A Structural Snapshot of Type II Pilus Formation in *Streptococcus pneumoniae*

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Background: PitB and SrtG are essential components of the pneumococcal PI-2 pilus.

Results: PitB assembles head-to-tail into fibers that do not require SrtG1 for stabilization.

Conclusion: PitB associates like beads on a string to form a fiber that is 35 Å in diameter.

Significance: The PI-2 pilus displays a simple architecture composed of a single protein with adhesive properties.

Pili are fibrous appendages expressed on the surface of a vast number of bacterial species, and their role in surface adhesion is important for processes such as infection, colonization, and biofilm formation. The human pathogen *Streptococcus pneumoniae* expresses two different types of pili, PI-1 and PI-2, both of which require the concerted action of structural proteins and sortases for their polymerization. The type PI-1 streptococcal pilus is a complex, well studied structure, but the PI-2 type, present in a number of invasive pneumococcal serotypes, has to date remained less well understood. The PI-2 pilus consists of repeated units of a single protein, PitB, whose covalent association is catalyzed by cognate sortase SrtG-1 and partner protein SipA. Here we report the high resolution crystal structures of PitB and SrtG1 and use molecular modeling to visualize a “trapped” 1:1 complex between the two molecules. X-ray crystallography and electron microscopy reveal that the pneumococcal PI-2 backbone fiber is formed by PitB monomers associated in head-to-tail fashion and that short, flexible fibers can be formed even in the absence of coadjuvant proteins. These observations, obtained with a simple pilus biosynthetic system, are likely to be applicable to other fiber formation processes in a variety of Gram-positive organisms.

*Streptococcus pneumoniae* is a frequent colonizer of the human upper respiratory tract and is one of the main causative agents of pneumonia, meningitis, and septicemia (1). Despite the development of multi-valent polysaccharide-based vaccines, pneumococcal infection still continues to be a worldwide problem, affecting mostly the elderly and the very young, notably in developing countries (2, 3). One of the key steps for infection initiation is adherence of pneumococci to eukaryotic surfaces, which is mostly achieved through the interaction between bacterial surface-exposed proteins and host carbohydrates in a lectin-like fashion or through the direct action of adhesins that recognize the extracellular matrix (4–6). Some adhesins have been shown to be located at the most tip of pili, hair-like, proteinaceous surface structures that aid in bacterial adhesion to host molecules but also play key roles in biofilm formation and evasion from the immune system (7).

The study of the molecular machineries from both Gram-positive and Gram-negative pathogens have revealed not only key differences in the mechanisms of pilus formation by the two different bacterial types (8) but have also underscored the importance of the detailed comprehension of the pilus formation systems for the exploration of novel anti-adhesion strategies that could lead to the development of new antimicrobials.

The pilus-forming building blocks (pilins) of Gram-positive bacterial species are covalently associated by dedicated sortases, cysteine transpeptidases whose genes are located within the same pathogenicity islet as the pilin-encoding genes. Pili have now been extensively studied in a number of organisms, including *Corynebacterium*, *Enterococcus*, and *Streptococcus* species (9). Notably, genome sequencing studies revealed the existence of more than one pilus islet in many species (10). Specifically, *S. pneumoniae* carries two pilus-encoding pathogenicity islets, PI-1 and PI-2. PI-1, also known as the *rlrA* islet, spans a 14-kb region and encodes three pilins (RrgA, the adhesin; RrgB, the backbone; RrgC, the bridging element) and three class C sortases (4, 11–16). The PI-1 islet is present in ∼30% of all pneumococcal strains (50% among antibiotic-resistant strains); a tendency toward the spreading of PI-1 clones has been reported, indicating that pilus expression may provide a fitness advantage to the bacterium (17). The PI-2 islet encodes two pilins (PitA and PitB), two sortases (SrtG1 and SrtG2) and one signal peptidase-like protein (SipA) (18). Notably, both PitA and SrtG2 are dispensable for PI-2 formation, indicating that PitB is the backbone pilin, and its covalent association is catalyzed by SrtG1 (19). The function of SipA is more controversial despite the fact that its homologs are present in pilus-related pathogenicity islets of a number of streptococcal species (*Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus agalactiae*) (20). How-

The atomic coordinates and structure factors (codes 4Y4Q and 4Y4R) have been deposited in the Protein Data Bank (http://wwpdb.org/).  

*This work was supported by ANR-11-BSV8-005-02 (including a postdoctoral fellowship to M. M. S.), the Laboratoire International Associé BACWALL, and FAPESP Grant 11/52067-6 (to A. D.). The authors declare that they have no conflicts of interest with the contents of this article.*  

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Architecture of the Pneumococcal Type II Pilus

however, it has been suggested to function as a chaperone for pilus polymerization (19), notably by associating with a partially unfolded pilin to facilitate folding and subsequent polymerization (21).

The catalytic mechanism of sortases as well as their structures and those of pilin molecules have been the object of much interest in the past few years. Sortases have been classified into four classes (A-D) based on their primary sequences and substrate recognition motif (22). Their three-dimensional folds all display a central β-barrel motif, but they are involved in processes that range from iron uptake to pilus polymerization and covalent attachment of virulence factors to the bacterial cell wall (23). Most pilus-forming sortases (class C), in addition to the classic β-barrel, also carry "lid" sequences that cover the active site and could play a role in substrate recognition (24–28).

A notable exception is the pilus-related sortase from Streptococcus pyogenes, which lacks a lid and was shown to be structurally similar to class B sortases (29), which have been reported to be involved in anchoring virulence factors to the cell wall in the case of iron acquisition (22, 30). Sortases recognize the cell wall sorting signal of a target protein, notably an LPXTG-like motif, and associate it to an amino group located on the cell surface. In the case of pilin substrates, the active site Cys residue of the sortase enzyme performs a nucleophilic attack on the link between Thr and Gly of the target motif, resulting in the formation of an amide bond joining the newly formed C-terminal Thr carboxylate to the ε-amino group of a Lys residue from the adjacent pilin subunit (31–33). Thus, the identity of the surface-exposed Lys that is involved in covalent association between pilins is relevant for the understanding of the pilus formation process at a mechanistic level.

On the other hand, the building blocks responsible for pilus formation have been reported to fold into independent domains associated to each other as beads on a necklace, which provides the necessary flexibility for pilus recognition of target surfaces (24, 34, 35). One notable exception, however, is the adhesin RrgA, which displays interdependent domains, one of which mimics the eukaryotic VWA motif (4).

