The *Corynebacterium diphtheriae* shaft pilin SpaA is built of tandem Ig-like modules with stabilizing isopeptide and disulfide bonds

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Cell-surface pili are important virulence factors that enable bacterial pathogens to adhere to specific host tissues and modulate host immune response. Relatively little is known about the structure of Gram-positive bacterial pili, which are built by the sortase-catalyzed covalent crosslinking of individual pilin proteins. Here we report the 1.6-Å resolution crystal structure of the shaft pilin component SpaA from *Corynebacterium diphtheriae*, revealing both common and unique features. The SpaA pilin comprises 3 tandem Ig-like domains, with characteristic folds related to those typically found in non-pilus adhesins. Whereas both the middle and the C-terminal domains contain an intramolecular Lys–Asn isopeptide bond, previously detected in the shaft pilins of *Streptococcus pyogenes* and *Bacillus cereus*, the middle Ig-like domain also harbors a calcium ion, and the C-terminal domain contains a disulfide bond. By mass spectrometry, we show that the SpaA monomers are cross-linked in the assembled pili by a Lys–Thr isopeptide bond, as predicted by previous genetic studies. Together, our results reveal that despite profound dissimilarities in primary sequences, the shaft pilins of Gram-positive pathogens have strikingly similar tertiary structures, suggesting a modular backbone construction, including stabilizing intermolecular and intramolecular isopeptide bonds.

crystal structure | polymerization | bacterial pilus | mass spectrometry | pilin motif

Pili are long, thin protein assemblies that extend from the cell surface of many bacteria and play pivotal roles in colonization and pathogenesis. Like Gram-negative bacteria, many Gram-positive pathogens express pili on their surface (1–3). These structures have aroused great interest because of their direct roles in infection and pathogenesis and their importance as vaccine candidates (2, 3). They also use covalent isopeptide (amide) bonds, both intermolecular and intramolecular, to give strength and stability, and thus present a new paradigm among protein polymers (4–6).

Unlike Gram-negative pili, whose subunits associate via non-covalent interactions, these Gram-positive pili are formed by covalent polymerization of pilin subunits, orchestrated by transpeptidase enzymes called sortases (6, 7). The general principles of assembly were first established through studies on the SpaA pili expressed by *Corynebacterium diphtheriae*. These pili, encoded by the gene cluster *spaA-spaB-srtA-spaC*, comprise a polymeric shaft formed by SpaA, SpaC located at the tip, and SpaB found at the base and occasionally along the shaft (6, 8–10). In our current model, successive major pilin SpaA subunits are joined by the action of the pilus-specific sortase SrtA. Cleavage between Thr-494 and Gly-495 of the LPXTG motif near the SpaA C terminus is followed by presumed amide bond formation between the new C terminus and Lys-190 from a conserved YPKN pilin motif in the next subunit (6). Finally, the entire assembly is covalently attached to the cell wall peptidoglycan by a housekeeping sortase (9, 11). Presumably, the tip pilin SpaC is linked to the SpaA shaft via the same reaction as recently

identified for the tip pilin BcpB of *Bacillus cereus* (12). It remains unclear how the minor pilin SpaB is incorporated into the pilus structure, although recent evidence indicates that SpaB forms the basal subunit for tethering the pilus to the cell wall and that it, too, is attached to the polymer via a specific Lys residue (9).

A major step forward in understanding Gram-positive pilus structure and assembly came with the structural analysis of the major pilin Spy0128 from *Streptococcus pyogenes* (5). Spy0128 does not have a recognizable pilin motif, but the crystal structure revealed columns of molecules resembling a putative polymer assembly and identified a candidate lysine; this was then confirmed by mass spectral analysis of native *S. pyogenes* pili. The structure also revealed unexpected internal crosslinks in the form of self-generated isopeptide bonds, 1 in each domain of the 2-domain structure, joining Lys and Asn side chains. These are strategically located to give strength and stability to the pilus assembly.

The major pilins of different Gram-positive bacteria show wide variations in size and sequence, making it difficult to predict whether the structural principles seen for *S. pyogenes* apply also to other Gram-positive pili. Here we present the high-resolution crystal structure of SpaA, the archetypal major pilin from *C. diphtheriae*. This reveals a modular structure comprising 3 tandem Ig-like domains, 2 of which contain internal Lys–Asn isopeptide bonds like those in Spy0128. We also confirm, by mass spectrometry, the identity of the lysine used in polymerization and note a pilus-like assembly of SpaA molecules in the crystal. These results point to a likely common architecture for the backbones of many Gram-positive pili and consolidate a new paradigm for the structure, stability, and assembly of these remarkable covalent polymers.

Results

Structure Determination. A construct comprising residues 53–486 of *C. diphtheriae* SpaA was expressed in *Escherichia coli*, purified, and crystallized. This lacks residues 1–52 encompassing the signal peptide and ends 4 residues before the sortase-recognition LPXTG motif. The crystal structure, with 1 SpaA molecule per asymmetric unit, was solved by single wavelength anomalous dispersion methods and refined at 1.6-Å resolution ($R = 19.3\%$, $R_{\text{free}} = 22.0\%$) [supporting information (SI) Table S1]. Only the

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tococci, *Streptococcus pneumoniae*, and *B. cereus* (19–21), but the pilin subunits involved show large variations in size and sequence that mask any possible structural homology.

