoding proteins with LPXTG sorting signals, alongside a plethora of sortase genes[22–24], suggesting that these enzymes cannot all act on the same substrates[25,26]. Therefore, sortases have been classified into four groups (sortase classes A–D) on the basis of sequence homology, the substrate for sortase cleavage and the nucleophile accepted by the sortase[27] (TABLE 1). Note that the sortase class A–D classification is used throughout this manuscript. However, many sortases have received designations that relate to the order of their discovery in various Gram-positive bacteria; to avoid confusion, these enzymes are described with the acronyms SrtA–F.

Sortase class A enzymes are present in all Gram-positive bacteria and are often referred to as housekeeping sortases. They recognize the sequence LPXTG at the carboxyl terminus of surface protein precursors[7,9–11,28]. The product of the sortase class A reaction is a surface protein that is covalently linked to lipid II and is then incorporated into the cell wall envelope (FIG. 1). Sortase class A substrates include surface proteins from the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family that have been implicated in the virulence of multiple Gram-positive bacterial species[22,29,30].

Sortase class B enzymes are encoded by several *Staphylococcus* spp., *Bacillus* spp., *Listeria* spp. and *Clostridium* spp.[17,31–34]. Sortase class B enzymes recognize a unique NP(Q/K)TN sorting signal in proteins that are involved in haem-iron scavenging, and cross-link the anchored haem-containing products near membrane

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Table 1 | **Sortase classes, substrate specificity and nucleophiles**

Sortase class	Substrates	Substrate motif	Nucleophile
A	Surface proteins	LPXTG	Lipid II[9,28,10]
B	Haem transport factor	NP(Q/K)TN	Peptidoglycan crossbridge[33,34]
C	Pilin proteins	(I/L)(P/A)XTG	Lys residue of pilins[27,35]
D	Mother cell and endospore envelope proteins	LPNTA	Lipid II[37,38]

transporters. The β-barrel structure of sortase class B enzymes is similar to that of the class A enzymes but encompasses additional α-helices[17,18].

The pilin-specific class C sortases polymerize pili by catalysing transpeptidation reactions that form covalent links between individual subunits of these fibrous structures[35]. In contrast to sortases that only accept single cell wall peptides as nucleophiles, pilin-specific sortases recognize multiple pilin proteins both as substrates for cleavage of the LPXTG-like motif and as nucleophiles. Pilin-specific class C sortases are encoded in pilin gene clusters with their cognate substrates and often contain a C-terminal hydrophobic domain not found in sortases from other classes. The crystal structures of the three pilin-specific sortase class C enzymes from *Streptococcus pneumoniae* (SrtB, SrtC and SrtD (also known as SrtC1, SrtC2 and SrtC3, respectively)) revealed that these enzymes adopt structures comparable to those of sortase class A and sortase class B proteins[14,15]. Interestingly, the α3 helix forms a flexible lid over the active site; this is a unique feature of pilin-specific sortases and is required for efficient polymerization of pili and for enzyme stability[36].

Last, sortase class D enzymes are expressed in spore-forming microorganisms. In *Bacillus* spp. and *Streptomyces* spp., sortase class D enzymes recognize and cleave the sorting signals of select substrates to immobilize their anchored products in the cell wall envelope during spore formation[37,38]. To date, no crystal structure of a sortase class D enzyme has been solved.

Here, we review mechanisms of sortase action and the role of sortases in the formation of pili, as well as the development of vaccines to prevent infections by Gram-positive bacteria.

Mechanism of cleavage

All sortases recognize a specific sorting signal at the C terminus of their target protein. The specificity of cleavage is determined by recognition of sortase-specific sorting signals (TABLE 1); each sortase cleaves its specific sorting signal and subsequently forms an acyl (thioester) bond between the sortase active site and a residue in the sorting signal. This acyl intermediate is then resolved by a specific nucleophilic amino acid, thus specifying the cell wall component to which the protein becomes linked. For example, in *Staphylococcus aureus* the class A sortase cleaves the LPXTG sorting signal between the Thr and Gly residues to form an acyl intermediate between the Thr residue of the surface protein and a reactive Cys in the TLXTC catalytic pocket of the sortase (FIG. 1). Subsequently, the acyl intermediate is resolved by nucleophilic attack by the cell wall precursor lipid II. In *S. aureus*, lipid II consists of an undecaprenylpyrophosphate anchor linked to *N*-acetylmuramyl (MurNAc), which is in turn bound to *N*-acetylglucosamine (GlcNAc) and to the cell wall pentapeptide L-Ala–γ-D-Gln-L-Lys–D-Ala–D-Ala; the L-Lys is bound to a short peptide (a pentaglycine in *S. aureus*) that will normally form the crossbridge between two pentapeptides of different peptidoglycan strands[28]. However, the terminal amino acid of the pentaglycine can also 'attack' the linkage between the sortase and the surface protein, forming an amide bond that tethers the surface protein to lipid II in the bacterial plasma membrane. Subsequent steps then transfer the polysaccharide residues and the attached protein to the peptidoglycan cell wall. In order to solubilize sortase-anchored proteins, murein (peptidoglycan) must therefore be treated with peptidoglycan hydrolases[39,40].

SrtD, the pilin-specific class C sortase of *Bacillus cereus*, cleaves the (I/L)(P/A)XTG sorting signal of pilin precursors[41]. The acyl intermediate between SrtD and pilins is resolved by nucleophilic attack by the side chain ε-amino group of Lys from within another pilin subunit. The result of this reaction is two covalently linked surface proteins, which can be extended by further polymerization into a pilus structure. Similarly to cell wall-anchored surface proteins, the polymerized pilin subunits are immobilized within the murein sacculus (the peptidoglycan cell wall) by the housekeeping class A sortase[8,42–44].

Linking protein secretion to cell wall synthesis

Protein secretion and cell wall sorting of surface proteins by sortases appear to be coordinated with sites of cell wall synthesis, as polypeptides can be linked to lipid II. Penicillin-binding proteins assemble the bacterial murein sacculus using lipid II as a substrate for transglycosylation and transpeptidation reactions[45,46]. These enzymes first polymerize the glycan strands (MurNAc–GlcNAc–MurNAc–GlcNAc)$_n$ and subsequently catalyse cross links between the amino groups of the crossbridges and the carboxyl groups of D-Ala residues at position four in the cell wall pentapeptides[47,48]. The subcellular location of cell wall synthesis varies between bacterial species. In Gram-positive cocci, including staphylococci and streptococci, bulk cell wall synthesis occurs at the site of cell division[49], whereas rod-shaped bacilli and listeriae catalyse *de novo* peptidoglycan synthesis mostly in helical arrangements along the cylindrical cell[50]. Within a fully assembled cell wall, crossbridges cross-link neighbouring wall peptides[51].

