Molecular architecture of *Streptococcus pneumoniae* TIGR4 pili

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Although the pili of Gram-positive bacteria are putative virulence factors, little is known about their structure. Here we describe the molecular architecture of pilus-1 of *Streptococcus pneumoniae*, which is a major cause of morbidity and mortality worldwide. One major (RrgB) and two minor components (RrgA and RrgC) assemble into the pilus. Results from TEM and scanning transmission EM show that the native pili are approximately 6 nm wide, flexible filaments that can be over 1 µm long. They are formed by a single string of RrgB monomers and have a polarity defined by nose-like protrusions. These protrusions correlate to the shape of monomeric RrgB–His, which like RrgA–His and RrgC–His has an elongated, multi-domain structure. RrgA and RrgC are only present at the opposite ends of the pilus shaft, compatible with their putative roles as adhesin and anchor to the cell wall surface, respectively. Our structural analyses provide the first direct experimental evidence that the native *S. pneumoniae* pilus shaft is composed exclusively of covalently linked monomeric RrgB subunits oriented head-to-tail.

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Introduction

Various types of filamentous surface appendages, pili, have been identified in Gram-negative and Gram-positive bacteria (Wu and Fives-Taylor, 2001). Pili fulfill manifold functions during bacterial life cycles, such as host cell invasion, biofilm formation, cell aggregation, DNA transfer and twitching motility (Proft and Baker, 2009). Their structure has to withstand both environmental stress and the activities of

the host immune system. The role of pili as adhesive organelles is crucial to the survival of pathogenic bacteria, which have to attach to specific host cells for colonisation and to establish an infection. While many Gram-negative pili have been studied in detail over the last decades (Fronzes *et al*, 2008), the majority of Gram-positive pili have been discovered only recently and their study, initiated through pioneering work by Schneewind and co-workers on *Corynebacterium diphtheriae* pili (Ton-That and Schneewind, 2003; Ton-That *et al*, 2004), is in its infancy. In contrast to Gram-negative pili, which are typically formed by non-covalently linked subunits, Gram-positive pili are extended polymers assembled from covalently cross-linked pilin subunits and tethered to the cell wall peptidoglycan (reviewed by Ton-That and Schneewind, 2004; Telford *et al*, 2006; Mandlik *et al*, 2008b). As demonstrated for the major pilin subunit of *C. diphtheriae*, the conserved genetic requirements necessary for pilus formation include the pilin motif (WXXXVXVPKN), the E-box domain (YXLXETXAPXGY) and the cell wall sorting signal (LPXTG), followed first by hydrophobic and then by charged amino acids (Ton-That and Schneewind, 2003, 2004; Ton-That *et al*, 2004). Mass spectrometric studies of pilus fragments of *Bacillus anthracis* have confirmed the existence of intermolecular amide bonds between the C-terminal threonine of cleaved sorting signals and the conserved lysine residue (YPKN) within the pilin motif (Budzik *et al*, 2008). However, the structure of the backbone pilin Spy0128 of *Streptococcus pyogenes* and mass spectrometric analysis of pilus fractions showed the isopeptide bond to link the threonine of the sorting signal EVPTG with a conserved lysine that is close to but not within the pilin-like motif (Kang *et al*, 2007). Sortases catalyse the reaction between the threonine of the LPXTG motif and the conserved lysine of the next backbone-forming protein (Marraffini *et al*, 2006; Manzano *et al*, 2008; Neiers *et al*, 2009), and also anchor pili in the cell wall, as demonstrated for several bacterial genera (Swaminathan *et al*, 2007; Budzik *et al*, 2008; Mandlik *et al*, 2008a; Nobbs *et al*, 2008; Neiers *et al*, 2009).

The Gram-positive bacterium *Streptococcus pneumoniae*, also known as pneumococcus, is a major human pathogen (Lode, 2009). The clinical serotype-4 strain *S. pneumoniae* TIGR4 (TIGR4) forms long pili (Barocchi *et al*, 2006; Hilleringmann *et al*, 2008) and its virulence depends on them (Barocchi *et al*, 2006; Rosch *et al*, 2008). However, the second pneumococcal pilus found recently (Bagnoli *et al*, 2008) has not been detected. The long *S. pneumoniae* TIGR4 pili are encoded by the *rtrA* pathogenicity islet that includes a Rof-A-like transcriptional regulator (RlrA), three sortases (SrtC-1, SrtC-2 and SrtC-3) and three structural proteins RrgA (Swiss-Prot Q97SC3), RrgB (Swiss-Prot Q97SC2) and RrgC (Swiss-Prot Q97SC1), all of which contain an LPXTG motif (or variants thereof). As pneumococcal RrgB possesses the conserved motifs necessary for pilus formation (Ton-That and Schneewind, 2003, 2004; Ton-That *et al*, 2004), it has been suspected to form the backbone of the pneumococcal

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TIGR4 pilus, as indeed implied by immunoelectron microscopy (immuno-EM; Barocchi *et al*, 2006; LeMieux *et al*, 2006; Hilleringmann *et al*, 2008). This supposition was recently proved correct by the work of Falcker *et al* (2008), which revealed that only RrgB is required for pilus formation. Roles as ancillary proteins have been suggested for the two other components, RrgA and RrgC (Barocchi *et al*, 2006; LeMieux *et al*, 2006, 2008; Nelson *et al*, 2007; Hilleringmann *et al*, 2008).

