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# RrgA and RrgB Are Components of a Multisubunit Pilus Encoded by the *Streptococcus pneumoniae rlrA* Pathogenicity Islet

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**The *rlrA* pathogenicity islet in *Streptococcus pneumoniae* TIGR4 encodes three surface proteins, RrgA, RrgB, and RrgC, and three sortase enzymes. Using transmission electron microscopy, cell fractionation, cell wall sorting signal domain swapping, and Western blotting, we show that RrgA and RrgB are incorporated into a multisubunit pilus in *S. pneumoniae*.**

Gram-positive bacteria have evolved a number of mechanisms to localize proteins to their surfaces. One mechanism of surface display is through the covalent linkage of proteins to cell wall stem peptides, a process mediated by sortases. Sortases are membrane-anchored transpeptidases that recognize a C-terminal cell wall sorting signal (CWSS) characterized by the presence of an LPXTG motif. Sortase covalently links the threonine of this motif to cell wall precursors, which are then incorporated into the cell wall (19). Most gram-positive bacteria contain at least one sortase orthologue, SrtA, which has recently been shown to be required for colonization in *Streptococcus pneumoniae* (3, 10). Additionally, SrtA has been shown to be required for virulence in several other pathogens (2, 4, 5, 11, 26); however, multiple sortase paralogues have also been found in the genomes of a subset of these species (17). In many cases, the additional sortase paralogues are genetically linked to cell wall-anchored proteins with variant CWSSs that serve as substrates for the linked sortase, including the *rlrA* pathogenicity islet in *S. pneumoniae* TIGR4 (1, 8, 14, 16, 19, 22).

We sought to characterize the *rlrA* pathogenicity islet further for the following reasons: RrgA is a virulence factor in a murine lung infection model (8), yet the islet has a varied distribution among serotypes (18), and the RrgA, RrgB, and RrgC proteins exhibit homology to ones that were recently described to form pili in other gram-positive organisms (20–22). The most well-studied model of gram-positive pilus formation is *Corynebacterium diphtheriae*, where there are four motifs within the major pilin subunit, SpaA, that are necessary for pilus assembly. These motifs are an N-terminal signal peptide, a CWSS, a pilin motif, and an E-box motif (20–22). The identification and characterization of these motifs have led to a model of assembly wherein SpaA is polymerized into a pilus structure by covalent linkage between subunits catalyzed by the linked sortase, and the base of the pilus is covalently attached to the cell wall (20, 22). In addition, there appear to be minor subunit proteins that are incorporated along the pilus shaft and at the tip, one of which contains a variant CWSS. Incorporation of these minor subunits has been shown to require additional sortases that are also genetically linked; however, the mechanisms of their incorporation into the pilus are poorly understood. All four of the required motifs found in SpaA are present in RrgB, suggesting that the latter is the major subunit of a pilus in *S. pneumoniae* and that the other surface proteins and sortases in the islet are involved in pilus formation. Although a pilus has never been identified in *S. pneumoniae*, it has been previously reported in the literature that other streptococcal species, including *S. parasanguis*, *S. salivarius*, and *S. sanguis*, produce adhesive organelles such as fimbriae (6, 7, 13, 23–25), and it has recently been shown that *S. agalactiae* and *S. pyogenes* produce pili (12, 15).

To determine if RrgB forms a pilus structure analogous to that of SpaA, cell wall-anchored proteins were isolated from bacteria from the mid-exponential phase expressing a hemagglutinin (HA)-tagged RrgB (RrgB-HA) (inserted at residue 133) after cell wall digestion of whole cells with 10 mM Tris-HCl (pH 8.0), 30% raffinose, 100 U mutanolysin, 1 mg lysozyme, and EDTA-free protease inhibitor cocktail (Roche). The soluble molecules from this digest were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and RrgB-HA was detected by Western blotting using a polyclonal anti-HA antibody (Santa Cruz Biotech). This analysis revealed multiple high-molecular-weight species of RrgB-HA, the majority of which are present in the stack of the gel (Fig. 1A), similar to that seen for SpaA in *C. diphtheriae* cell wall extracts. This ladder of bands is hypothesized to represent pili of various lengths that are composed of covalently linked major and minor pilin subunits (22). A similar banding pattern was observed for RrgA-HA (HA tag inserted at residue 224 to replace the nine amino acids at residues 224 to 232) (Fig. 1A) as well as for native RrgA immunoblotted with anti-RrgA polyclonal antiserum (Covance Research Products Inc.) (data not shown). The reduction in RrgB-HA signal relative to that for RrgA-HA was consistently observed by Western blotting, suggesting partial obscuring of HA epitopes or, alternatively,