Nevertheless, despite the advances in structural and functional characterization, a complete understanding of the association between sortases and pilins as well as pilins themselves in atomic detail is still lacking due to the complexity of the multiprotein pilus systems and the difficulty in "trapping" a complex between an active sortase and a pilin molecule. In this work we have solved the high resolution structures of PitB and SrtG1, the backbone pilin, and cognate sortase from the type II pilus (P1-2) of S. pneumoniae and used molecular docking to understand the first step before nucleophilic attack; that is, association of the target LPXTG motif onto the sortase recognition platform. In addition, we have also visualized the type II pilus fiber using x-ray crystallography and electron microscopy. This work thus allows a more complete comprehension of pilus biosynthesis in Gram-positive organisms in that it provides a snapshot of a key step of an essential reaction in atomic detail as well as insight into the mechanism of type II pilus formation.

Experimental Procedures

Cloning, Expression, and Purification of PitB—The pitB gene was amplified by PCR from S. pneumoniae clinical strain 19F Taiwan 14 (a gift from M. Barocchi, Novartis) using conventional PCR techniques. The amplified fragment was cloned into pET151 (Invitrogen) in-frame with an N-terminal His tag using a TOPO® cloning kit (Invitrogen), generating pET151-PitB. Subsequently, to perform phasing experiments, two methionine residues were incorporated into the sequence by site-specific mutagenesis. We thus generated mutants PitB-L242M/L307M, which were produced using the QuikChange site-directed mutagenesis kit (Stratagene). The mutated amino acids, Leu-242 and Leu-307, were selected based on a homology model built using MODELLER (36). Escherichia coli BL21(DE3) cells (Novagen) were transformed with pET151-PitB and grown in LB medium supplemented with ampicillin (100 μg/ml) at 37°C to an optical density value (A600 nm) of 0.6 absorbance units. Protein expression was triggered by the addition of 0.5 mM isopropyl-D-thiogalactoside (IPTG), and cells were incubated for 4 h at 25°C with shaking. Cultures were centrifuged and resuspended in buffer A (30 mM Tris-HCl, pH 8.0, 150 mM NaCl) and then disrupted by sonication. The lysate was centrifuged to remove cell debris (18,000 × g for 25 min) and loaded onto a 5-ml Ni2⁺ Chelating Sepharose™ column (GE Healthcare). After extensive washing using buffer A supplemented with 20 mM imidazole, PitB was eluted using a linear gradient from 80 to 300 mM imidazole. Fractions were pooled and dialyzed for 2h at 4°C against buffer A. The N-terminal His tag was removed by incubating the protein overnight with tobacco etch virus protease; uncleaved PitB and tobacco etch virus protease were removed by reloading the sample onto the Ni2⁺ column. PitB was further purified by gel filtration using a Superdex 200™ 16/60 GL (GE Healthcare) column equilibrated in buffer A.

Selenomethionylated PitB was expressed in E. coli BL21 (DE3) cells in M9 minimal medium supplemented with thiamine (0.2 mg/ml). Thirty minutes before induction with 0.3 mM isopropyl-D-thiogalactoside, a solution of leucine (50 mg/ml), valine (50 mg/ml), isoleucine (50 mg/ml), lysine (100 mg/ml), phenylalanine (100 mg/ml), threonine (100 mg/ml), and selenomethionine (60 mg/ml) was added to the culture to inhibit the methionine biosynthesis pathway and to drive the incorporation of selenomethionine. Expression and purification of selenomethionylated PitB were performed as for the native protein; 1 mM DTT was added to the gel filtration buffer to prevent sample oxidation.

PitB Crystallization and Structure Determination—Native PitB crystals were obtained at 20°C by vapor diffusion using a 20 mg/ml protein stock solution and, as precipitant, a solution containing 3 M NaCl, 0.1 M HEPES, pH 7.0. Native data were collected at beamline ID14–4 of the European Synchrotron Radiation Facility (ESRF)2 in Grenoble, France. Crystals belonged to space group P41_21_21 with unit cell dimensions a = b = 54.21 Å and c = 361.41 Å. Two molecules were present in the

2The abbreviations used are: ESRF, European Synchrotron Radiation Facility, Grenoble, France; r.m.s., root mean square.
TABLE 1
Data collection and refinement statistics
Thir highest resolution shell is shown in parentheses.

|                | PitB native | PitB - SeMet | SrtG1 |
|----------------|-------------|-------------|-------|
| Data set       |             |             |       |
| Wavelength (Å) | 0.9393      | 0.9784      | 1.0045|
| Space group    | P4_2        | P4_2        | C222  |
| Cell dimensions (Å) | a = b = 54.21, c = 361.41 | a = b = 55.22, c = 362.22 | a = 74.15, b = 101.14, c = 143.29 |
| Resolution (Å) | 54.21-2.80 (2.95-2.80) | 50.22-3.50 (3.69-3.50) | 41.31-2.16 (2.24-2.16) |
| Rmerge (%)     | 0.195 (0.583) | 0.270 (0.634) | 0.078 (0.675) |
| Completeness (%) | 99.9 (100) | 98.0 (97.9) | 95.70 (83.41) |
| Redundancy     | 4.8 (4.9)   | 12.5 (12.5) | 3.5 (3.3) |
| Refinement     |             |             |       |
| No. reflections | 25,500 (2543) | 27,931 (2414) |       |
| Rwork (%)      | 0.2393 (0.3833) | 0.1882 (0.2939) |       |
| Rmerge (%)     | 0.2977 (0.4825) | 0.2318 (0.3593) |       |
| No. atoms      | 5,275       | 5,196       | 2,960 |
| Protein        | 5,196       | 2,960       | 155   |
| Solvent        | 79          | 349         | 0.008 |
| Protein residues | 676        | 1,33        | 1.17  |
| r.m.s. bond length deviation (Å) | 0.010 | 0.010 |       |
| r.m.s. bond angle deviation (Å) | 1.33 | 1.33 |       |
| Ramachandran plot (%) | 93 | 96 | 96 |
| Favored        | 21.06       | 11.72       |       |
| Clashscore     | 37.10       | 49.40       | 49.3/50.7 |
| Average B-factor | 37.3/21.0 | 37.3/21.0 |       |

Architecture of the Pneumococcal Type II Pilus

asymmetric unit, leading to $V_M = 3.59^{+1}/\text{Da}$, corresponding to an approximate solvent content of 65.72%. Data were indexed and integrated with software Mosflm (37) and merged and scaled with Scala (38). Crystals of selenomethionine PitB-L242M-307M were grown in the same condition as the native variant, and a single wavelength anomalous dispersion experiment was performed on the selenium edge on beamline BM14 of the ESRF. Crystals diffraction to 3.3 Å and were in space group P4_2; data were treated using the same software as for the native form.