Because of the link between cell wall synthesis and anchoring of proteins to the cell wall by sortases, it seems plausible that there are assembly sites at which protein secretion and cell wall synthesis machines can

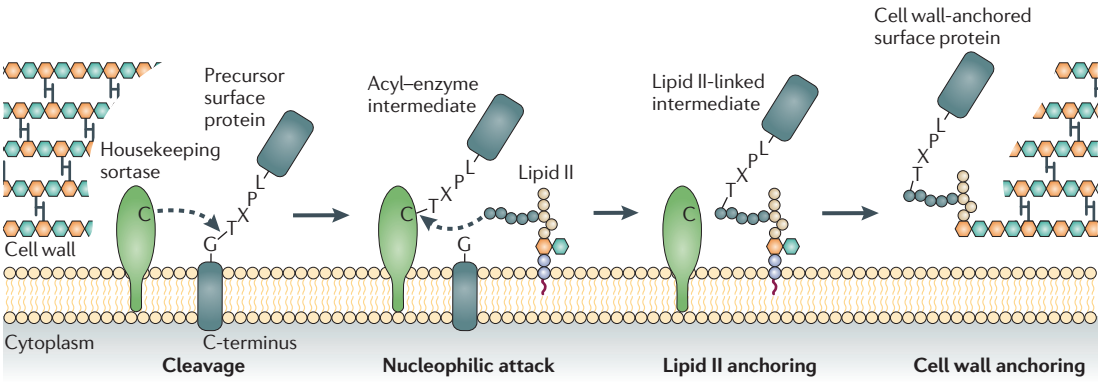

Figure 1 | **Mechanism of sortase-mediated surface protein anchoring to the cell wall.** Surface proteins are synthesized in the cytoplasm as precursor proteins with an amino-terminal signal sequence and a carboxy-terminal sorting signal. The C-terminal sorting signal consists of a positively charged tail, a hydrophobic region and a LPXTG motif. Following secretion by the Sec secretion system, signal peptidases cleave the signal peptides of surface proteins, thereby producing the precursor surface proteins. The active-site Cys of the sortase cleaves the amide bond between Thr and Gly of the C-terminal pentapeptide LPXTG motif and generates an acyl–enzyme (thioester) intermediate. Nucleophilic attack by the amino group within the pentaglycine crossbridge of lipid II links the C-terminal Thr of the surface protein to lipid II. Penicillin-binding proteins incorporate the precursor into the cell wall as a mature cell wall-anchored surface protein for surface display by catalyzing a transpeptidation reaction.

interact with sortases. These sites would ensure rapid incorporation of surface proteins into the bacterial envelope. Although such assemblies have thus far not been characterized, the localization of pili and the pilin-specific class C sortases at discrete sites near cross walls (the cell walls that separate enterococcal cells) has been reported in *Enterococcus faecalis*[52]. Future work will need to unravel whether these sites include an aggregation of protein secretion and cell wall synthesis machinery.

Sortase substrate specificity and pilus design

Polymerization of pili in Gram-positive bacteria requires cross-linking of the pilin subunits by sortases. This process is initiated through the formation of sortase class C–acyl intermediates with minor or major pilin subunits. For example, the initial step in the assembly of the *B. cereus* pilus is cleavage of the sorting signal of the pilus minor subunit, BcpB, by SrtD[41] (FIG. 2a). The resulting acyl intermediate can only be resolved by nucleophilic attack by the major subunit, BcpA, ensuring that BcpB caps the pilus tip. Subsequent nucleophilic attacks by BcpA–SrtD at BcpB–BcpA$_n$–SrtD intermediates promote elongation of the pilus fibre (BcpB–BcpA$_{n+1}$–SrtD)[35]. Unlike BcpB, the sorting signal of which can only be cleaved by SrtD, the sorting signal of BcpA is a substrate for both SrtD and the *B. cereus* housekeeping sortase, SrtA. Pilus assembly is terminated when SrtA cleaves BcpA at its sorting signal so that BcpA–SrtA can serve as the nucleophile acceptor for the elongated pilus[53]. The acyl intermediate of this reaction, BcpB–BcpA$_n$–SrtA, can only be relieved by lipid II, thus the final product can be incorporated into the cell wall envelope.

A similar paradigm applies for the assembly of pili that contain three different pilin subunits (FIG. 2b). For example, the synthesis of one type of pilus in *C. diphtheriae* involves a minor subunit, SpaC, that forms the adhesive tip[8,54]. SpaC is cleaved by the pilin-specific class C sortase (SrtA in *C. diphtheriae*)[55,56]. The SpaC–SrtA intermediate is only relieved via nucleophilic attack by the major pilin SpaA. SpaC–SpaA$_n$–SrtA intermediates are relieved in turn by SpaA–SrtA or by SpaB–SrtF, as SpaB, one of the minor pilin subunits, also harbours a Lys ε-amino group nucleophile for SpaC–SpaA$_n$–SrtA[57]. Work on a different pilus of *C. diphtheriae* revealed that the level in that pilus of the major pilin subunit, SpaH, determines pilus length[58]. The SpaB sorting signals can only be cleaved by the housekeeping class A sortase (SrtF in *C. diphtheriae*), and then the pilus is transferred to lipid II[59]. In agreement with this model, *C. diphtheriae spaB*-null mutants form SpaC–SpaA$_n$ pili but cannot immobilize these pili in the cell wall envelope[57].

Structure of recombinant pilin subunits

In 2007, the first crystal structure of a pilin protein from a Gram-positive bacterium was reported: that of Gbs52, a minor pilin that mediates attachment of pili to the cell wall envelope in *Streptococcus agalactiae* (group B *Streptococcus*; GBS)[60]. The recombinant pilin folds into two adjacent immunoglobulin (Ig)-like domains with seven anti parallel β-strands (FIG. 3). The C-terminal Ig-like domain is stabilized by an isopeptide bond that is autocatalytically formed between a Lys and an Asn side chain[61]. A similar domain arrangement had been previously observed for the B domain of *S. aureus* collagen adhesin (Cna)[62], a surface protein that is linked to the cell wall by a class A sortase. These Ig-like folds containing an intramolecular isopeptide bond are called Cna-B domains. In addition to two Cna-B domains stacked on top of one another, Gbs52 carries an extended Pro-rich linker in close proximity to its C-terminal sorting signal[61]. This feature is conserved among minor pilin proteins that function as cell wall-anchoring subunits for those pili that are assembled from three different subunits in Gram-positive bacteria[63].