Immuno-EM has been used to unravel the location of the various pilus proteins since the first molecular description of Gram-positive pili (Ton-That and Schneewind, 2003). Antibodies targeted to RrgB, the major pilus building block of pneumococcal TIGR4 pili, were consistently found to bind along the filaments (Barocchi *et al*, 2006; LeMieux *et al*, 2006; Hilleringmann *et al*, 2008). Results concerning the location of the ancillary proteins RrgA and RrgC are less consistent: anti-RrgA is reported to bind in regularly spaced clusters along the pili (LeMieux *et al*, 2006) and close to the cell surface in the absence of both RrgB and RrgC (LeMieux *et al*, 2006; Nelson *et al*, 2007). Triple labelling has shown RrgA clusters to decorate pilus assemblies randomly and independent of RrgC distribution (Nelson *et al*, 2007; Hilleringmann *et al*, 2008), whereas a colocalisation of RrgA and RrgC in clusters has been observed by double-labelling experiments (Falcker *et al*, 2008). In addition, surface topographs acquired by atomic-force microscopy allowed identification of RrgC at the ends of detached pili (Falcker *et al*, 2008). Given these, in part contradictory, data the localisation of the three proteins in the pilus is still uncertain. In fact, both local association of RrgA and RrgC to the pilus shaft and their incorporation within it have been proposed (LeMieux *et al*, 2006; Nelson *et al*, 2007; Falcker *et al*, 2008; Hilleringmann *et al*, 2008).

The pilus-specific TIGR4 sortases, SrtC-1, SrtC-2 and SrtC-3 (formerly SrtB, SrtC and SrtD) diverge in sequence from the housekeeping sortase, SrtA, the latter being dispensable for pilus assembly and localisation to the cell wall (LeMieux *et al*, 2008). Class-C sortases exhibit functional redundancy concerning pilus assembly and cell wall localisation (LeMieux *et al*, 2008; Manzano *et al*, 2008; Neiers *et al*, 2009). One study showed SrtC-1 and SrtC-3 to be required for incorporation of the ancillary subunits (LeMieux *et al*, 2008), while another report suggested SrtC-1 and SrtC-2 to differ only in their ability to incorporate RrgC (Neiers *et al*, 2009). Manzano *et al* (2008) demonstrated that SrtC-1 assembles RrgB fibres with high efficiency *in vitro*, whereas SrtC-3 has a much smaller fibre-assembling capacity and SrtC-2 has none at all. Negative-stain EM showed that these *in vitro* fibres mimic the pneumococcal pilus backbone with its beaded appearance. The structures of SrtC-1 and SrtC-3 both exhibit a flexible lid that shields the active site (Manzano *et al*, 2008). Based on this information a universal mechanism for pilus biogenesis was proposed, where class-C sortases with encapsulated active sites require activation by their specific substrate for pilus assembly (Manzano *et al*, 2008). Most recently, the structure of SrtC-2, showing the lid for this sortase as well, corroborated this hypothesis (Neiers *et al*, 2009).

Although genetically based functional and epidemiological studies have substantially increased our understanding of Gram-positive pili (Telford *et al*, 2006), information on their

native structure is lacking. Crystal structures of single pilus subunits of *Streptococcus agalactiae* (GBS) and *S. pyogenes* (GAS) have given novel insights into Gram-positive pilus structure: Krishnan *et al* (2007) describe the ancillary protein GBS52 as having a typical adhesin fold with two immunoglobulin-like domains. The first crystal structure of a Gram-positive pilus backbone protein showed the GAS shaft subunit, Spy0128, as an extended protein comprising two Ig-like domains and two intramolecular isopeptide bonds (Kang *et al*, 2007). These intramolecular bonds are likely to dictate pilus integrity, and a model based on Spy0128 describes the pilus fibre as a chain of individual subunits covalently linked head-to-tail by intermolecular peptide bonds (Kang *et al*, 2007; Kang and Baker, 2009).

Given the fundamental structural difference between Gram-positive pili and their Gram-negative counterparts, and the significance these pili have during the bacterial life cycle, the elucidation of their native structure is of importance, not only to increase our understanding of the biology of Gram-positive bacteria, but also of related human disease. Here we study the molecular architecture of a Gram-positive pilus. We visualise native pneumococcal TIGR4 pili using a combination of electron microscopic techniques and show the pilus as a chain of RrgB proteins covalently linked head-to-tail, with the ancillary proteins RrgA and RrgC at its distal and proximal ends, respectively. Our results provide the first direct electron microscopic evidence for a simple Gram-positive pilus architecture, and resolve some of the open questions concerning the location and function of RrgA and RrgC.