Four selenium positions were identified in the PitB-L242M-307M data by AutoSharp (39), and further model building, including phase extension to the higher resolution native dataset (2.8 Å), was performed with Autobuild (40). Model building was further extended with Buccaneer (41) and manual rebuilding with Coot (42). Refinement was carried out using the Phenix package (43) and Refmac (44). Solvent molecules were added by using the automated procedure in Phenix. Geometrical parameters of the model were verified by Procheck (45). Data collection and refinement statistics are presented in the Table 1.

Generation of PitB Active Site Mutants—Primer pairs were designed to modify Lys-53, Lys-179, Lys-194, and Lys-203 into alanines based on the QuikChange™ Site-Directed Mutagenesis kit (Stratagene). The pET151-PitB construct was used as a template, and a two-step protocol was employed for the mutagenesis kit (Invitrogen), generating pET151-SrtG1.

E. coli BL21(DE3) cells (Novagen) were transformed with pET151-SrtG1 and grown in LB media at 37 °C, supplemented with ampicillin (100 µg/ml). Protein expression was triggered by 1 mM isopropyl-$\beta$-D-thiogalactoside (Inalco) until the culture reached an optical density ($A_{600}$) of 0.7. After a 4 h incubation at 28 °C, bacteria were collected and resuspended in lysis buffer (30 mM Tris, pH 8.0, 150 mM NaCl) and then disrupted by sonication. The lysate was centrifuged to remove cell debris (18,000 × g for 25 min) and loaded onto a column containing 5 ml of Ni$^{2+}$-charged Chelating Sepharose™ (GE Healthcare). After extensive washing using the lysis buffer supplemented with 20 mM imidazole, SrtG1 was eluted by a linear gradient from 80 to 300 mM imidazole. Fractions were pooled and dialyzed for 2 h at 4 °C against dialysis buffer (30 mM Tris pH 8.0, 150 mM NaCl). The His tag was removed by incubating the protein overnight with tobacco etch virus protease; uncleaved SrtG1 and tobacco etch virus protease were removed by reloading onto the Ni$^{2+}$ column. SrtG1 was further purified by gel filtration using a Superdex 200™ 16/60 GL (GE Healthcare) equilibrated in 30 mM Tris, pH 8.0, 150 mM NaCl.

Cloning, Expression, and Purification of SrtG1—The srtg1 gene was amplified by PCR from S. pneumoniae clinical strain 19F Taiwan 14. The amplified fragment (encompassing residues 46–260) was cloned into the pET151 vector (Invitrogen) in-frame with an N-terminal His tag using a TOPO® Cloning kit (Invitrogen), generating pET151-SrtG1.

DNA microarrays were adsorbed onto the clean face of a carbon film on a mica sheet (carbon/mica interface) and negatively stained with 2% (w/v) neutral sodium silicotungstate, pH 7.5. Micrographs were taken under low dose conditions with a Tecnai12 LaB6 electron microscope working at 120 kV and with nominal magnifications of 23,000× and 49,000× using a Gatan Orius™ SC1000 CCD camera. For experiments involving SrtG1 addition, purified PitB and SrtG1 were incubated at a molar ratio of 1:2. Four µl of this mix (leading to a final concentration of 0.05 mg/ml of PitB) were loaded onto grids and observed by negative staining electron microscopy as described above.
with unit cell dimensions $a = 74.15$ Å, $b = 101.14$ Å, and $c = 143.29$ Å and two molecules in the asymmetric unit ($V_m = 2.69\text{Å}^3/Da$, corresponding to an approximate solvent content of 54.22%). A diffraction data set was measured at beamline ID 23–2 of the ESRF. The dataset was indexed and integrated with Mosflm (37), merged, and scaled with Scala (38). The structure was solved by molecular replacement using BALBES (47) using the crystal structure of Spy0129, a *S. pyogenes* class B sortase (PDB code 3PSQ) as template. Model building and refinement were performed using the same software as for PitB, described above. Data collection and refinement statistics are presented in Table 1.

**Molecular Docking**—PitB and SrtG1 were docked by using the automated ClusPro docking server (48), which employs a range of multistage protocols for protein-protein docking. Initially, ClusPro performed rigid docking by using PIPER (49); subsequently, docked conformations were filtered and clustered, and selected conformations were stabilized using Monte Carlo simulations. The binding energy score was generated from an energy function of the PIPER docking program based on the conformation of the bound complex (49).

Docking was guided by assigning the attraction region around the active site cavity and substrate recognition motif of SrtG1 (loop regions between $\beta3/\beta4$, $\beta4/\beta5$, $\beta6/\alpha4$, $\beta8/\beta9$, $\beta4$, the C-terminal part of $\beta8$, and the N-terminal part of $\beta9$) and the C-terminal PitB VTPT sequence along with all of the loop regions in the vicinity (loop regions between $\beta13/\beta14$, $\beta19/\beta20$, and $\beta21/\beta22$). All other parameters were left as defaults. After following the recommended procedure in which 20,000 models were retained after initial docking and filtering, the top 2,000 solutions were clustered; the clusters were ranked by using the ClusPro algorithm (48). The top-scoring solution was checked manually by selecting docked complexes that belonged to the most populated clusters of interacting complexes but with the lowest binding energy scores. We assumed that the model with the lowest binding energy was the one with optimal conformation.