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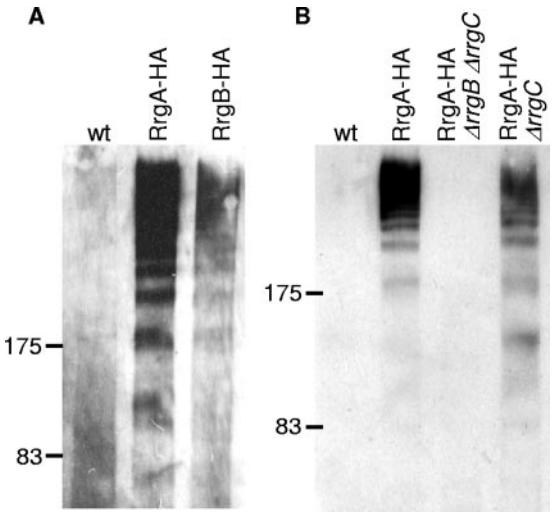


FIG. 1. RrgA and RrgB are polymerized into pili. (A) Cell wall fractions of RrgA-HA and RrgB-HA *S. pneumoniae* TIGR4 form high-molecular-weight complexes when immunoblotted with anti-HA antisera. Wild-type (wt) TIGR4 (lane 1), RrgA-HA (lane 2), and RrgB-HA (lane 3) are shown. (B) The high-molecular-weight RrgA-HA complexes are not present in a strain with *rrgB* deleted and *rrgC* but are present when only *rrgC* is deleted. Wild-type TIGR4 (lane 1), RrgA-HA (lane 2), RrgA-HA Δ*rrgB* Δ*rrgC* (lane 3), and RrgA-HA Δ*rrgC* (lane 4) strains are shown.

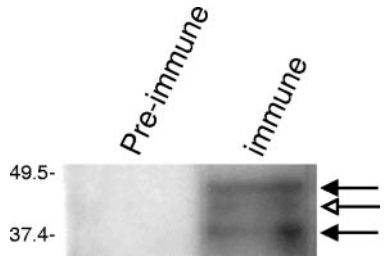


FIG. 2. RrgB antiserum is specific to RrgB. (A) Two RrgB peptides were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed separately with mouse preimmune serum (lane 1) or mouse anti-RrgB serum (lane 2). The two RrgB peptides (filled arrow) and a breakdown product (open arrow) were detected by the antiserum.

reduced assembly of this mutant protein into pili. These results suggest that the *rlrA* pathogenicity islet encodes a pilus similar to that described for *C. diphtheriae*. Furthermore, the similar banding patterns exhibited by RrgA and RrgB suggested two models: RrgA and RrgB are incorporated into a single multimeric pilus, or RrgB and RrgA form two independent pili. The absence of a consensus pilin motif and E box in RrgA favors the former hypothesis. To test these possibilities, we analyzed the migration of RrgA-HA by SDS-PAGE and Western blotting using cell wall preparations from double- and single-gene in-frame deletion strains, Δ*rrgB* Δ*rrgC* and Δ*rrgC*. We were unable to construct a deletion of *rrgB* by itself. RrgA-HA is undetectable in the cell wall fraction by Western blotting in the Δ*rrgB* Δ*rrgC* strain background, but the wild-type-like high-molecular-weight banding pattern is maintained in the Δ*rrgC* strain background (Fig. 1B). These data demonstrate a dependence of RrgA pilus formation on RrgB.

**Visualization of a pilus structure on the surface of *S. pneumoniae*.** The conserved motifs found in RrgB, in addition to the Western blot data, led us to hypothesize that RrgB is the major pilus subunit. We sought to visualize a pilus structure by specifically looking at the RrgB protein via immunogold transmission electron microscopy. To this end, mouse antiserum raised against two peptides comprising the central portion of RrgB between the signal sequence and the C-terminal CWSS (N-terminal peptide at residues 44 to 341; C-terminal peptide at residues 343 to 624) was used. This antiserum was specific for RrgB, since it detected RrgB in cell wall extracts via Western analysis and did not recognize cell wall extract from an Δ*rrgB* Δ*rrgC* strain (data not shown). Finally, the antiserum but not the preimmune serum recognized the RrgB peptides that were used to raise the antiserum (Fig. 2).