Results

Overall morphology of native pneumococcal TIGR4 pili

As documented earlier, the surface of the *S. pneumoniae* TIGR4 bacteria examined was covered by a non-homogenous distribution of pili (Barocchi *et al*, 2006). Different phenotypes of varying complexity have been described for these long structures (Barocchi *et al*, 2006; LeMieux *et al*, 2006; Hilleringmann *et al*, 2008). When TIGR4 bacteria were imaged by negative-stain EM after minimum perturbation (see section Materials and methods), the pili were seen as

Figure 1 The pili of *S. pneumoniae* TIGR4 bacteria imaged by negative-stain TEM. (A) Fine pili protruding from the bacterial surface (arrowheads). (B) Second example illustrating the variability in pilus number. Note that the individual pili (arrowhead) appear wider close the cell surface (arrow) due to different negative staining. (C) Third example illustrating the pilus bundles (#) and tangles (*) that can form. Some individual pili can also be detected (arrowhead). Pili can be followed back to the bacterial surface. Negative stain: 2% phosphotungstic acid (PTA). A high-pass filter was applied to increase pilus visibility. Scale bar: 100 nm.

fine flexible filaments ~ 6 nm in diameter (Figure 1A–C, arrowheads) that could be at least $1.5\ \mu\text{m}$ long. In some cases changes in the degree of negative staining caused them to appear wider close to the bacterial surface (Figure 1B, arrow). A distinct tendency of the pili to associate into bundles of various diameters (Figure 1C, #) or, at larger distances from the bacterium, to intertwine to form tangles was also detected (Figure 1C, *). Both types of association were random and probably dependent on the bacterium, the negative-staining agent and grid handling. The resulting aggregates correlate well with the phenotypes of varying complexity detected previously primarily by immuno-EM (Barocchi *et al*, 2006; Hilleringmann *et al*, 2008), a technique that cannot match the structural resolution obtained by negative-stain transmission EM (TEM). Our results suggest that pneumococcal TIGR4 pili are single filaments and that the aggregates and super-helical assemblies previously observed are sample preparation artefacts. The degree to which the bacterial capsule and attached filaments could be visualised in the present experiments depended on the negative stain used. Under optimum staining conditions the fine ~ 6 -nm-wide pili could be followed all the way to the cell boundary (Figure 1C).

The pilus shaft is formed by RrgB and has RrgA and RrgC at its ends

TIGR4 bacteria were treated with the murein-hydrolysing enzyme mutanolysin to release their peptidoglycan-anchored pili into the supernatant. Using a modified procedure of Hilleringmann *et al* (2008), the liberated pili were then isolated in 10 mM Tris–HCl (pH 8), 1 mM EDTA and 1 mM DTT (see section Materials and methods). Under the transmission electron microscope TIGR4 pili appeared as long, flexible, ~ 6 -nm-wide filaments (Figure 2A), with the general morphology observed *in situ* (Figure 1). The structure revealed in enlarged views (Figure 2A, inset) bears some similarity to that of RrgB filaments assembled *in vitro* (Manzano *et al*, 2008). Analysis of the same high-molecular-weight (HMW) fractions by SDS–PAGE and Western blotting documented the presence of all three pilus proteins, RrgA, RrgB and RrgC (Figure 2B). In agreement with previous reports (LeMieux *et al*, 2006, 2008; Falke *et al*, 2008; Hilleringmann *et al*, 2008) RrgB was found to be the major constituent and RrgA and RrgC only accounted for a minor fraction of the total protein present. The HMW fractions observed for RrgB confirm the covalent association of Gram-positive pilus subunits previously reported (Telford *et al*, 2006).

Immunolabelling was used to localise all three proteins within the pilus structure. In contrast to previous reports, we directly visualised primary antibodies to overcome the resolution limit imposed by the size of the primary and secondary gold-bearing antibody complex (Barocchi *et al*, 2006; LeMieux *et al*, 2006; Falke *et al*, 2008; Hilleringmann *et al*, 2008). The polyclonal antibodies generally used for Western blots were purified further according to the protocol of Mueller *et al* (2005), were highly specific and did not show cross-reactivity (Supplementary Figure S1). Their concentrations were adjusted to show binding but minimise the number of free antibodies in the solutions. Anti-RrgB–His antibodies decorated the pilus shaft at irregular intervals and, having two binding sites, often linked pili together forming

ladder- and net-like assemblies depending on the degree of lateral cross-linking (Figure 2C and Supplementary Figure S2). Views of single antibodies are shown in the inset of Figure 2D for comparison. In contrast to anti-RrgB–His antibodies, antibodies against RrgA–His only bound at the end of pili, generally clustering and, as they are divalent, frequently linking two pili together in typical v-shaped assemblies not otherwise observed (Figure 2D and Supplementary Figure S3). Anti-RrgC–His also bound at the end of the pilus shaft and sometimes clustered there (Figure 2E and Supplementary Figure S4). As RrgC is two times smaller than RrgA (see below and Figure 3), the capacity of anti-RrgC–His to link two fibres appears to be much diminished. As polyclonal antibodies were used, the formation of antibody clusters does not necessarily mean that there is more than one copy of the labelled protein present. Neither anti-RrgA–His nor anti-RrgC–His decorated the pilus shaft, and the ladder-like assemblies typical of anti-RrgB–His were not formed. Occasionally an antibody was extremely close to the side of a pilus, but this was a chance occurrence rather than specific binding as it was rare and reflected the free antibody concentration and distribution on the grid. Accordingly, the results show that RrgA and RrgC are present at the ends of the pilus shaft formed by RrgB, and strongly imply that these two ancillary proteins are neither incorporated in nor associated with it. In confirmation, mutant bacteria lacking either RrgA or, RrgC or both (Supplementary Figure S5) still form long pili with the shaft morphology of the wild type (see below; Supplementary Figure S6 and reference Falke *et al*, 2008).