The present structure for the *C. diphtheriae* major pilin SpaA resolves this question, revealing a modular assembly that utilizes Ig-like domains similar to those used in the *S. pyogenes* major pilin Spy0128, despite very low sequence identity. These domains correspond to 2 types of Ig-like domain, the CnaB and CnaA folds that are widely used in the cell-surface adhesins known as MSCRAMMS (microbial surface components recognizing adhesive matrix molecules) (22). Prototype CnaB and CnaA domains are present in the multidomain *S. aureus* Cna protein, which has a structural B-region with repeating doublets of CnaB domains, preceded by a collagen-binding A-region with 2 CnaA domains (22, 23).

Spy0128, the major pilin of *S. pyogenes*, is one of the smallest shaft pilins (32.5 kDa) and comprises 2 tandem CnaB domains (5). SpaA, in contrast, is significantly larger (47 kDa) and has a single CnaA-type domain, the M-domain, inserted between 2 CnaB-type domains. This mosaic architecture suggests an evolutionary process in which copies or pieces of older genes are assembled to form new genes. It seems likely that all of the major pilins of sortase-assembled Gram-positive pili conform to the same structural principles. In many cases, for example the major pilins SpaD and SpaH that form the 2 other types of pilus produced by *C. diphtheriae*, sufficient sequence identity exists to infer similar structures; these share $\approx 24\%$ identity with SpaA, including the intramolecular isopeptide bond-forming residues, the pilin motif, and the Cys residues. In others, such as Spy0128, there is much less sequence similarity.

Sequence comparisons with the major pilins from other Gram-positive bacteria, such as *S. pneumoniae*, *S. agalactiae*, and *B. cereus*, reveal N- and C-terminal regions that are similar to the N- and C-domains of SpaA, including the N-domain pilin motif and the C-domain isopeptide bond-forming residues. The middle regions are more variable; some may form CnaA-type domains like the SpaA M-domain, with substantial insertions/deletions, whereas others may have CnaB-type middle domains or adopt entirely different folds.

There is growing evidence that this modular architecture extends also to the minor pilin subunits. GBS52, a minor pilin from *S. agalactiae* that seems to correspond functionally to SpaB, comprises 2 CnaB-type domains (15), and its C-terminal N2 domain closely resembles the SpaA C-domain, including the internal isopeptide bond. Sequence comparisons show that the *S. pyogenes* minor pilin Cpa also has a C-terminal domain homologous with the C-domain of the *S. pyogenes* major pilin Spy0128, again including an internal isopeptide bond (5). This leads to the concept that incorporation of the minor pilins is facilitated by their structural resemblance to the pilins that comprise the polymeric shaft.

The 2 internal isopeptide bonds in SpaA confirm that such crosslinks are a common feature of Gram-positive pili. First discovered in the crystal structure of Spy0128 and confirmed by mass spectral analysis of native GAS pili (5), they have also been found in the *B. cereus* major pilin BcpA, which has 3 such bonds (4). The combined sequence and structural data from these 3 characterized major pilins now enable internal isopeptide bonds to be inferred from the sequences of other major pilins. Re-evaluation of the crystal structures of GBS52 and the A- and B-domains of *S. aureus* Cna further showed that similar isopeptide bonds are also present in minor pilins and adhesins (5).

The SpaA structure further defines the determinants of intramolecular isopeptide bond formation. Mutagenesis has shown that a catalytic carboxyl group is essential for bond formation (4, 5, 24); this can be Asp or Glu, as shown by the use of Asp-241 in the M-domain and Glu-446 in the C-domain. The isopeptide moiety can have either *cis* or *trans* configuration, with

corresponding bidentate or monodentate interaction with the essential carboxyl side chain. The main requirement is proximity of the Lys–Asn pair and a hydrophobic environment in which both the Lys and Asp/Glu are uncharged. The locations of these isopeptide bonds seem to be characteristic of the folds of the domains in which they occur, and hence probably reflect their evolutionary history. In the CnaB-like C-domain of SpaA, the isopeptide bond joins the first and last β -strands, as in both CnaB-type domains of Spy0128. In contrast, in the CnaA-type M-domain, the isopeptide bond bridges the first and second-last strands, linking 2 opposing β -sheets.

Given the widespread occurrence of internal isopeptide crosslinks in the cell-surface proteins of Gram-positive bacteria, what is their structural and functional importance? We have shown that the intramolecular isopeptide bonds in Spy0128 strongly enhance thermodynamic stability and resistance to proteolysis (24). SpaA additionally contains a disulfide bond, conserved in the other *C. diphtheriae* major pilins, which might be expected to further enhance stability. The major pilins of *S. pyogenes*, *B. cereus*, and *S. pneumoniae* lack Cys residues, however, and given that many Gram-positive bacteria lack the disulfide formation machinery of Gram-negative bacteria (25, 26), we speculate that isopeptide bonds have evolved as an alternative means of stabilization. As amide bonds they would also be less prone to chemical disruption than disulfide bonds, a property that may be important for such thin, exposed assemblies, which do not seem to form higher-order bundles.