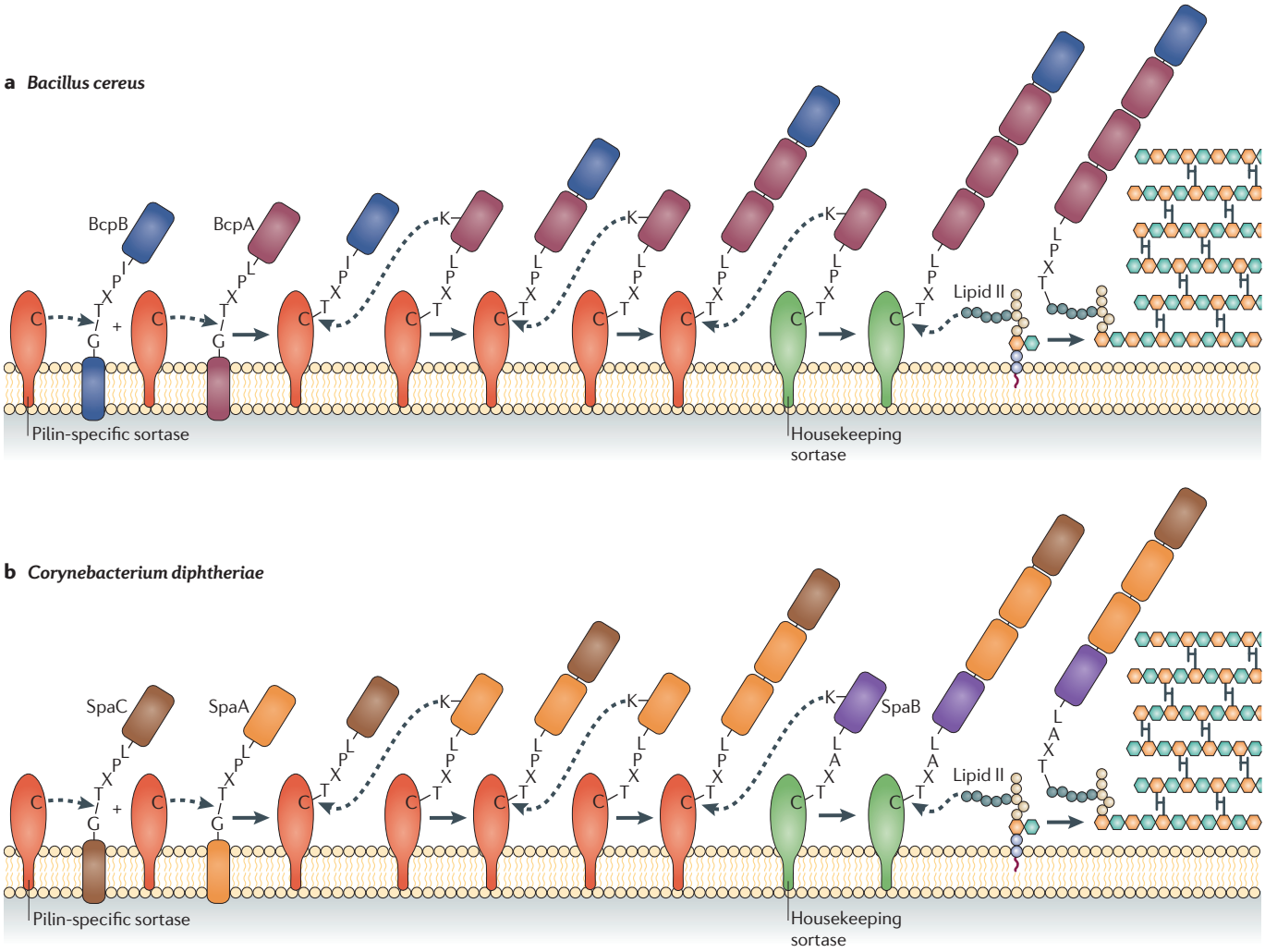

Figure 2 | **Assembly mechanisms for heterodimeric and heterotrimeric pili. a** | The pilin-specific sortase cleaves the sorting signals of BcpA and BcpB of *Bacillus cereus* at the Thr of the LPXTG motif, generating an acyl–enzyme intermediate. Nucleophilic attack by the side chain of Lys in the YPKN motif of BcpA forms an intermolecular isopeptide bond between BcpB and BcpA or BcpA and BcpA, and catalyses pilus polymerization. Nucleophilic attack of lipid II on the acyl intermediate formed by the housekeeping sortase and BcpA transfers the pilus to the cell wall envelope and terminates pilus assembly. **b** | A similar mechanism occurs with SpaCAB pili of *Corynebacterium diphtheriae*. An internal isopeptide bond is formed between SpaC and SpaA and subsequently between the SpaC–SpaA dimer and additional SpaA subunits to allow pilus polymerization. The pilus is anchored to the cell wall via a SpaB subunit, which is already linked to the housekeeping sortase. Nucleophilic attack by Lys139 of SpaB incorporates this minor subunit into the pilus. Nucleophilic attack of lipid II at the acyl intermediate of the housekeeping sortase with SpaB transfers the pilus to the cell wall envelope and terminates pilus assembly.

Since the first description of the structure of Gbs52, several reports have described crystal structures of other recombinant pilin proteins. The major pilin subunit of the M1-type *Streptococcus pyogenes* (group A *Streptococcus*; GAS) str. SF370 pilus backbone structural protein (BP; encoded by the Spy0128 locus)[64], comprises two domains with irregular all-β structures that also represent variants of the Ig-like fold (the Cna-B domain) (FIG. 3a). In the assembled pilus, the elongated subunits are thought to be stacked head to tail, which would juxtapose the two residues involved in the intermolecular isopeptide bond, the C-terminal Thr311 of the cleaved sorting signal and the nucleophile Lys161 used in the transpeptidation reaction. BP contains two intramolecular isopeptide bonds, one per Ig-like domain[64], which contribute to protease resistance and the overall stability of folded pilin subunits[64,65]. Both of these bonds have been identified within assembled pili, but it remains unclear whether they are formed before or after the sortase-mediated assembly of the intermolecular isopeptide bond (Thr311–Lys161).