**Results and Discussion**

**PitB Structure Determination**—A construct comprising residues 46–386 of PitB (PitB$_{46–386}$) from *S. pneumoniae* strain 19F Taiwan 14 was expressed in *E. coli*, purified, and crystallized. The N-terminal 45-residue signal peptide and the C-terminal 27-residue transmembrane-anchoring domain were not included in the construct (Fig. 1A). PitB crystals diffracted to 2.8 Å at the ESRF. Molecular replacement attempts using the structure of the major pilin from *S. pyogenes* as a model (which was the closest homolog and shared 28% sequence identity with PitB) did not yield solutions. We thus decided to attempt to solve the structure by single/multiwavelength anomalous diffraction phasing, but PitB only contains 2 methionines out of a total of 341 residues, and this ratio does not suffice for providing sufficient phasing power. To phase the data, we mutated two leucines (Leu-242, Leu-307) into methionines, generating PitB-L242M/L307M (PitB-2M). The candidates for mutation were selected based on the homology model built using MODELLER (36) and evaluated as substitution sites by taking into account several factors such as predicted flexibility and structural compatibility. Once PitB-2M was expressed and crystallized, phases were experimentally
obtained using single wavelength anomalous dispersion on the selenomethionine variant. The asymmetric unit contains two monomers of PitB, which are related to each other by a non-crystallographic 2-fold axis. The excellent quality of the electron density map allowed for the clear tracing of the entire chain, with the exception of three C-terminal residues for each of the two independent PitB molecules. The two monomers were almost identical; when the two molecules were superimposed, the root mean square (r.m.s.) difference in Cα atom positions was 0.15 Å over 316 aligned residues.

**Structure and Stabilization of PitB**—PitB consists of two domains, D1 (46–222) and D2 (223–386), and presented overall dimensions of 110 Å (length) and 40 Å (width) (Fig. 1B). The two domains displayed irregular structures that are modified variants of the immunoglobulin fold. D1, the N-terminal domain (green in Fig. 1B) comprised 12 β strands and formed an extended form of β sandwich in which the individual strands were progressively arranged in semi-helical fashion, forming a rather “flat” platform at the furthermost N-terminal end of the molecule. Notably, this platform was highly basic (Fig. 2A), which could be linked to the fiber-forming characteristics of PitB (see below). The C-terminal domain, D2 (cyan in Fig. 1B), comprised 11 β strands and formed a compact β sandwich. The core of D2 was formed by a five-stranded β sheet packed against another four-stranded sheet, whereas the other two anti-parallel β-strands (β15/β16) were somewhat more exposed. The overall structure of PitB is thus reminiscent of that of other pilus-forming molecules, which consist of domains associated as “strings of pearls,” linked by flexible loops; the difference here seems to be that the two domains of PitB are rather stably associated, suggesting a rigid, rod-like structure. In fact, analyses of the different domains of PitB using DALI (50) confirm that the folds of D1 and D2 are similar to those of the N-terminal and C-terminal domains of the major pilin protein Spy0128 of *S. pyogenes* (35), respectively, as predicted by sequence comparisons. Other similar molecules are the minor pilin FctB (51) (D1) and Spy0125 (52, 53) (D2), also from the same organism; however, r.m.s. deviations are in the range of 2.8–3.2 Å for all
with most of the differences coming from loop regions and minor positional differences between strands.

The resistance and stability of Gram-positive pili has been shown to require the formation of isopeptide amide bonds within the pilin monomers themselves. Isopeptide bonds, products of an intramolecular reaction between the side chains of lysine and asparagine residues, are found to be present in all pilin molecules whose structures have been solved to date (4, 14, 35, 54). PitB harbors two isopeptide bonds, one in each domain (Fig. 2B); these had been originally identified by mass spectrometry and mutagenesis (20). The bond within D1 is formed between the side chains of Lys-66 and Asn-217 and is stabilized by the side chain of Glu-147, whereas in D2 it is formed between Lys-246 and Asn-375 and stabilized by Glu-328. As is the case for other pilins, the Lys-Asn isopeptide bonds present in PitB are located within hydrophobic cores and are important for stability of the protein in thermal denaturation experiments (20).

**Reconstitution of the Type II Pilus Backbone**—Associated PitB monomers generate columns of molecules extending through the z axis of the crystal by stacking head-to-tail with an approximate rotation angle of 120° between two successive PitB monomers. The head-to-tail organization places the C-terminal VTPTG sorting recognition motif of a D2 domain into a cavity of the successive D1 domain (Fig. 3A). The interaction interface between the C terminus of D2 (dark blue in Fig. 3A) and the N terminus of D1 (cyan in Fig. 3A) buries ~659 Å² of solvent-accessible surface; notably, interactions are mostly made with main chain atoms of the VTPTG sequence, suggesting that different sequences could potentially be accepted.

![Architecture of the Pneumococcal Type II Pilus](image-url)
Architecture of the Pneumococcal Type II Pilus

During the pilus formation process, SrtG1 recognizes VTPTG and forms a covalent bond with the side chain of a neighboring lysine residue of the adjacent domain. Analysis of the PitB structure reveals that four Lys residues (in D2) are located in the immediate neighborhood of VTPTG (from the adjacent D1) and could be involved in formation of the covalent bond: Lys-53, Lys-179, Lys-194, and Lys-203 (Fig. 3C). These residues are positioned within the “basic patch” mentioned above.

To address the issue of the identity of the Lys residue that participates in the covalent bond with VTPTG, we mutated all four abovementioned lysines in PitB into alanines, expressed and purified the variants as per the wild type molecule, and analyzed their capacity to generate fibers in the presence or absence of purified SrtG1 by negative staining electron microscopy (Fig. 4). Interestingly, PitB-K53A, PitB-K179A, and PitB-K194A were all able to form short fibers, much like wild type PitB (PitB-K203A could not be purified due to instability). This result was unchanged whether SrtG1 was incubated with the samples or not, indicating that in vitro, PitB molecules can associate as short fibers, but SrtG1 is not capable of polymerizing them into pili. This observation indicated that, contrary to type I pili, type II pili cannot be polymerized in an in vitro setting.

We subsequently attempted to study this issue in vivo by complementing the S. pneumoniae strain PN110 (that expresses PI-2 pili on its surface) from which the pitB gene was deleted (PN110ΔpitB) with plasmids expressing wild type and mutant PitBs. Interestingly, none of the complemented strains was able to display clearly identifiable fibers on their surface, contrary to wild type PN110, which showed clear high molecular weight bands on an SDS gel prepared from cell wall preparations (not shown). This suggests that when PitB is added exogenously, the SrtG1/SipA machineries are not capable of catalyzing pilus formation. These results further support the theory that all PI-2 proteins must be co-expressed from the same operon for PitB (potentially in semi-unfolded state, as suggested by Young et al. (21)) to be tackled by its partner peptidase and sortase for polymerization to occur successfully. These observations further strengthen the hypothesis that the polymerization mechanism of PI-2 pili, despite requiring the action of only three proteins, is highly regulated in vivo, requiring the three players to be expressed concomitantly from the same operon.