All three pilus components have an elongated structure with several domains

To acquire further structural information, the three pilus components were expressed in *Escherichia coli* with an engineered C-terminal His₆ tag but otherwise mimicking the predicted processed forms, which are lacking the N-terminal signal sequence and the C-terminal region starting from the respective LPXTG sorting motif (Supplementary Figure S7). The affinity purified RrgA–His (93.37 kDa), RrgB–His (66.29 kDa) and RrgC–His (40.26 kDa) proteins were examined by EM. First, the oligomeric state of RrgA–His was defined by scanning TEM (STEM) mass measurements. The measured mass of $108 (\pm 42)$ kDa ($n=319$) clearly showed the large majority of the protein to be monomeric (Supplementary Figure S8A). Also, all three proteins were imaged by negative-stain TEM. The average projections calculated by single-particle analysis of TEM electron micrographs and STEM single-shot images are shown in Figure 3. RrgA–His is a flexible, ~ 18 -nm-long, elongated macromolecule with four domains of unequal size. These domains give the structure a distinct taper, one end being ~ 5 nm wide and the other ~ 3 nm wide (Figure 3A). RrgB–His is an ~ 12 -nm-long, elongated particle (Figure 3B). Up to three domains can be detected and, depending on the orientation, a lateral protrusion is sometimes discernible. The domains are almost 5 nm wide without and ~ 6.5 nm wide with the protrusion. Being of about 2/3 the length of RrgA–His and roughly the same width, the imaged RrgB–His was monomeric. The overall shape of the RrgB–His particles is compatible with the partial 2.2- X-ray structure of Spy0128 (aa 18–308), the major pilin subunit of the Gram-positive human pathogen

Figure 2 Constituents of isolated TIGR4 pili and their structural significance. (A) TEM image of TIGR4 pili isolated in Tris buffer and negatively stained with 2% UAc. A beaded structure is evident in an enlarged view (inset). (B) Western blot analysis of the HMW pilus fractions. RrgB forms a ladder of HMW polymers (lane 1, anti-RrgB-His antibody). Loading equal volumes showed the HMW pilus material to contain different amounts of RrgA (lane 2), RrgB (lane 3) and RrgC (lane 4) as measured by Western blotting with anti-RrgA-His, anti-RrgB-His and anti-RrgC-His antibodies, respectively. (C) TEM images of TIGR4 pili after incubation with antibodies to RrgB-His. Single antibodies link the pili laterally to form ladder-like structures. (D) TEM image of TIGR4 pili after incubation with antibodies to RrgA-His. A cluster of antibodies (white arrow) links the pili together at their ends; the individual antibodies are difficult to distinguish. Such v-shaped pilus assemblies were typical. Inset: various orientations of individual antibodies and traces indicating their outer contours. (E) TEM image of a TIGR4 pilus after incubation with antibodies to RrgC-His. A single antibody is attached to the end of the pilus (white arrow). Note the absence of antibodies along the pilus shafts in panels D and E. Scale bars: panel A, 100 nm, inset 20 nm; panels C-E and inset in panel D, 20 nm.

Figure 3 EM of the purified pilus constituents. Representative negative stain (2% UAc) TEM averages (left gallery) and contrast reversed STEM single-shot, dark-field images (right gallery) are shown. (A) RrgA-His. The averages were calculated by sorting 592 single projections into 19 classes. (B) RrgB-His. The averages were calculated by sorting 706 single projections into 14 classes. (C) RrgC-His. The averages were calculated by sorting 256 single projections into 12 classes. Scale bar: 10 nm.

S. pyogenes (Kang *et al*, 2007). With 291 residues this construct is much smaller than RrgB, which comprises 608 residues in its predicted processed form. This explains the different dimensions of the two structures, the Spy0128 construct being 2–3 nm wide and 9.8 nm long, that is, half as wide and 20% shorter than the RrgB–His class averages. Images of RrgC–His, the smallest of the three pilus proteins, revealed up to 4-nm-wide and 10-nm-long elongated particles with 2–3 domains (Figure 3C). From these dimensions the protein was also monomeric, as confirmed by size-exclusion chromatography (data not shown).

The shaft subunits are RrgB monomers and these give the pilus a distinct polarity

The mass-per-length of freeze-dried, unstained, isolated TIGR4 wt pili (Figure 4A) was measured by STEM to determine the stoichiometry of their RrgB subunits. The 395 segments evaluated gave a histogram with a single peak at $6.4 (\pm 1.4)$ kDa/nm (standard error, 0.07 kDa/nm; Figure 4B). Given the $\pm 5\%$ overall precision of the STEM measurement (Müller and Engel, 2006), this indicates the presence of one 65.44-kDa RrgB monomer every $10.2 (\pm 0.5)$ nm on average, which is slightly shorter than the length determined for a recombinant RrgB–His monomer, ~ 12 nm. As expected the mass-per-length of TIGR4 Δ rrgA pili was comparable (Supplementary Figure S8B).