The minor pilin subunit Cpa (also known as ancillary protein 1; encoded by the Spy0125 locus) is located at the tip of M1-type pili in *S. pyogenes* str. SF370. This pilus is formed via the polymerization of BP[44] and mediates streptococcal adherence to host cells (BOX 1; TABLE 2)[66,67].

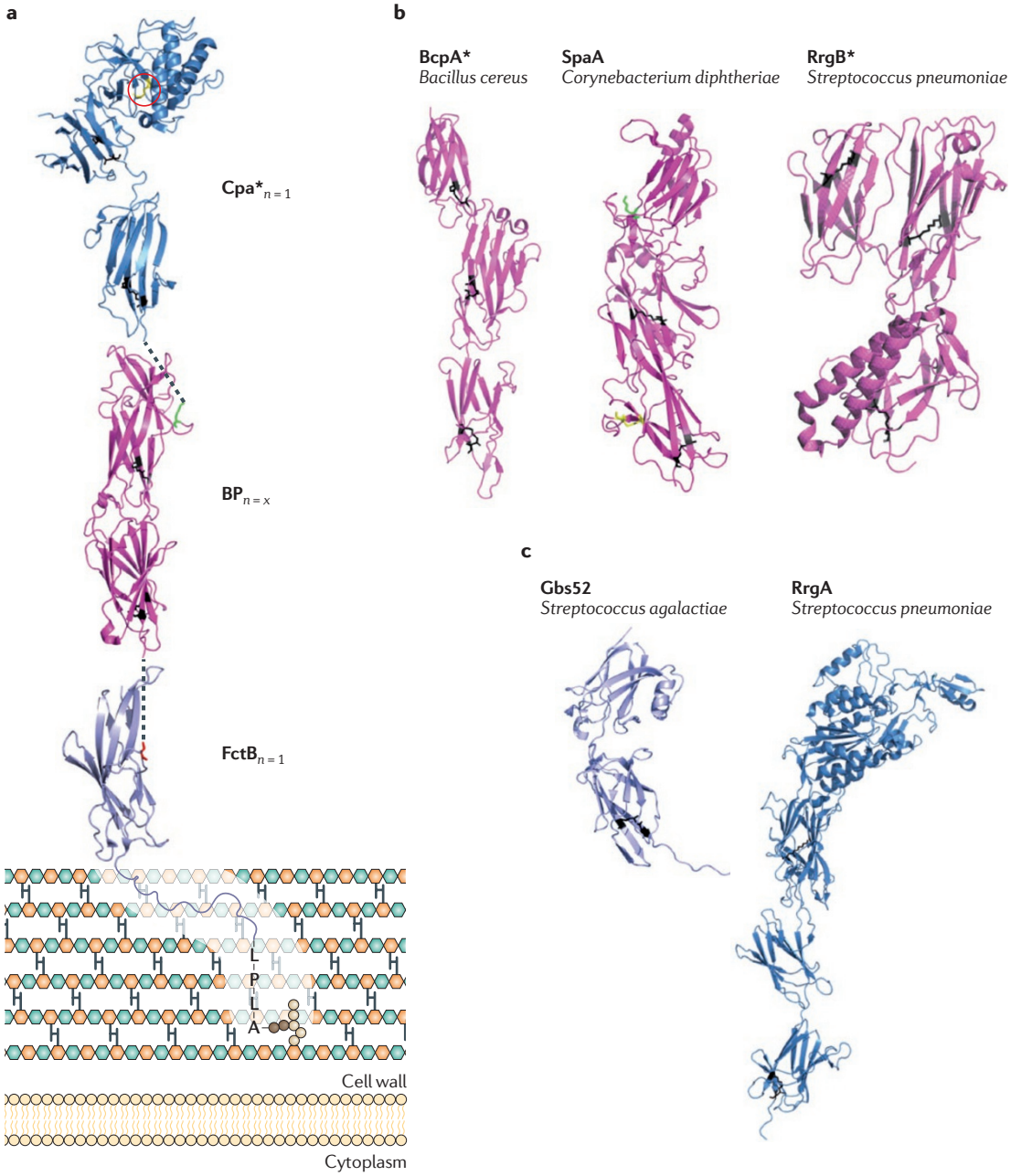

Figure 3 | **X-ray crystal structures of *Streptococcus pyogenes* pilins and pilin subunits from other Gram-positive bacteria. a** | Structural model of the *Streptococcus pyogenes* pilus formed by Cpa–BP–FctB (Cpa is also known as ancillary protein 1 and is encoded by the Spy0125 locus; backbone structural protein (BP) is encoded by the locus Spy0128; and FctB is also known as ancillary protein 2 and is encoded by the Spy0130 locus), showing that intermolecular isopeptide bonds (dashed lines) assemble the pilus, which is covalently connected to the cell wall. The tip pilin (Cpa) contains a thioester linkage (yellow, circle), in addition to intramolecular isopeptide bonds (black). **b**,**c** | Major pilin subunits (part **b**) and minor pilin subunits (part **c**) are composed of variants of Cna-B immunoglobulin-like folds that typically contain intramolecular isopeptide bonds (black); however, SpaA also contains a disulphide bond (yellow). The Lys implicated in intermolecular amide bond formation is depicted in stick mode in green and faces outward from the structure. Asterisks indicate crystal structures of truncated recombinant proteins.