To gain further insight into the polymerization mechanism itself, we solved the structure of SrtG1 and used molecular dynamics to “trap” a complex between the sortase and PitB. These experiments allowed us to create a snapshot of the first step in pilus formation.

**Structural Characterization of SrtG1—** SrtG1 is a 333-residue protein that comprises an N-terminal 45-residue region encompassing a signal peptide and an initial transmembrane region, a central sortase domain, and a 43-amino acid, C-terminal, 2nd transmembrane anchoring domain (Fig. 5A). This organization is in slight contrast with that of other pilus-catalyzing sortases that only present a predicted transmembrane region at their C termini. Notably, the structure reported here comprises residues 46–260, which represented the most stable form of the
Architecture of the Pneumococcal Type II Pilus

A

B

C

FIGURE 5. Structure of SrtG1. A, schematic image of SrtG1, which is composed of a signal peptide (white box), a catalytic domain (blue/yellow), and a C-terminal transmembrane (TM) region. The flexible region that was removed for crystallization purposes is indicated in yellow. B, structure of SrtG1, which displays the classical 9-stranded β-barrel also observed in other sortases. The dotted green line represents the region 110–119, which could not be traced in the electron density map. C, the catalytic site of SrtG1 carries a Cys-His-Arg triad.

molecule. SrtG1 also carries a characteristic sortase signature motif, LLSTC, which deviates from the classical motif (TLXTC).

SrtG1 was crystallized in space group C2221, with two molecules in the asymmetric unit using the structure of Spy0129 from S. pyogenes as a search model in a molecular replacement experiment. For each of the two independent SrtG1 molecules, residues 46–75 and 110–119 were not visible in the crystal experiment. For each of the two independent SrtG1 molecules, the two molecules in the asymmetric unit are related by a non-crystallographic 2-fold axis, and analyses performed with PISA (56) indicate that the dimeric arrangement is not suggestive of a physiological oligomer, as the buried surface area between the two molecules is only 1,718 Å2 of a total surface of 16,937 Å2 (CSS = 0.0). Superposition of the two monomers reveals that they are very similar, with an r.m.s. difference in Ca atom positions of 0.4 Å over 174 aligned residues.

SrtG1, a Pilus-forming Class B Sortase—The overall structure of SrtG1 conforms to the archetypal sortase fold in which a highly twisted, eight-stranded β-sheet generates a core β-barrel that is surrounded by four helices and one small strand (Fig. 5B). The central β-barrel is structurally reminiscent of those observed in other sortases, including enzymes not involved in pilus biosynthesis. However, the arrangement of secondary structure elements around the barrel is distinct from other molecules (Fig. 6). A DALI search (50) reveals that its closest homologs are Spy0129, an ancillary class B sortase from the pilus-forming machinery of S. pyogenes (29) as well as those from Staphylococcus aureus and Bacillus anthracis (57), all of which present r.m.s. deviations in the range of 2.5 Å for ~200 Ca atoms. The most noticeable differences between SrtG1 and its closest counterpart, Spy0129, are the positions and lengths of the α-helices that surround the β-barrel core (compare the two structures on the left half of Fig. 6). These findings are corroborated by the fact that SrtG1 displays sequence identity levels in the range of 36% with the abovementioned sortases and <20% with class A and class C enzymes. Importantly, despite the fact that SrtG1 is essential for the formation of the Type II pilus in S. pneumoniae, it does not display the prototypical lid found in pilin sortases involved in type I pilus biosynthesis (24, 27, 28). These observations strongly suggest that SrtG1 is also a class B sortase, as is the case for Spy0129, which is also involved in pilus biosynthesis in a Gram-positive organism but does not display the lid region observed in class C enzymes (29).

The transpeptidation reaction catalyzed by sortases requires the participation of a Cys-His-Arg catalytic triad. In SrtG1, the triad is composed of Cys-241 at the C terminus of β7, Arg-249 at the N terminus of β, and His-147 within the loop that follows β4 (Fig. 5C). Cys-241 points directly into the active site, but the closest interaction that it makes with other elements of the catalytic triad is a 4.4 Å interaction with the NH1 atom of Arg-249; the distance to His-147 is in the range of 6.0 Å. However, the side chain of Arg-249 faces the Cys nucleophile; as in other sortase active sites, NH1 makes a hydrogen bond with the backbone carbonyl of Gly-248, which immediately precedes it, thus guaranteeing that it is well positioned for stabilization of the charged intermediate that is formed upon nucleophilic attack of Cys-241 on the peptidic substrate.

The catalytic activity of sortases that form the pilus backbone has been characterized using biochemical and microscopy.
methods (24, 25, 28, 59–62). In the case of SrtG1, this reaction would correspond to the association of PitB monomers, but upon direct incubation of the two proteins in a number of conditions, we could not observe stable fiber formation (albeit the fact that PitB on its own can generate short fibers, as shown above). Thus, to gain insight into this SrtG1-catalyzed PitB association process, we used molecular modeling by employing our two high resolution structures of PitB and SrtG1, as described below.

Molecular Basis of the SrtG1-PitB Interaction—Details of the sortase-catalyzed reaction have been widely investigated thanks to extensive studies performed on sortases A and B (SrtA/SrtB; Refs. 33 and 63–69). It has been shown that upon cleavage of the LPXTG Thr-Gly bond by the catalytic cysteine, the formation of a stable thioacyl intermediate ensues. The role of the catalytic His has been linked to the donation of protons during the reaction, whereas that of the Arg is associated to stabilization of the negative charge of the intermediate (32). Despite these detailed advances, structural knowledge on how the substrates themselves are recognized (thus, the step that precedes covalent complex formation) is limited, largely due to the difficulty in obtaining complexes between sortases and their cognate substrates. In the case of sortases that catalyze the formation of the type I pilus backbone, for example, their structures reveal that they display lids in the vicinity of the active site, suggesting that specific regions on the surface must be recognized before the lid can be “opened” and the active site made accessible for the substrate (24, 25). We thus employed our structure of PitB (which harbors the LPXTG motif) and of SrtG1 (which does not display a lid) in the generation of a snapshot of the first step in type II pilus biogenesis using molecular modeling. The structure of PitB was used as a ligand to dock to that of SrtG1 using ClusPro v.2, an automated docking program that employs a multistage protocol (48); details of the procedure can be found under “Experimental Procedures.”