We further hypothesize that their strategic location gives mechanical (force-bearing) stability. Both in these pili, typified by Spy0128 and SpaA, and in multidomain adhesins such as Cna, an almost linear chain of covalent connectivity can be traced along the long axis (5, 27). In SpaA this begins with Lys-190, the site of attachment to the preceding subunit, and extends through the M- and C-domains to the next intermolecular linkage, possibly explaining why the N-domain does not require an isopeptide bond. The attachment of adhesins to host cells subjects them to significant tensile stress, and their structures are thought to have evolved both to withstand stress and to use it to optimize binding (28).

There is also growing evidence that the structural stabilization conferred by the internal isopeptide bonds can be critical for molecular recognition. Defective sortase recognition or failure to present the Lys residue of the pilin motif appropriately would explain the loss of pilus assembly when intramolecular isopeptide bonds in SpaA or BcpA are deleted by mutation of the catalytic Asp/Glu. The isopeptide bonds in CnaA and GBS52 may similarly influence the binding of partner molecules, because their isopeptide bond-containing domains are important for specific binding of collagen (CnaA) and capable of binding to human pulmonary epithelial cells (GBS52) (15, 29).

Finally, an intriguing feature of the crystal structures of both SpaA and Spy0128 is the way the pilin molecules pack end-to-end in columns in the crystal, resembling assembled pili. The intermolecular contacts do not seem to be particularly extensive (buried surface 850 Å² for Spy0128 and 814 Å² for SpaA), yet in each case the molecules pack such that the critical lysine residue (Lys-161 in Spy0128, Lys-190 in SpaA) is brought close to the C terminus of the next molecule. Importantly, in SpaA the conserved Trp-181 of the pilin motif forms part of this interface, consistent with a role in oligomer formation. The roles of the conserved Tyr-188 and Pro-189 of the pilin motif are less clear, but patches of electron density around them may represent parts of the unmodeled AB loop. These residues may interact transiently with this loop, which could in turn mediate the pilin–pilin and/or pilin–sortase interactions.

Materials and Methods

Cloning and Protein Purification. DNA encoding amino acids 53–486 of SpaA from *C. diphtheriae* was amplified by PCR from genomic DNA, cloned, over-

expressed in *E. coli* as an N-terminally His-tagged protein, and purified by nickel-affinity chromatography (30). After His-tag removal and final size-exclusion chromatography, the protein was concentrated to 100 mg/mL in 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl. Selenomethionine (SeMet)-substituted SpaA was produced using the methionine biosynthesis inhibition method (31) and similarly purified, but with 5 mM DTT and 1 mM EDTA in the final gel filtration buffer.

Crystallization and Structure Determination. Crystals were grown in sitting drops comprising 100 nL protein (100 mg/mL) and 100 nL precipitant. The best native SpaA crystals were obtained with 20% PEG 3350, 0.1 M NaI, and 0.1 M NaF as precipitant and SeMet-SpaA crystals with 20% PEG 3350 and 0.2 M Na formate. Crystals were flash-cooled without further cryoprotection. X-ray diffraction data were collected on beamline PX1 at the Australian Synchrotron, to 1.6 Å and 1.8 Å resolutions, respectively, for native and SeMet-SpaA crystals. Data were processed and scaled with MOSFLM and SCALA (32). All 4 Se atoms were located by SHELX (33) with refinement and phase determination in autoSHARP (34). Density modification and model building with PHENIX (35, 36) placed 408 of 436 residues, and model building was completed using COOT (37). The model was refined using REFMAC (38). Data collection, phasing, and refinement statistics are in Table S1. Structural superpositions were done with SSM (39).

Isolation of SpaA Pili. Engineered SpaA pili were produced by transformation of the plasmid pAG153, encoding SpaA and SrtA, into a *C. diphtheriae* strain

that lacks *spaA* and *srtA*, followed by expression and purification of the polymers (6). These procedures are described more fully in *SI Materials and Methods*. The use of a His-tagged SpaA construct with a SrtA construct lacking 13 C-terminal residues means that the engineered SpaA pili are secreted into the culture medium and can be purified by nickel-affinity chromatography as previously described (6).

Proteolytic Digestion and Mass Spectral Analyses. Purified SpaA pili and recombinant SpaA protein were digested and analyzed according to previous protocols (5). Briefly, SDS-PAGE gel bands containing recombinant SpaA or SpaA pili were cut out and incubated with trypsin (Promega) followed by AspN endopeptidase (Roche). Peptides in the *m/z* range 300–1,600 were analyzed using a Q-STAR XL Hybrid MS/MS system (Applied Biosystems). Searches against SpaA sequence using Mascot search engine version 2.0.05 (Matrix Science) identified linear peptides, and the unmatched peptides were then searched manually to identify those containing noncontiguous peptides crosslinked by isopeptide bonds, either intramolecular or intermolecular. Full details are in *SI Materials and Methods*.

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