The high signal-to-noise ratio of the STEM was also exploited to examine negatively stained TIGR4 pili. These images confirmed that the pilus is formed by a single string of subunits and revealed a well-defined protrusion extending at irregular intervals from the filament shaft like a ‘nose’ (Figure 4C and D). Most importantly, they showed that this ‘nose’ points in a defined direction giving the pilus a distinct polarity. Where the boundaries of single subunits could be discerned (Figure 4C and Supplementary Figure S9, lines), their shapes correlated well to those of the RrgB–His class averages and single projections implying that the pilus ‘nose’ is the protrusion observed in some orientations of the macromolecule. In agreement with the measured mass-per-length, the subunit spacing ($10.2 (\pm 0.6)$ nm; marked by lines in Figure 4C and Supplementary Figure S9) was somewhat less than the length of an RrgB–His monomer, implying that adjacent RrgB monomers may overlap slightly, as detailed in Figure 4E and F. Close examination of the pili formed by TIGR4 Δ rrgC, TIGR4 Δ rrgAC bacteria revealed the same substructure, that is, a single string of monomeric RrgB subunits, with ‘noses’ protruding at irregular intervals (Supplementary Figure S6, insets).

As the ‘nose’ was not always visible at regular intervals along the pilus shaft under the preparation conditions used, the RrgB monomers do not appear to assemble according to a defined helical rule. In any case, the covalent bonds formed between the monomeric RrgB subunits and further possible molecular interactions not only allow the filaments to bend freely, but also permit a degree of rotation around the long filament axis under the forces encountered on adsorption to the EM grid.

The ancillary proteins RrgA and RrgC are localised at opposite ends of the pilus shaft

Knowledge of its existence allowed the fine ‘nose’ feature to be detected on TEM images and showed it to consistently point in one direction for longer pilus stretches, indicating

that the individual RrgB pilus subunits are linked head-to-tail. Examination of the immunolabelling images with this information also clearly showed that anti-RrgA–His and anti-RrgC–His labelled opposite ends of the pilus shaft (Figure 5 and Supplementary Figure S10). The role of RrgA as a pilus-associated adhesin that is expected to locate at the distal end (Nelson *et al*, 2007; Hilleringmann *et al*, 2008), would suggest that RrgC is located at the proximal end of the pilus shaft. In accordance, the pili formed by a TIGR4 Δ rrgC genetic background (TIGR4 Δ rrgC and TIGR4 Δ rrgAC) detached more easily from the bacteria, resulting in the appearance of more HMW pili material in the culture supernatant (Supplementary Figure S11).

Discussion

Since Ton-That *et al*’s description of the pili of *C. diphtheriae* (Ton-That and Schneewind, 2003), many Gram-positive bacteria have been shown to possess such filamentous appendages, including group-A *Streptococci* (Mora *et al*, 2005), group-B *Streptococci* (Lauer *et al*, 2005), *S. pneumoniae* (Barocchi *et al*, 2006), *Enterococcus faecalis* (Nallapareddy *et al*, 2006), *Bacillus cereus* (Budzik *et al*, 2007) and actinomyces (Wu and Fives-Taylor, 2001; Ton-That *et al*, 2004). In addition, probable pilus loci have been identified by genome sequencing of *Streptococcus spp.* (Osaki *et al*, 2002; Xu *et al*, 2007). The adhesive function of pili (Barocchi *et al*, 2006; Dramsi *et al*, 2006) is critical for the attachment of pathogens to specific host cells during colonisation, and explains why pilus expression increases the pathogenicity of various Gram-positive bacteria in animal models (Hava and Camilli, 2002; Abbot *et al*, 2007; Maisey *et al*, 2007; Rosch *et al*, 2008). This central function has promoted several EM analyses of native pili attached to bacterial cells and after purification (LeMieux *et al*, 2006; Nelson *et al*, 2007; Fälder *et al*, 2008; Hilleringmann *et al*, 2008), as well as X-ray studies of Gram-positive pilus components (Kang *et al*, 2007; Krishnan *et al*, 2007) and of the pilus assembly machinery (Manzano *et al*, 2008; Neiers *et al*, 2009).

We have used TEM, antibody labelling and STEM to elucidate the structure of the native TIGR4 pilus. The shaft is an ~ 6 -nm-wide, single chain of slightly overlapping, head-to-tail covalently linked, monomeric RrgB subunits and can be at least $1.5 \mu\text{m}$ long. According to the mass-per-length measurements by STEM, a $1.5\text{-}\mu\text{m}$ -long pilus comprises approximately 150 RrgB monomers. TIGR4 pili assembled in a Δ rrgA, Δ rrgC or Δ rrgAC genetic background were also examined after purification and exhibit the same length and morphology as the wt pilus. The two ancillary proteins RrgA and RrgC are found at opposite ends of the shaft. RrgA is distal and consequently, RrgC proximal to the bacterium, as illustrated in Figure 6.

The proposed model agrees with functional implications derived from the structures of the *S. pneumoniae* sortases SrtC-1 and SrtC-3 (Manzano *et al*, 2008), and SrtC-2 (Neiers *et al*, 2009). All of them possess an encapsulated active site that is postulated to be activated by the specific LPXTG-like motifs found in RrgA, RrgB and RrgC. These motifs are known to have profound consequences for catalysis (LeMieux *et al*, 2008), and could be indicative of a controlled sequential pilus assembly process. Although the sequence of this process remains to be unveiled, it would ensure RrgA to