The LPXTG motif of PitB, located at its extreme C terminus, corresponds to the VTPTG sequence; however, the C-terminal Gly residue could not be modeled in the electron density map (potentially due to flexibility), so only the VTPT sequence was used. The interaction region is vast and involves 850 Å² in SrtG1 (17 residues) and 950 Å² in PitB (18 residues), corresponding to 13 direct H-bonds and 206 non-bonded contacts.
Architecture of the Pneumococcal Type II Pilus

(Fig. 7). The VTPT motif is recognized within an elongated cleft that harbors the active site in one of its extremities, and most contacts between VTPT and SrtG1 are made between the sortase and backbone atoms of VTPT. The SH group of Cys-198 from SrtG1, which should cleave the covalent bond between Thr-383 and Gly-384 within the LPXTG motif of PitB, is H-bonded to the backbone carbonyl of Pro-382 of PitB. It is of note that the side chains of the two other SrtG1 residues involved in catalysis, His-104 and Arg-206, also interact with VTPT backbone atoms, thus explaining the interchangeability in LPXTG-like sequences accepted for catalysis by pilus-biosynthesis sortases (24–26). Interestingly, the mode of recognition between SrtG1 and the VTPTG motif of PitB is reminiscent of that shown for complexes between SrtA and SrtB bound to their substrate peptides (64, 66); one major difference, however, is the absence of the “L” shape for the substrate, which is induced by the presence of a Pro residue in the P3 position of the peptide in the case of SrtA/SrtB. In SrtG1, position P3 is occupied by a Thr, and thus the conformation of the substrate peptide follows a more linear path.

Nevertheless, the large amount of interactions that the C terminus of PitB makes with the elongated cleft of SrtG1 brings into question how such substrates are recognized in lid-carrying sortases such as SrtC-1, SrtC-2, and SrtC-3 from S. pneumoniae. If the entire cleft is used for binding, as is the case here, then not only must the lid itself undergo a conformational modification but also the loop that precedes it (Fig. 6), allowing the main β-sheet of the sortase to be contacted by the substrate molecule; this possibility is supported by mutagenesis and structural data obtained for group B Streptococcus sortases (58, 59). It is not clear why SrtG1 and Spy0129 from S. pyogenes (29) lack lid regions, but this could potentially be related to the lack of necessity of strict discrimination of different substrates, as the biosynthesis of the respective pili is simple compared with other multicomponent fibers, such as the type 1 Streptococcus pneumococcal pilus, for example.

These studies also indicate that the VTPTG sequence of PitB is recognized in a very similar fashion by both SrtG1 (Fig. 7) and a neighboring PitB molecule (Fig. 3). This observation suggests that it is improbable that SrtG1 recognizes both PitB monomers concomitantly (generating a ternary complex) due to the probability of the generation of steric clashes. In addition, in vivo it is likely that this reaction also requires the contribution not only of SipA (as indicated above) but also of the lipidic environment on the surface of the bacterium. However, the C-terminal Thr residue of the LPXTG sequence remains exposed once bound to SrtG1 (Fig. 7) and is thus “available” for recognition by an incoming Lys residue from a partner PitB molecule (as highlighted above; Fig. 3C). Thus, it is tempting to hypothesize that SrtG1 positions the thio-acyl intermediate in an exposed location that allows for direct attack from an incoming Lys residue; formation of the final covalent bond engenders release of SrtG1 and a slight rotation of the two associated PitB molecules occurs for the C terminus of D2 to be stably associated to the neighboring D1. Delineation of the precise mechanism of pilus formation will require the combination of further biochemical, microbiological, and structural studies.

Author Contributions—M. M. S., A. M. D. G., and A. D. conceived the study and analyzed the results. M. M. S. purified and crystallized PitB and SrtG1 and solved the crystal structures and also performed the modeling experiments. C. L., D. F., and G. S. performed all electron microscopy experiments. D. M. T. designed and constructed vectors for expression of mutant proteins. A. M. D. G. constructed mutant strains and performed in vivo trials. M. M. S. and A. D. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank J. Marquez and the HTX Laboratory team for access to and help with high throughput crystallization and the ESRF for access to beamlines. This project used the platforms of the Grenoble Instruct Center (ISBG; UMS 3518 CNRS-CEA-UJF-EMBL) with support from FRISBI (ANR-10-INSB-05-02) and GRAL (ANR-10-LABX-49-01) within the Grenoble Partnership for Structural Biology (PSB). The electron microscopy facility is supported by the Rhône-Alpes Region, the Fondation pour la Recherche Medicale (FRM), and the FEDER funds.

References

1. Mehr, S., and Wood, N. (2012) Streptococcus pneumoniae: a review of carriage, infection, serotype replacement, and vaccination. Paediatr. Respir. Rev. 13, 258–264
2. Bogaert, D., Hermans, P. W., Adrian, P. V., Rümke, H. C., and de Groot, R. (2004) Pneumococcal vaccines: an update on current strategies. Vaccine 22, 2209–2220
3. Feldman, C., and Anderson, R. (2014) Recent advances in our understanding of Streptococcus pneumoniae infection. F1000Prime Rep. 6, 82
4. Izoré, T., Contreras-Martel, C., El Mortaji, L., Manzano, C., Terrasse, R., Vernet, T., Di Guilmi, A. M., and Dessen, A. (2010) Structural basis of host cell recognition by the pilus adhesin from Streptococcus pneumoniae. Structure 18, 106–115
5. Kline, K. A., Falker, S., Dahlberg, S., Normark, S., and Henriques-Normark, B. (2009) Bacterial adhesins in host-microbe interactions. Cell Host Microbe 5, 580–592
6. Hament, J. M., van Dijk, H., Fleer, A., Aerts, P. C., Schoenmakers, M., de Snoo, M. W., Dekker, B. H., Kimpen, J. L., and Wolfs, T. F. (2003) Pneumococcal immune adherence to human erythrocytes. Eur. J. Clin. Invest. 33, 169–175
7. Chagnot, C., Zorgani, M. A., Astruc, T., and Desvaux, M. (2013) Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. Front. Microbiol. 4, 303
8. Proft, T., and Baker, E. N. (2009) Pili in Gram-negative and Gram-positive bacteria: structure, assembly, and their role in disease. Cell. Mol. Life Sci. 66, 613–635
9. Kang, H. J., and Baker, E. N. (2012) Structure and assembly of Gram-positive bacterial pilus: unique covalent polymers. Curr. Opin. Struct. Biol. 22, 200–207
10. Danne, C., and Drämsli, S. (2012) Pili of Gram-positive bacteria: roles in host colonization. Res. Microbiol. 163, 645–658
11. Barocchi, M. A., Ries, I., Zogaj, X., Hemsley, C., Albiges, B., Kanth, A., Dahlberg, S., Fernebro, J., Moschioni, M., Masignani, V., Hultenby, K., Taddei, A. R., Beiter, K., Wartha, F., von Euler, A., Covacci, A., Holden, D. W., Normark, S., Rappuoli, R., and Henriques-Normark, B. (2006) A pneumococcal pilus influences virulence and host inflammatory responses. Proc. Natl. Acad. Sci. U.S.A. 103, 2857–2862
12. Hava, D. L., and Camilli, A. (2002) Large-scale identification of serotype 4 Streptococcus pneumoniae virulence factors. Mol. Microbiol. 45, 1389–1406
13. LeMieux, J., Hava, D. L., Basset, A., and Camilli, A. (2006) RrgA and RrgB are components of a multisubunit pilus encoded by the Streptococcus pneumoniae thrA pathogenicity islet. Infect. Immun. 74, 2453–2456
14. El Mortaji, L., Contreras-Martel, C., Moschioni, M., Ferlenghi, I., Man-
zano, C., Vernet, T., Dessen, A., and Di Guilmi, A. M. (2012) The full-length Streptococcus pneumoniae major pilin RrgB crystallizes in a fiber-like structure. Biochem. J. 441, 833–841