The C-terminal domain of Cpa, residues 286–723, mediates its adhesive properties and contains three domains: the middle domain (residues 291–372 and 590–597), the top domain (residues 390–583) and the bottom domain (residues 603–719)[68]. Of these, the middle domain and the bottom domain each adopt an Ig-like all-β fold with an intramolecular isopeptide bond involving residues Lys297 and Asp595 in the middle domain and residues Lys610 and Asn715 in the bottom domain, similar to those found in other pilins. Remarkably, in contrast to most of these domains, which have a Lys–Asn isopeptide bond, the isopeptide bond of the Cpa

Box 1 | **The function of pili in Gram-positive bacteria**

Pili are involved in adherence to host epithelial tissues, an important first step in colonization, biofilm formation and subsequent initiation of the infection processes of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Corynebacterium diphtheriae*. *S. pneumoniae* pili adhere to lung epithelial cells and are implicated in pneumococcal colonization of the host respiratory tract[87,96]. Piliated *S. pneumoniae* strains appear to be more virulent than non-piliated strains in an invasive disease model in mice[87]. *S. pyogenes* pili bind to human tonsil epithelial cells, primary human keratinocytes[97] and pharyngeal cells[67], whereas the minor pilin subunits of *C. diphtheriae*, SpaB and SpaC, are important for adhesion to pharyngeal epithelial cells[54]. *S. agalactiae* pili are involved in adhesion to and invasion into brain microvascular endothelial cells[98] and are important for adherence to respiratory epithelial cells[99]. Furthermore, *S. agalactiae* variants lacking the major pilin subunit were less virulent than the wild-type parent in a mouse bacteraemia model[100]. Interestingly, pili encoded by pilus islets 1 (PI-1) and PI-2a gene clusters of *S. agalactiae* are involved in epithelial translocation[101,102], an invasive mechanism that contributes to the dissemination of *S. agalactiae* through host tissues. The pili of *S. pyogenes* and *S. agalactiae* contribute to biofilm formation[67,99], whereas the Ebp pili of *Enterococcus faecalis* are involved in adherence to host cells and in biofilm formation[85] and are very similar to the Ebp (PilB) pilus proteins from pilin gene cluster 3 of *Enterococcus faecium*[86]. These pili of *E. faecium* are implicated in enterococcal primary attachment and biofilm formation[103]. Competitive index analysis of mouse urinary tract infections showed that wild-type piliated *E. faecalis* or *E. faecium* outcompete non-piliated variants[91,103,104]. Similar results were obtained when comparing piliated and non-piliated *E. faecalis* strains in a rat endocarditis model[85].

Pilin proteins of pathogenic streptococci exhibit sequence similarities to microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), a class of LPXTG-containing surface proteins that are found in Gram-positive bacteria and interact with components of the extracellular matrix[105] (TABLE 2). In agreement with this finding, the pilus tip proteins RrgA of *S. pneumoniae* and Acb of *Streptococcus gallolyticus* showed concentration-dependent binding to specific extracellular matrix molecules[29,106]. *S. pyogenes* pili are encoded in the fibronectin-binding, collagen-binding T antigen (FCT) region[107]. The FCT region of some streptococci encodes the collagen type I-binding minor pilin protein Cpa (also known as ancillary protein 1; encoded by the Spy0125 locus), which is deposited at the tip of pili, and the fibronectin-binding proteins PrtF2 and PrtF[107–109].

middle domain is formed between a Lys and an Asp. The top domain of Cpa harbours an internal thioester bond between Cys426 and Gln575; this type of thioester bond has also been observed in mammalian complement components and complement-like proteins that contribute to insect innate immune functions by linking to microbial elements[69,70]. Although the presence of the thioester bond is a compelling argument for a model whereby streptococcal pili may also form covalent links with their host cell receptors, direct proof for this conjecture is still missing.

FctB (also known as ancillary protein 2; encoded by the Spy0130 locus) is thought to be located at the base of the pilus and is required for the incorporation of the assembled fibre into the cell wall envelope[63]. The crystal structure of FctB of *S. pyogenes* str. 90/306S revealed an Ig-like fold within the N-terminal domain and a C-terminal helix similar to a polyproline II helix (PP-II)[63,71]. Recombinant FctB does not contain the intramolecular isopeptide bond that is seen in BP and Cpa; instead, a hydrogen bond network at the hydrophobic core of FctB may form a functional replacement for the stabilizing force exerted by intramolecular isopeptide bonds in other pilin domains. The N-terminal Ig-like domain contains a region referred to as the Ω-loop, which is not present in the Cna-B domain of staphylococcal Cna[63]. This loop contains a Lys residue that is conserved among members of the FctB family of streptococcal ancillary pilin subunits[63]. A similarly positioned residue, Lys161, in the Ω-loop of the major pilin BP serves as the nucleophile in sortase-mediated assembly of these pili[61]. A covalent link between the Lys120 of FctB forms an intermolecular amide with the C-terminal Thr311 of BP[63]. The C-terminal PP-II helix appears to be a conserved feature of other minor pilin proteins[63].

Crystal structures have been solved for recombinant forms of all three protein subunits of the *S. pyogenes* pilus[61,63,68]. These provide the opportunity to derive an atomic model for assembled pili[72] (FIG. 3a). Two questions about the structure of the pili of *S. pyogenes* and other Gram-positive bacteria remain to be answered. First, what is the role, if any, of post-translational modifications such as glycosylation of subunits? The presence of multiple *N*-glycosylation sites and the aberrant migration of *Enterococcus faecium* pilus components through SDS–PAGE suggests that such modifications may occur[73]. However, as these modifications cannot occur within the recombinant subunits, biochemical and/or mass spectrometry analysis of the entire pilus may uncover additional modifications in the pilins. Second, is the atomic model of the pilus based on crystal structures correct? Future high-resolution imaging of native pili should uncover the repeated head-to-tail organization of major and minor subunits in the assembled fibre.

Crystal structures of pilus subunits from other bacteria — including BcpA, the major pilin of *B. cereus*[74], SpaA, the major pilin of *C. diphtheriae*[75], RrgA, the tip adhesin of *S. pneumoniae*[76], and Gbs52, the anchoring subunit of *S. agalactiae*[61] — have been solved. Although the N-terminal Ig-like domain of BcpA (Cna_1) could not be crystallized, the structure of the remainder of the molecule showed that it comprises three domains designated Cna_2 and Cna_3, for their Ig-like Cna-B structures, and Xna, which contains a jelly-roll fold[74]. Within the assembled pilus, each of the four domains of BcpA harbours an intramolecular isopeptide bond derived from Lys and Asn (via a catalytic Glu in Cna_1, Cna_2 and Cna_3 and via an Asp residue in Xna), but the recombinant BcpA protein lacks the bond in the Cna_1 domain; this bond is formed only after BcpA has been polymerized by SrtD[74].

SpaA, the major pilin of *C. diphtheriae*, is composed of three tandem Ig-like domains in which the middle and C-terminal domains are stabilized by intramolecular Lys–Asn isopeptide bonds[75]. Surprisingly, the C-terminal Ig-like domain of recombinant SpaA is stabilized by an internal disulphide bond; disulphide isomerization as a source for disulphide bonds in secreted proteins has not been reported for other Gram-positive bacteria[77].