Wild-type *S. pneumoniae* strain TIGR4 was incubated with mouse anti-RrgB serum, and bound antibody was visualized with 18-nm Colloidal Gold-AffiniPure anti-mouse immunoglobulin G (IgG) and negatively stained with uranyl acetate. The presence of numerous pili was detected on the surface of the bacteria (Fig. 3A and B). No labeling was observed with the mouse preimmune serum (Fig. 3H) or when the primary antibody was not used (data not shown). To test the hypothesis that RrgA is also incorporated into pili, wild-type *S. pneumoniae* and a strain expressing a second HA-tagged RrgA (HA inserted at residue 820 to replace the nine amino acids at residues 820 to 828) were incubated with rabbit anti-HA antibody and visualized with 12-nm Colloidal Gold-AffiniPure anti-rabbit IgG. RrgA-HA was detected within "patches" that were equally spaced along fibers, which are presumably pili (Fig. 3C). In the *RrgA*-HA background, if *rrgB* and *rrgC* are deleted, the pilus structure is not observed and the gold particles are seen close to the surface of the cell, indicating that RrgA-HA cannot be assembled into pili in the absence of RrgB and/or RrgC (Fig. 3D). To determine which of these two proteins is required for incorporation of RrgA-HA into pili, an RrgA-HA Δ*rrgC* strain was examined. RrgA-HA labeling in pili was observed (Fig. 3E), suggesting that RrgB is the pilin subunit, which is consistent with the Western blot results and the presence of pilin motifs in RrgB. The presence of both RrgA and RrgB in the same pilus structure was detected by double labeling with two different-sized gold particles (Fig. 3F and G).

This is the first report where an external structure of this nature has been visualized on the surface of *S. pneumoniae*. These data, together with the Western blot and homology data, suggest that pili are assembled on the surface of *S. pneumoniae* TIGR4. RrgB is the major pilin subunit that forms the length of the pilus into which RrgA is incorporated as a minor subunit at periodic intervals. The precise locations of RrgA, RrgB, and possibly RrgC in the pilus are an interesting area of study, and the mechanism of their incorporation will require further investigation.

**Incorporation of RrgA into pili requires *srtD*.** Due to the genetic linkage of *rrgA*, *rrgB*, and *rrgC* with *srtB*, *srtC*, and *srtD*, and the variant LPXTG motifs in each Rrg protein, we previously hypothesized that each sortase would direct the cell wall anchoring of one Rrg protein (8). To test this and to begin to analyze the mechanism of incorporation of RrgA into pili, the cell wall localization of RrgA was examined in previously de-