15. Paterson, N. G., and Baker, E. N. (2011) Structure of the full-length major pilin from Streptococcus pneumoniae: implications for isopeptide bond formation in Gram-positive bacterial pilis. PLoS ONE 6, e22095

16. Shaik, M. M., Maccagni, A., Tourcier, G., Di Guilmi, A. M., and Dessen, A. (2014) Structural basis of pilus anchoring by the ancillary pilin RrgC of Streptococcus pneumoniae. J. Biol. Chem. 289, 16988–16997

17. Sjöström, K., Blomberg, C., Fernenbo, J., Dagerhamn, J., Mordhorst, E., Barocci, M. A., Browall, S., Moschioni, M., Andersson, M., Henriques, F., Albiger, B., Rappuoli, R., Norman, S., and Henriques-Normark, B. (2007) Clonal success of pilated penicillin nonsusceptible pneumococci. Proc. Natl. Acad. Sci. U.S.A. 104, 12907–12912

18. Zähner, D., Gudlavalleti, A., and Stephens, D. S. (2010) Increase in pilus islet 2-encoded pilin among Streptococcus pneumoniae isolates, Atlanta, Georgia, USA. Emerg. Infect. Dis. 16, 955–962

19. Bagioni, F., Moschioni, M., Donati, C., Dimitrovska, V., Ferlenghi, I., Facchetti, C., Muzzi, A., Giusti, F., Emolo, C., Sinisi, A., Hilleringmann, M., Pansegrau, W., Cessini, S., Rappuoli, R., Covacci, A., Masiagnini, V., and Barocci, M. A. (2008) A second pilus type in Streptococcus pneumoniae is prevalent in emerging serotypes and mediates adhesion to host cells. J. Bacteriol. 190, 5480–5492

20. Zähner, D., Gandhi, A. R., Yi, H., and Stephens, D. S. (2011) Mitis group streptococci express variable pilus islet 2 pilis. PLoS ONE 6, e25124

21. Young, P. G., Proft, T., Harris, P. W., Brimble, M. A., and Baker, E. N. (2014) Structure and activity of Streptococcus pyogenes SipA: a signal peptide-like protein essential for pilus polymerisation. PLoS ONE 9, e99135

22. Dramsi, S., Trieu-Cuot, P., and Bierne, H. (2005) Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. EMBO J. 24, 289–297

23. Schneewind, O., and Missiakas, D. M. (2012) Protein secretion and surface display in Gram-positive bacteria. Annu. Rev. Microbiol. 66, 133–156

24. Caradoc-Davies, T. T., Proft, T., and Baker, E. N. (2010) Crystal structure of the major (Spy0128) and minor (Spy0130) pili subunits from Streptococcus pneumoniae. J. Mol. Biol. 393, 1043–1054

25. Cozzati, R., Vannini, C., Adami, S., Adami, C., Marques, P., Reis, A., and Raposo, G. (2010) Tethering proteins to the surface of living cells. J. Cell Biol. 191, 1037–1048

26. Clancy, W. K. W., Melvin, J. A., and McCafferty, D. G. (2010) Sortase transpeptidases: insights into mechanism, substrate specificity, and inhibition. Biopolymers 94, 385–396

27. Marraffini, L. A., Dedent, A. C., and Schneewind, O. (2006) Sortases and the art of anchoring proteins to the envelopes of Gram-positive bacteria. Microbiol. Mol. Biol. Rev. 70, 192–221

28. Hilleringmann, M., Ringler, P., Müller, S. A., De Angelis, G., Rappuoli, R., Ferlenghi, I., and Engel, A. (2009) Molecular architecture of Streptococcus pneumoniae TIGR4 pili. EMBO J 28, 3921–3930

29. Kung, H. I., Coulibaly, F., Clow, F., Proft, T., and Baker, E. N. (2007) Stabilizing isopeptide bonds revealed in Gram-positive bacterial pilus structure. Science 318, 1625–1628

30. Webb, B., and Sali, A. (2014) Protein structure modeling with MODELLER. Methods Mol. Biol. 1137, 1–15

31. Evans, P. (2006) Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82

32. Vonrhein, C., Blanc, E., Roversi, P., and Bricogne, G. (2007) Automated structure solution with autoSHARP. Methods Mol. Biol. 364, 215–230

33. Terwilliger, T. C., Grosse-Kunstleve, R. W., Afonine, P. V., Moriarty, N. W., Adams, P. D., Read, R. J., Zwart, P. H., and Hung, L. W. (2008) Iterative-build OMIT maps: map improvement by iterative model building and refinement without model bias. Acta Crystallogr. D Biol. Crystallogr. 64, 515–524

34. Cowtan, K. (2006) The Buccaneer software for automated model building. 1. Tracing protein chains. Acta Crystallogr. D Biol. Crystallogr. 62, 1002–1111

35. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

36. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

37. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 53, 240–255

38. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291

39. Wang, W., and Malcolm, B. A. (1999) Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange site-directed mutagenesis. BioTechniques 26, 680–682