Figure 4 STEM analysis of isolated TIGR4 pili. (A) STEM dark-field image of unstained, freeze-dried TIGR4 pili; the regions selected for mass-per-length measurement are indicated. (B) The mass-per-length histogram obtained from this sample. The peak at $6.4 (\pm 1.4)$ kDa/nm ($n = 395$) shows the pilus filament to be a single string of RrgB monomers and predicts the presence of one monomer every $10.2 (\pm 0.5)$ nm. (C, D) Contrast-reversed STEM dark-field images recorded at a magnification of $\times 10^6$ from negatively stained TIGR4 (2% PTA). A nose-like protrusion is present at irregular intervals and gives the filaments a defined polarity. Indeed, individual pilus subunits can be distinguished and have the same shape as RrgB-His monomers; compare with Figure 3B. Subunit boundaries are indicated by white lines in panel C (analysed in Supplementary Figure S9) and for panel D by the model in panel E. (E) Highly contoured TEM images of RrgB-His monomers (average length $12.2 (\pm 0.5)$ nm) matched, without straightening, to the subunits of pilus (D), illustrating that its RrgB subunits overlap; the RrgB monomers are shown in alternating shades of grey and their ends are marked by dotted lines to facilitate visualisation. (F) Outline of the outer contours of panel E superimposed on panel D. Scale bar: panel A, 30 nm and panels C–F, 10 nm.

be at the distal tip of the pilus shaft assembled from RrgB, which is the only one of the three pilins to have both the pilin and the LPXTG domain (Supplementary Figure S7B). Incorporation of the RrgC protein at the proximal end would then terminate the pilus assembly process and

induce cell wall linkage similar to that observed for SpaB in *C. diphtheriae* (Mandlik *et al*, 2008a) or GBS150 in *S. agalactiae* (Nobbs *et al*, 2008).

In spite of this agreement our model is in contrast to other reports, which state that ancillary proteins RrgA and RrgC are

Figure 5 Localisation of the ancillary proteins, RrgA and RrgC. TEM of negatively stained (2% UAc) immunolabelled TIGR4 pili, enlarged to show pilus polarity. (A) Pili labelled with anti-RrgA-His; the noses point away from the antibody cluster. The antibody cluster is indicated by a white arrow. (B) Pilus labelled with anti-RrgC-His; the noses point towards the antibody. The antibody is indicated by a white arrow. As RrgC anchors the pilus to the cell wall, as suggested by the release of pili in the absence of RrgC (Supplementary Figure S11), the black arrows in panels A and B point away from the bacterium. Scale bar: panels A and B, 20 nm.

either incorporated in or associated with the pilus shaft (LeMieux *et al*, 2006; Nelson *et al*, 2007; Falcker *et al*, 2008; Hilleringmann *et al*, 2008). Immuno-EM showed RrgC and RrgA to be in clusters along the length of the pilus shaft (LeMieux *et al*, 2006; Hilleringmann *et al*, 2008), sometimes together as indicated by double-labelling studies (Falcker *et al*, 2008). The discrepancy to the observations presented here is explained by the lower resolution of the previous immuno-EM studies, which could not resolve single pili with certainty, but rather visualised immunogold-labelled pilus bundles. LeMieux *et al* (2008) came to a similar conclusion speculating that the observed clustering of RrgA simply manifests the bundling of different-length pili with RrgA at their tips. Immunogold labelling cannot prove this hypothesis because single pili within a bundle cannot be resolved. Negative-stain EM can achieve higher resolution. Using this technique we first demonstrate that pili emerge from the cell surface as single, ~6-nm-wide filaments, which then form bundles or tangle at random (Figure 1). Second, by isolation of native pili using a modified protocol we obtain a higher-resolution definition of the pilus shaft by STEM dark-field imaging

Figure 6 Model of *S. pneumoniae* pilus. (A) The TIGR4 pilus consists of a shaft composed of RrgB, with RrgA at its distal and RrgC at its proximal end. Sortase SrtC-1 mediates the polymerisation of RrgB (red) via the LPXTG motif (*, IPQTG) and pilin motif (#) (Manzano *et al*, 2008) into a single string of monomers, covalently linked head-to-tail. The nose-like feature of RrgB gives the pilus a clear polarity. C-type sortases also control the addition of the ancillary proteins RrgA and RrgC, and anchor RrgC to the peptidoglycan cell wall, depending on the recognition of the respective LPXTG motifs YPRTG (RrgA) and VPDTG (RrgC; LeMieux *et al*, 2008; Neiers *et al*, 2009). RrgA is located at the distal end of the pilus, while RrgC is proximal to the bacterium. This is in agreement with the proposed role of RrgA as adhesin (Nelson *et al*, 2007; Hilleringmann *et al*, 2008) and the release of pili into the supernatant when RrgC is not present. RrgA and RrgC do not localise together in these single pili and are neither incorporated into the pilus shaft nor present along its length as proposed earlier (LeMieux *et al*, 2006; Falcker *et al*, 2008; Hilleringmann *et al*, 2008). (B) The ability of RrgA and RrgC to form a heterodimer (LeMieux *et al*, 2008) suggests a site in RrgC that can be covalently linked to an LPXTG motif from either RrgA or RrgB. The inability of RrgA and/or RrgC to form polymers may indicate that (i) RrgA has no site to interact with an LPXTG motif and (ii) RrgC has an LPXTG motif that activates only the C-type sortase, which links it to the cell wall.

of single pili, and show their previously observed beaded structure (Manzano *et al*, 2008) to arise from single RrgB monomers linked head-to-tail with a periodicity of about 10 nm (Figure 4C–F and Supplementary Figure S9). This periodicity is compatible with STEM mass-per-length measurements; 6.4 ± 1.4 kDa/nm (Figure 4B) also translates to about 1 RrgB monomer/10 nm. Third, the well-defined protrusions on the filaments, the ‘noses’, result in clear polarity and indicate a head-to-tail subunit assembly (Figure 4C–F).