The major pilin of *S. pneumoniae*, RrgB, is composed of four domains, designated D1–D4. A crystal structure of residues 184–627, which includes D2–D4, showed that each of these domains assembles into a β-sandwich structure with an intramolecular isopeptide bond reminiscent of the Ig-like fold of other pilin proteins[76]. Fitting of the partial RrgB fragment crystal structure into the overall structure of assembled RrgB pili, as analyzed by

Table 2 | **The roles of Gram-positive pili**

Bacterium	Pilins	Pilus function	Refs
Staphylococcus intermedius	Saf2 and Saf3	• Adherence to salivary agglutinin	124
Actinomyces naeslundii T14V	FimP and FimQ (type 1), and FimA and FimB (type 2)	• Adherence to tooth enamel • Formation of surface glycoconjugates	10,125-128
Corynebacterium diphtheriae NCTC13129	SpaA, SpaB, SpaC, SpaD, SpaE, SpaF, SpaH, SpaI and SpaJ	• Adherence to epithelial cells	54
Enterococcus faecalis OG1RF	EbpA, EbpB and EbpC	• Adherence during biofilm formation • Adherence during urinary tract infection	85,91,104
Enterococcus faecium TX0082	EbpA, EbpB and EbpC (also known as PilB)	• Adherence during biofilm formation • Adherence during urinary tract infection	103
Lactobacillus rhamnosus GG	SpaA, SpaB, SpaC, SpaD, SpaE and SpaF	• Adherence to human intestinal mucus	129,130
Streptococcus agalactiae 2603V/R or COH1	Gbs80, Gbs52 and Gbs104 (encoded by PI-1)	• Adherence to respiratory and intestinal epithelial cells • Adherence during biofilm formation	60,101
Streptococcus agalactiae 2603V/R or 515	Gbs59 (BP-2a), Gbs67 (AP1-2a) and Gbs150 (AP2-2a) (all encoded by PI-2a)	• Adherence to respiratory and intestinal epithelial cells • Adherence during biofilm formation	101,131
Streptococcus agalactiae NEM316	Gbs1477 (PilB), Gbs1478 (PilA) and Gbs1474 (PilC) (all encoded by the *srtC3-c4* locus)	• Adherence to respiratory and intestinal epithelial cells • Adherence during biofilm formation	88
Streptococcus agalactiae NCTC10/84	PilA, PilB and PilC	• Adherence to and invasion of epithelial cells, resistance to phagocytosis, and increased virulence in a mouse model • Mutants lacking PilA are defective in adherence to host cells • Mutants lacking PilB are defective in invasion of host cells, less virulent in mice, and more susceptible to killing by macrophages and neutrophils • PilB protects against the antimicrobial peptide cathelicidin	98,100
Streptococcus gallolyticus subsp. *gallolyticus*	Sbs7 and Acb	• Adherence to collagens type I, IV and V	106
Streptococcus pneumoniae TIGR4	RrgA and RrgB (encoded by *rlrA*, also known as PI-1)	• Adherence to respiratory epithelial cells, modulation of inflammatory responses and virulence in mice • The tip adhesin binds fibronectin, collagen I and laminin I	29,87,96
Streptococcus pneumoniae PN110	PitA and PitB (encoded by PI-2)	• Adherence to respiratory epithelial cells	121
Streptococcus pyogenes M1	BP (encoded by the Spy0128 locus), Cpa (also known as ancillary protein 1; encoded by the Spy0125 locus) and FctB (also known as ancillary protein 2; encoded by the Spy0130 locus) (all encoded in the FCT2 region)	• Adherence to tonsilar epithelial cells and keratinocytes • Adherence during biofilm formation • Binding to salivary gp340 protein	67,97,132
Streptococcus pyogenes M49 or Alab49	BP, Cpa and FctB (all encoded in the FCT3 region)	• Virulence in a skin infection model • Adherence to and bacterial internalization of Hep2 cells	108,133,134

BP, backbone structural protein; FCT, fibrinogen-binding, collagen-binding T antigen; Fim; type 1 fimbrial major subunit, PI; pilus islet

transmission electron microscopy, suggests that the pilin subunits are stacked head to tail during sortase-mediated assembly[76].

The structure of the recombinant RrgA adhesin of pneumococcal pili also consists of four domains (D1–D4)[78]. The C-terminal D4 domain is stacked against the D1 and D2 domains, each of which assumes an Ig-variant all-β fold. These three domains are likely to function as a stalk for the presentation of the D3 domain at the tip of the assembled pilus. The folded structure of the large D3 domain, residues 219–586, resembles that of the A3 domain of human von Willebrand factor, a molecule known to mediate association between platelets and collagen during haemostasis (the formation of a blood clot)[79–81]. As is the case in the Cpa adhesin of *S. pyogenes*, but not in the major pilins and ancillary pilins that are involved in anchoring pilus fibres in the bacterial envelope, the residues forming the tertiary structure of the RrgA D1 and D2 domains are interspersed throughout the primary amino acid sequence of the protein[78]. Thus, the structure does not follow a design plan laid down as a sequence of independent folding events in the primary structure of the protein. Two intramolecular isopeptide bonds stabilize the folded structure of recombinant RrgA. The D2 domain (residues 133–220 and 587–722) is stabilized by a bond between Lys191 and Asn695, and the D4 domain (residues 731–859) is stabilized by a bond between Lys742 and Asn854.