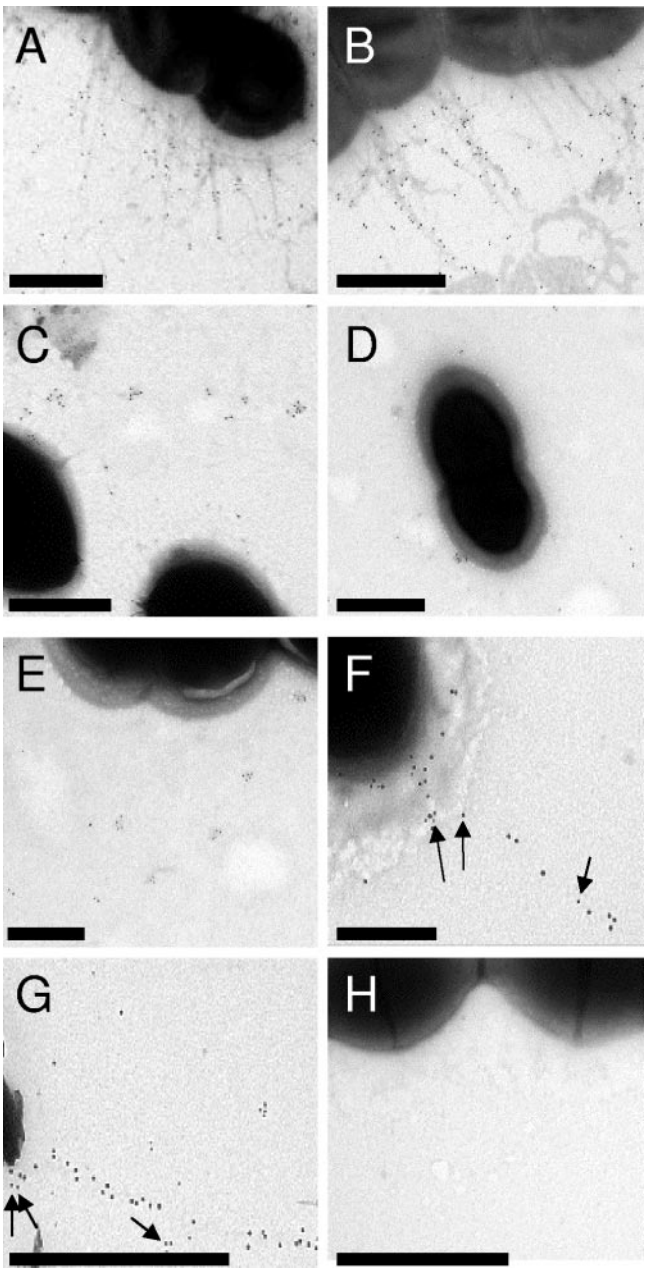


FIG. 3. Immunogold transmission electron microscopy visualization of RrgB and RrgA-HA incorporated into pilus-like structures on the surface of *S. pneumoniae* TIGR4. (A and B) Representative micrographs of TIGR4 labeled with mouse anti-RrgB antiserum and visualized with anti-mouse IgG Colloidal Gold 18-nm particles. (C to E) Representative micrographs of TIGR4 labeled with rabbit polyclonal anti-HA antibody and visualized with anti-rabbit IgG Colloidal Gold 12-nm particles. (C) RrgA-HA; (D) RrgA-HA Δ*rrgB* Δ*rrgC*; (E) RrgA-HA Δ*rrgC*. (F and G) *S. pneumoniae* RrgA-HA labeled with both mouse anti-RrgB antiserum and anti-mouse IgG Colloidal Gold 18-nm particles as well as rabbit polyclonal anti-HA antibody and anti-rabbit IgG Colloidal Gold 12-nm particles. RrgA, labeled with 12-nm gold particles (arrows), is found periodically within the pilus. (H) TIGR4 labeled with mouse preimmune serum. Magnifications: (A) ×10,500 (scale bar, 1 μm); (B, G, and H) ×19,000 (scale bar, 1 μm); (C to F) ×25,000 (scale bar, 0.5 μm).