These 'noses' relate to lateral protrusions on images of negatively stained, recombinantly expressed RrgB–His monomers (Figure 3B). In agreement, antibody labelling demonstrated the pilus shaft to be exclusively comprised of RrgB proteins (Figure 2C–E and Supplementary Figure S2–4). Fourth, once these basic simple features of the TIGR4 pilus had been defined by high-resolution STEM, we could also recognise them on close inspection of TEM images recorded at lower magnification. Based on the polarity imposed by the 'noses' and primary antibody labelling it can be said that RrgA and RrgC are located at opposite ends of the pilus shaft (Figure 5 and Supplementary Figure S10). In contrast to immuno-EM, where a secondary, gold-labelled antibody is necessary, direct visualisation of the primary antibody provides the resolution required to locate a specific protein associated with a single pilus shaft. With the new higher resolution data obtained, the previously reported clusters of surface-located RrgA can be interpreted as the distal ends of several interacting individual pili that may emerge from different sites of the cell wall or have different length.

The location of RrgA is assigned as distal as this protein has recently been described as an adhesin (Nelson *et al*, 2007; Hilleringmann *et al*, 2008). This implies that RrgC is at the proximal end of the pilus, which must be anchored in the bacterial cell wall. In *C. diphtheriae* the protein SpaB is proposed to act as the terminal subunit and cell wall anchor in pilus assembly, and in its absence the pili formed are largely found in the medium (Mandlik *et al*, 2008a). In *S. agalactiae* the pilus is covalently linked to the cell wall via the ancillary pilus subunit GBS150, and its absence provokes the release of pili into the culture supernatant (Nobbs *et al*, 2008). Similarly, less HMW pneumococcal TIGR4 pili are found in the supernatant of wt *S. pneumoniae* liquid cultures than in the supernatants of Δ rrgC and Δ rrgAC mutants, the apparent enhanced pilus loss of the latter over the Δ rrgC mutant probably resulting from the missing adhesive function of RrgA (Supplementary Figure S11). Accordingly, RrgC is likely to be the terminal pilus subunit and to warrant cell wall anchoring. The proposed pilus model (Figure 6) agrees with the fact that RrgB assembles *in vitro* in the presence of the pilus-polymerising transpeptidase SrtC-1 (Manzano *et al*, 2008) and *in vivo* in the absence of both RrgA and RrgC (Supplementary Figure S6C and Falker *et al*, 2008; LeMieux *et al*, 2008). The observation that the accessory subunits RrgA and RrgC are found in similar quantities within each 'rung' of the ladder of bands arising from pili of different lengths on Western blots corroborates this model as well (LeMieux *et al*, 2008). Our results confirm the observation that RrgA and RrgC can form covalent heterodimers, but not higher order polymers in the Δ rrgB background. As indicated in our model, this implies that the IPQTG motif in the RrgB protein can be covalently linked to a critical lysine in RrgC, and that in the absence of RrgB the same site in RrgC is linked to the YPRTG motif of RrgA. Whereas RrgC must have another site to be anchored to the cell wall, RrgA appears to expose a single motif for sortase action (Figure 6 and Supplementary Figure S7).

Our model of the pilus shaft (Figure 6) extends the previous model of the *S. pyogenes* pilus created on the basis of the 2.2-Å Spy0128 structure and mass spectrometry (Kang *et al*, 2007). No overlap of subunits is proposed in the Spy0128 filament model. In contrast, we predict an overlap of

about 1 nm for RrgB, in accordance with the sequence-based pneumococcal pilus assembly model (Telford *et al*, 2006). The overlap is based on pilus images recorded at $\times 10^6$ magnification, the mass-per-length values provided by STEM and the dimensions of RrgB–His monomers. A major difference in the mass of Spy0128 (aa 18–308; Kang *et al*, 2007) and the integrated RrgB monomer (608 aa) may explain this overlap, but a high-resolution structure of full-length RrgB is required to prove our hypothesis.

Using quantitative EM techniques we have visualised the molecular details of a Gram-positive pilus for the first time. Together with the sequence analysis presented by Manzano *et al* (2008), our observations suggest a pilus architecture that is likely to be valid for other Gram-positive pili. It implies a simple pilus assembly mechanism, and indicates novel sites for therapeutic intervention.

Materials and methods

Bacterial strains and culture conditions

S. pneumoniae type-4 strain TIGR4 was used (Tettelin *et al*, 2001). The TIGR4 Δ rrgA mutant used initially was kindly donated by B Henriques-Normark (Karolinska Institutet, Stockholm). Later TIGR4 Δ rrgA, TIGR4 Δ rrgB, TIGR4 Δ rrgC and TIGR4 Δ rrgAC mutants were created by PCR-based overlap extension (Supplementary data and Supplementary Tables S1–S2). The pneumococcal strains were stored at -80°C in 12% glycerol and routinely grown at 37°C under 5% CO_2 on Tryptic Soy Agar (Becton Dickinson) supplemented with 5% defibrinated sheep blood or in Todd–Hewitt Yeast Extract (THYE) broth. When appropriate, erythromycin and kanamycin (Sigma-Aldrich) were used as selection markers.