Isopeptide bonds within assembled pili

Evidence suggests that pilin-specific sortases mediate the assembly of pili in Gram-positive bacteria through

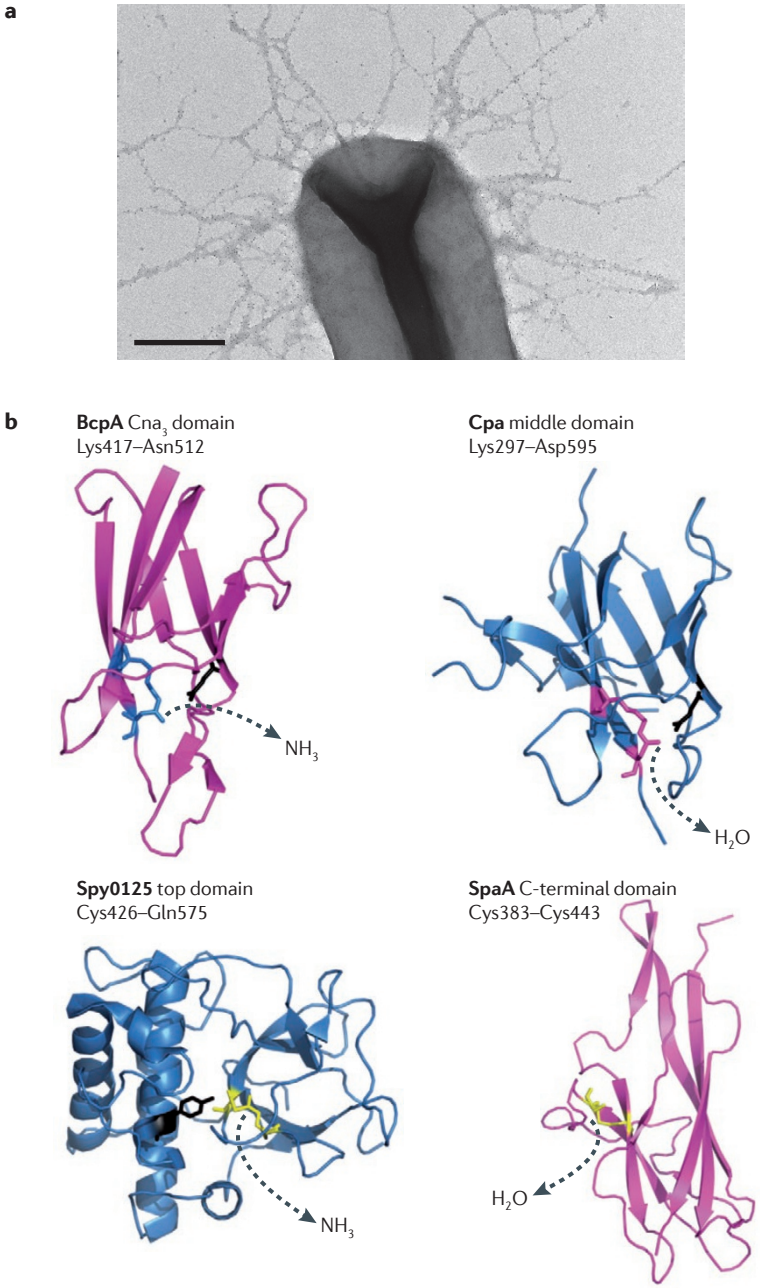

Figure 4 | **Intramolecular isopeptide bonds assemble pili. a** | Electron micrograph of protease resistant pili of *Bacillus cereus* in which the major subunit, BcpA, is labelled with 5 nm gold particles. The scale bar represents 200 nm. **b** | Covalent intramolecular bonds discovered in pilin subunits. Autocatalytic generation of Lys–Asn and Lys–Asp isopeptide amide bonds in domains of pilin subunits eliminates an NH_3 or H_2O molecule, respectively. X-ray crystallography also showed the presence of intramolecular thioester and disulphide bonds, which are accompanied by the loss of an NH_3 or H_2O molecule. Domains of major pilins are shown in magenta and those of minor subunits are shown in blue. (Part **a** electron micrograph courtesy of A.P.A.H.)

the formation of intermolecular isopeptide bonds. This hypothesis stimulated the bioinformatic approaches that not only identified major pilins with the YPKN pilin motif and the C-terminal sorting signal as putative pilus determinants, but also found gene clusters encompassing sortases[82]. These predictions were tested by examining bacterial surfaces for the presence of pili, leading to the identification of pili on *Actinomyces naeslundii*[83,84], *B. cereus*[42], *C. diphtheriae*[8], *E. faecalis*[85], *E. faecium*[86], *S. agalactiae*[43], *S. pneumoniae*[87] and *S. pyogenes*[44]. As additional evidence to support the pilin-specific-sortase hypothesis, assembly of these pili requires the expression of a major pilin and a pilin-specific sortase[14,55,56,85,88–92].

Nevertheless, these findings do not constitute definitive proof that pili of Gram-positive bacteria, other than *B. cereus* and *S. pyogenes*, are assembled from intermolecular and intramolecular isopeptide bonds. Using novel *in vivo* technology, Budzik and colleagues[53] showed that the C-terminal carboxyl group of Thr522 of BcpA is linked by SrtD via an intermolecular isopeptide bond to the ε-amino group of Lys162 in the YPKN pilin motif of the next BcpA subunit. Four intramolecular isopeptide bonds are also formed, one for each of the four folded domains of BcpA: Cna_1 (a Lys37–Asn163 bond), Cna_2 (a Lys174–Asn265 bond), Xna (a Lys273–Asn383 bond) and Cna_3 (a Lys417–Asn512 bond)[35,74] (FIG. 4). By contrast, recombinant BcpA cannot form the intramolecular isopeptide bond Lys37–Asn163, and its Cna_1 domain is sensitive to proteases. Upon SrtD-mediated assembly of the pilus, BcpA acquires protease resistance and the ability to form the Cna_1 intramolecular isopeptide bond[74]. BcpB, the tip protein of BcpA pili, is also linked to the ε-amino group of Lys162 in the YPKN pilin motif of the neighbouring BcpA subunit. This process is catalysed by SrtD, which cleaves the IPNTG sorting signal of BcpB between Thr and Gly[41]. Interestingly, the LPVTG sorting signal of BcpA (but not the IPNTG of BcpB) can be recognized and cleaved by two enzymes, SrtD (the pilin specific class C sortase) and SrtA (the housekeeping class A sortase). Depending on the availability of these enzymes and the resulting acyl intermediates, pili are either extended via the incorporation of BcpA or transferred to lipid II for incorporation into the cell wall envelope. Recognition of polypeptide substrates for cleavage by SrtA or SrtD requires only the C-terminal sorting signal[53]. However, completion of the transpeptidation reaction by a pilin-specific class C sortase requires larger segments of the major pilin protein and cannot be accomplished with the YPKN peptide alone. The autocatalytic assembly of three intramolecular isopeptide bonds in BcpA is likely to occur through a mechanism requiring catalytic Glu or Asp residues (Glu223 in Cna_2, Glu472 in Cna_3 and Asp312 in Xna). The assembly of the fourth intramolecular isopeptide bond remains an enigma. Autocatalysis of Lys37–Asn163 in the Cna_1 domain of recombinant BcpA does not occur. Formation of the intermolecular isopeptide bond immediately adjacent to Asn163 — between subunits in pili — is accompanied by the stabilization of Cna_1, and by the acquisition of the Lys37–Asn163 intramolecular isopeptide bond[74].