40. Long, F., Vagin, A. A., Young, P., and Murshudov, G. N. (2008) BALBES: a molecular replacement pipeline. Acta Crystallogr. D Biol. Crystallogr. 64, 125–132

41. Comeau, S. R., Gateliac, D. W., Vajda, S., and Camacho, C. I. (2004) ClusPro: an automated docking and discrimination method for the prediction of protein complexes. Bioinformatics 20, 45–50

42. Kozakov, D., Brenke, R., Comeau, S. R., and Vajda, S. (2006) PIPER: an FFT-based protein docking program with pairwise potentials. Proteins 65, 392–406

43. Holm, L., and Rosenström, P. (2010) DalI server: conservation mapping in 3D. Nucleic Acids Res. 38, W545–W549

44. Linke, C., Young, P. G., Kang, H. J., Bunker, R. D., Middelditch, M. J., Caradod-Davies, T. T., Proft, T., and Baker, E. N. (2010) Crystal structure of the minor pilin FcbR reveals determinants of Group A streptococcal pili anchoring. J. Biol. Chem. 285, 20381–20389

45. Solovyova, A. S., Pointon, J. A., Race, P. R., Smith, W. D., Keohoe, M. A., and Banfield, M. J. (2010) Solution structure of the major (Spy0128) and minor (Spy0129) pilus subunits from Streptococcus pyogenes. Eur. Biophys. J. 39, 469–480

46. Pointon, J. A., Smith, W. D., Saalbach, G., Crow, A., Keohoe, M. A., and Banfield, M. J. (2010) A highly unusual thioester bond in a pilus adhesin is required for efficient host cell interaction. J. Biol. Chem. 285, 33858–33866

47. Kang, H. I., and Baker, E. N. (2009) Intramolecular isopeptide bonds give thermodynamic and proteolytic stability to the major pilin protein of Streptococcus pneumoniae. J. Biol. Chem. 284, 20729–20737

48. Hilleringmann, M., Giusti, F., Baudner, B. C., Masiagnini, V., Covacci, A., Rappuoli, R., Barocci, M. A., and Ferlenghi, I. (2008) Pneumococcal pili are composed of protofilaments exposing adhesive clusters of RrgA. PLoS
Pathog. 4, e1000026

56. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797

57. Zhang, R., Wu, R., Joachimiak, G., Mazmanian, S. K., Missiakas, D. M., Gornicki, P., Schneewind, O., and Joachimiak, A. (2004) Structures of sortase B from Staphylococcus aureus and Bacillus anthracis reveal catalytic amino acid triad in the active site. Structure 12, 1147–1156

58. Cozzi, R., Zerbini, F., Assfalg, M., D’Onofrio, M., Biagini, M., Martinelli, M., Nuccitelli, A., Norais, N., Telford, J. L., Maione, D., and Rinaudo, C. D. (2013) Group B Streptococcus pilus sortase regulation: a single mutation in the lid region induces pilin protein polymerization in vitro. FASEB J. 27, 3144–3154

59. Khare, B., Fu, Z. Q., Huang, I. H., Ton-That, H., and Narayana, S. V. (2011) The crystal structure analysis of group B Streptococcus sortase C1: a model for the “lid” movement upon substrate binding. J. Mol. Biol. 414, 563–577

60. Wu, C., Mishra, A., Reardon, M. E., Huang, I. H., Counts, S. C., Das, A., and Ton-That, H. (2012) Structural determinants of Actinomyces sortase SrtC2 required for membrane localization and assembly of type 2 fimbriae for interbacterial coaggregation and oral biofilm formation. J. Bacteriol. 194, 2531–2539

61. El Mortaji, L., Fenel, D., Vernet, T., and Di Guilmi, A. M. (2012) Association of RrgA and RrgC into the Streptococcus pneumoniae pilus by sortases C-2 and C-3. Biochemistry 51, 342–352

62. Nielsen, H. V., Flores-Mireles, A. L., Aparicio, M., Kline, K. A., Pinkner, J. S., Neiers, F., Normark, S., Henriques-Normark, B., Caparon, M. G., and Hultgren, S. (2011) Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in Enterococcus faecalis. J. Bacteriol. 195, 4484–4495

63. Ilangovan, U., Ton-That, H., Iwahara, J., Schneewind, O., and Clubb, R. T. (2001) Structure of sortase, the transpeptidase that anchors proteins to the cell wall of Staphylococcus aureus. Proc. Natl. Acad. Sci. U.S.A. 98, 6056–6061

64. Jacobitz, A. W., Wereszczynski, J., Yi, S. W., Amer, B. R., Huang, G. L., Nguyen, A. V., Sawaya, M. R., Jung, M. E., McCammon, J. A., and Clubb, R. T. (2014) Structural and computational studies of the Staphylococcus aureus sortase B-substrate complex reveal a substrate-stabilized oxyanion hole. J. Biol. Chem. 289, 8891–8902

65. Naik, M. T., Suree, N., Ilangovan, U., Liew, C. K., Thieu, W., Campbell, D. O., Clemsen, J. J., Jung, M. E., and Clubb, R. T. (2006) Staphylococcus aureus sortase A transpeptidase. Calcium promotes sorting signal binding by altering the mobility and structure of an active site loop. J. Biol. Chem. 281, 1817–1826

66. Suree, N., Liew, C. K., Villareal, V. A., Thieu, W., Fadeev, E. A., Clemsen, J. J., Jung, M. E., and Clubb, R. T. (2009) The structure of the Staphylococcus aureus sortase-substrate complex reveals how the universally conserved LPXTG sorting signal is recognized. J. Biol. Chem. 284, 24465–24477

67. Ton-That, H., Mazmanian, S. K., Alksne, L., and Schneewind, O. (2002) Anchoring of surface proteins to the cell wall of Staphylococcus aureus. J. Biol. Chem. 277, 7447–7452

68. Frankel, B. A., Kruger, R. G., Robinson, D. E., Kelleher, N. L., and McCafferty, D. G. (2005) Staphylococcus aureus sortase transpeptidase SrtA: insight into the kinetic mechanism and evidence for a reverse protonation catalytic mechanism. Biochemistry 44, 11188–11200

69. Frankel, B. A., Tong, Y., Bentley, M. L., Fitzgerald, M. C., and McCafferty, D. G. (2007) Mutational analysis of active site residues in the Staphylococcus aureus transpeptidase SrtA. Biochemistry 46, 7269–7278