Expression and purification of RrgA, RrgB and RrgC

Recombinant expression and purification of His₆-tagged pilus proteins was performed as described previously (Hilleringmann *et al*, 2008). When necessary a size-exclusion chromatography step was performed after affinity purification. Purified proteins were finally dialysed against 10 mM Tris–HCl (pH 8), 1 mM EDTA and 0.5 mM DTT.

TIGR4 pilus purification

The native pili of TIGR4 wt and TIGR4 Δ rrgA, TIGR4 Δ rrgC and TIGR4 Δ rrgAC were purified essentially according a protocol described by Hilleringmann *et al* (2008) treating bacteria with mutanolysin, a murein-hydrolysing enzyme (Sigma M9901), to liberate covalently peptidoglycan-anchored pili into the supernatant, but using a Tris buffer (10 mM Tris–HCl (pH 8), 1 mM EDTA, 1 mM DTT)-based procedure. In addition the following modifications were applied: harvested bacteria were washed in Tris buffer and resuspended in Tris containing protoplast buffer (Tris buffer, 20% sucrose). Final sample dialysis against Tris buffer was performed using a molecular weight cut-off of 300 kDa (Spectra/Por Biotech cellulose ester).

SDS–PAGE and Western blot analysis

SDS–PAGE analysis was performed using NuPAGE 3–8% Tris Acetate Gels (Invitrogen) according to the instructions of the manufacturer. HiMark pre-stained, HMW protein standard (Invitrogen) served as the protein standard. Western blot analysis was performed using standard protocols. Unless otherwise stated, antibodies against recombinant RrgA–His and RrgB–His were used at 1:10 000 dilution, and against RrgC–His at 1:2000 dilution. Secondary goat anti-mouse HRP antibodies were diluted 30 000 $\times$.

Animal sera and antibodies

Polyclonal mouse antibodies against recombinant RrgA–His, RrgB–His and RrgC–His were produced in our laboratory. For immuno-labelling of isolated native pili, antibodies were purified to 100% specificity against their respective proteins using the protocol described by Mueller *et al* (2005).

Immunolabelling of the isolated pili

The respective affinity-purified antibodies were individually incubated with wt or Δ rrgA TIGR4 pili (control experiments) overnight or during 50 min at 4°C in a series of runs covering a range of concentrations. Samples were inspected by negative-stain TEM. The conditions were optimised to yield good labelling and have the minimum number of free antibodies on the EM grids.

Transmission electron microscopy

For TEM of whole bacteria, 100–200 µl of PBS was added to the blood agar growth plate and agitated gently to delicately remove bacteria from the agar. The plate was tilted and an aliquot of the resulting bacterial suspension was removed from close to the liquid surface. Small aliquots of this stock suspension were then directly loaded onto carbon-coated Parlodion microscopy grids. The bacteria were allowed to settle (5 min) and then stabilised by addition of 2% paraformaldehyde (40 s). Grids were washed on droplets of water, negatively stained and examined. As dictated by grid quality, the stock was sometimes centrifuged gently for several minutes (3000 r.p.m. for 5–10 min), the pellet was then gently resuspended in PBS and grids were prepared; if necessary these steps were repeated.

Samples of the isolated pili were diluted in buffer as required and adsorbed for 1 min to glow discharged 400 mesh carbon-coated Parlodion or STEM grids (see below). These were washed and negatively stained with 2% (w/v) uranyl acetate (UAc) or 2% (w/v) phosphotungstic acid (PTA) and imaged with a CM 100 transmission electron microscope (Philips, Eindhoven, the Netherlands) operating at 80 kV. Electron micrographs were recorded with a 2000 by 2000 pixel, charge-coupled device camera (Veleta; Olympus soft imaging solutions GmbH, Münster, Germany) at a nominal magnification of $\times 130\,000$, yielding a final pixel size corresponding to 0.36 nm on the specimen scale. Particles were manually selected for single-particle analysis and averaged using the EMAN software (Ludtke *et al*, 1999).

Scanning TEM

Samples were prepared on glow-discharged, thin carbon films coating a perforated carbon layer on gold-coated copper grids, washed and either freeze-dried for mass measurement or negatively stained as above. Mass measurements were performed on pilus and recombinant RrgA–His samples as described (Broz *et al*, 2007),

except that the grids were washed on eight drops of quartz double-distilled water. Images were recorded from the TIGR4 pili at doses ranging from 400 to 950 electrons/nm² and from pili of the TIGR4 Δ rrgA mutant at a dose of 700 ± 56 electrons/nm², and evaluated using the MASDET program package (Krzyżanek *et al*, 2009). A linear regression describing the dose dependence of the mass-per-length values determined for the former sample defined beam-induced mass-loss. Both the TIGR4 data set and the Δ rrgA pilus data were corrected accordingly and scaled to the mass-per-length determined in the same run for tobacco mosaic virus (TMV). Images were recorded from the RrgA–His sample at a dose of 1170 ± 100 electrons/nm² and evaluated using MASDET (Krzyżanek *et al*, 2009). Beam-induced mass-loss was corrected according to the behaviour of proteins in the mass range 120–190 kDa (Müller and Engel, 2001 and unpublished results) and the data were scaled according to the mass-per-length measured for TMV. The corrected data sets were binned into histograms and described by Gauss curves.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

MH, GD, RR and IF are employees of Novartis Vaccines and Diagnostics s.r.l.

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