Studies of *C. diphtheriae* and *S. pneumoniae* pili that are assembled from three different subunits revealed that the tip and major pilin subunits are cleaved by pilin-specific class C sortases and not by the housekeeping class A sortase, whereas the ancillary subunit that forms

the base of the pilus is cleaved by the class A sortase[57,89,90]. These findings explain why expression of an ancillary pilin gene is essential for the cell wall anchoring of some pili in Gram-positive bacteria.

Outlook on the frontiers of pilus research

The hypothesis that sortases mediate formation of the pilus enabled predictions that allowed the identification and characterization of pili in the envelope of Gram-positive bacteria. The resulting discoveries closed much of the knowledge gap about pilus structure and function between Gram-negative and Gram-positive bacteria (BOX 1; TABLE 2). Recent advances have described the involvement of minor (ancillary) subunits that serve either as adhesins at the tip of pili or as anchoring units for completed fibres in the cell wall envelope, enabling the predictions of the sortase–pilus hypothesis to be further refined. When combined with detailed views on the structure of pilin subunits and the protective value of antibodies directed at specific elements of assembled pili, this field aims towards a rational appreciation of the molecular events underlying the pathogenesis of infections caused by Gram-positive bacteria, and the design of vaccines that seek to prevent them (BOX 2).

Pilus research has also generated unexpected results, such as the discovery of intramolecular isopeptide bonds, disulphide bonds and thioester bonds in pilin subunits[61,68,75]. Future work in this area will seek to understand how these bonds are formed and what their specific contributions are to the biology of bacteria. Research into Gram-positive pili challenges chemists to synthesize organic compounds that may function as pilicides[93]. Following the discovery of the structural elements and construction principles for pili in Gram-negative bacteria, the field is now moving towards *in vitro* assembly reactions with purified subunits[94,95]. Scientists working on the pili of Gram-positive bacteria are confronted with a similar challenge: the *in vitro* reconstruction of pilus fibres with the proper intramolecular and intermolecular amide bonds. Much work remains to be carried out, although a recent report on *in vitro* fibre polymerization by a pilin-specific sortase from *S. pneumoniae* is an exciting advance[14]. Last, the dynamics of pilus assembly in Gram-positive bacteria, and the interaction of these pili with host cells, have not been fully imaged. Sophisticated light, fluorescence and high-resolution electron microscopy techniques should provide new insights into these fascinating structures that have so long been invisible to microbiologists.

Box 2 | **Deriving vaccines from pili**

The exciting advances in the past decade of research into the pili of Gram-positive bacteria have laid the foundation for exploring the vaccine potential of pili. Several Gram-positive bacteria that have pili and cause severe human disease warrant vaccine development: *Streptococcus agalactiae* (the cause of neonatal meningitis), *Streptococcus pneumoniae* (the cause of pneumonia, meningitis and otitis media) and *Streptococcus pyogenes* (the cause of pharyngitis, soft tissue infections and rheumatic fever)[23]. In addition, both *Entercoccus faecalis* and *Entercoccus faecium* (the causes of urinary tract infections, bacteraemia and endocarditis) have emerged as multidrug-resistant nosocomial pathogens, emphasizing the need for new antibiotics or vaccine development to prevent enterococcal infections[22,110,111]. Licensed vaccines against bacterial infections typically achieve antibody-mediated neutralization of key virulence traits associated with the pathogens, such as the inactivation of secreted toxins (tetanus or diphtheria vaccines) or the deposition of antibodies on capsular polysaccharides to overcome the anti-phagocytic attributes of these structures[112,113]. Examples of important diseases caused by sugar-coated microorganisms with capsular polysaccharides that have played a role in vaccine development are pneumococcal pneumonia[114], meningococcal meningitis[115], or neonatal meningitis that is caused by *Haemophilus influenzae*[116]. Is it possible to derive vaccines against bacterial pili for specific human diseases in which pilus expression is a prerequisite for pathogenesis? This has certainly been attempted, with pilus subunit vaccines to protect women against piliated *Escherichia coli* urinary tract infection, for example[117,118].

S. agalactiae tip pilins and major pilins have been used as subunit vaccines in mice. Maternal pilin-specific antibodies are transferred to the fetus and confer protection to newborn animals against lethal *S. agalactiae* challenge[119]. These streptococci produce pili from any one or two of the three pilus gene clusters (pilus islet 1 (PI-1), PI-2a and PI-2b). Although the PI-1 and PI-2b gene clusters are well conserved, the third p`ine development currently focuses on the two major pilins of the conserved islands, PI-1 and PI-2b, as well as the tip protein from PI-2a. Most *S. agalactiae* strains isolated from human neonatal meningitis cases express capsular polysaccharide, and antibodies against capsular material can confer protective immunity against neonatal meningitis. However, about 10% of *S. agalactiae* strains are non-encapsulated, and vaccine protection against this population could be addressed by developing pilus-specific vaccines.

One problem in the design of anti-pilin vaccines is the variability in the pilin subunits of a species. For example, all *S. pyogenes* strains examined produce pili from nine different chromosomal pathogenicity islands. These pili are assembled from three different pilin subunits: the major pilin, of which there are 15 variants; the tip pilin, of which there are 16 variants; and the ancillary pilin at the pilus base, of which there are five variants[23,120]. A vaccine derived from the 12 most frequently isolated variants of the major pilin may confer protection against about 90% of all clinical isolates[120].

Two basic *S. pneumoniae* pilus types have been identified, each containing three pilin subunits. Pneumococcal isolates express neither, one or both of the corresponding pilus gene clusters on the bacterial surface[121]. Vaccine studies of the purified pilin subunits (RrgA, RrgB and RrgC), of clinical serotype 4 strain *S. pneumoniae* TIGR4 indicated that any one pilus subunit can raise humoral immune responses with significant levels of vaccine protection[122], although it is currently not clear whether pilus antigens offer an advantage over pneumococcal capsular polysaccharide vaccines that are already licensed by the US Food and Drugs Agency (FDA).

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Competing interests statement
The authors declare no competing financial interests.

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