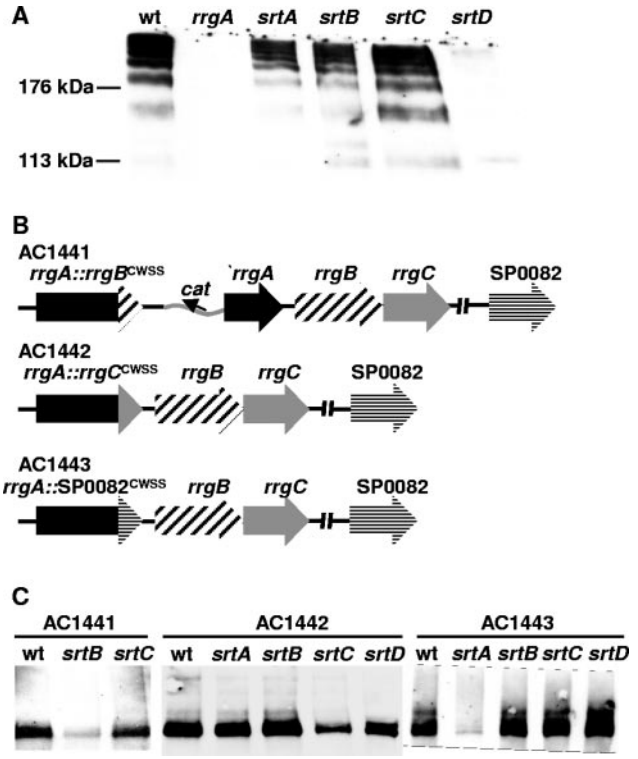


FIG. 4. Incorporation of RrgA into pili requires SrtD. (A) Cell wall fractions of wild-type and sortase mutant strains were examined by Western blotting with polyclonal anti-RrgA antiserum. Fractions were from wild-type TIGR4 (lane 1) and strains carrying mutations in *rrgA* (lane 2), *srtA* (lane 3), *srtB* (lane 4), *srtC* (lane 5), and *srtD* (lane 6). (B) Schematic of the RrgA chimeras examined by Western blotting. RrgA (black arrow and rectangles) containing the C-terminal CWSS of each gene listed (arrowheads) is shown. The RrgA-RrgB$^{CWSS}$ chimera was generated by a suicide plasmid insertion-duplication mutation as illustrated. The integrated plasmid is a derivative of pAC1000 (9) containing *rrgA′* (bases 1 to 2586) joined to *′rrgB* (bases 1902 to 1999). The RrgA-RrgC$^{CWSS}$ and RrgA-SP0082$^{CWSS}$ chimeras were generated by double-crossover allelic exchange using pAC1000 derivatives, resulting in the replacement of the 3′ end of *rrgA* (bases 2586 to 2683) with *′rrgC* (bases 1084 to 1150) and ′SP0082 (bases 2461 to 2574), respectively. (C) Western blots of the cell wall fractions of chimeric strains of AC1441, AC1442, and AC1443 in an otherwise-wild-type background (lane 1 in each panel) or in the sortase mutant backgrounds indicated above each lane. The cell wall fractions were immunoblotted with anti-RrgA polyclonal antibody. The intense band at approximately 120 kDa consists of two poorly resolved bands.

scribed strains in which each sortase was mutated (8). Cell wall extracts were prepared from identical amounts of mid-exponential-phase bacteria and analyzed by Western blotting using rabbit anti-RrgA antiserum. The high-molecular-weight ladder of RrgA was absent in the cell wall fraction of the *rrgA* and *srtD* strains, whereas a wild-type banding pattern was observed in each of the other mutants (Fig. 4A). The *srtD* mutation did not affect the expression of RrgA, since RrgA was detected in large amounts in the protoplast and secreted fractions (data not shown). This result suggests that SrtD is required for the incorporation of RrgA into pili.

To confirm the specificity of SrtD for incorporating RrgA into pili, we replaced the CWSS of RrgA with that of RrgB and RrgC and examined the RrgA chimeras in strains deleted for

individual sortases by Western blotting as described above. The chimeras contained the entire sorting signal of the indicated protein (from the first residue of the LPXTG motif to the end of the protein) in place of the analogous segment of RrgA (Fig. 4B). In addition, we created an RrgA chimera using a canonical (SrtA-recognized) LPXTG CWSS from an unrelated protein (SP0082). All three chimeras (RrgA-RrgB$^{CWSS}$, RrgA-RrgC$^{CWSS}$, and RrgA-SP0082$^{CWSS}$) were present in the cell wall fraction in the wild-type strain background (Fig. 4C); however, they were no longer present in the high-molecular-weight ladder exhibited by the wild type (compare Fig. 4A and C). Thus, although the chimeras are localized to the cell wall fraction, the wild-type RrgA CWSS is required for incorporation of the protein into pili in an SrtD-dependent manner.

The above-described results suggest that RrgB and RrgC require other sortases (presumably SrtB and SrtC) for their incorporation into the cell wall compartment. To examine this, the sortase mutations used as described above were introduced into the RrgA chimeric strains by natural transformation, and cell wall extracts were analyzed by Western blotting as described above. Cell wall localization of RrgA-RrgB$^{CWSS}$ was greatly reduced in the *srtB* strain, whereas that of RrgA-RrgC$^{CWSS}$ was only moderately reduced in the *srtC* strain (Fig. 4C). Consistent with a previous report of canonical LPXTG-anchored proteins in *S. pneumoniae* (10), cell wall localization of the RrgA-SP0082$^{CWSS}$ chimera required *srtA* (Fig. 4C). Note that the absence of a high-molecular-weight banding pattern in these experiments is due to use of RrgA in these chimeras, which apparently is unable to be incorporated into pili by SrtA, SrtB, or SrtC. This is consistent with RrgA lacking the consensus pilin and E-box motifs. Cumulatively, these data indicate that there is a one-to-one sortase-to-surface-protein correspondence for gene products in the *rlrA* pathogenicity islet and further suggest that this relationship is essential for the formation of multisubunit pili on the cell surface.

To date, pili have been shown to be present in several gram-positive organisms. In this study, we show evidence of similar pili in *S. pneumoniae* TIGR4 encoded by the *rlrA* pathogenicity islet and additionally show that RrgA is incorporated into pili as a minor subunit in an SrtD-dependent manner.

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