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Two Crystal Structures of Pneumococcal Pilus Sortase C Provide Novel Insights into Catalysis and Substrate Specificity

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The respiratory tract pathogen *Streptococcus pneumoniae* is a primary cause of morbidity and mortality worldwide. Pili enhance initial adhesion as well as the capacity of pneumococci to cause pneumonia and bacteremia. Pilus-associated sortases (SrtB, SrtC, and SrtD) are involved in the biogenesis of pneumococcal pili, composed of repeating units of RrgB that create the stalk to which the RrgA adhesin and the preferential pilus tip subunit RrgC are covalently associated. Using single sortase-expressing strains, we demonstrate that both pilin-polymerizing sortases SrtB and SrtC can covalently link pili to the peptidoglycan cell wall, a property shared with the non-pilus-polymerizing enzyme SrtD and the housekeeping sortase SrtA. Comparative analysis of the crystal structures of *S. pneumoniae* SrtC and SrtB revealed structural differences explaining the incapacity of SrtC, but not of SrtB, to incorporate RrgC into the pilus. Accordingly, site-directed mutagenesis of Thr^{160} in SrtB to an arginine as in SrtC (Arg^{160}) partially converted its substrate specificity into that of SrtC. Solving two crystal structures for SrtC suggests that an opening of a flexible lid and a concomitant cysteine rotation are important for catalysis and the activation of the catalytic cysteine of pilus-associated sortases.

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Introduction

The human-specific pathogen *Streptococcus pneumoniae* (pneumococcus) is a primary cause of morbidity and mortality worldwide and represents one of the four major infectious disease killers, together with HIV, malaria, and tuberculosis.[1,2] *S. pneumoniae* is the main cause of respiratory tract infections such as otitis media, sinusitis, and community-acquired pneumonia, and it is also an important pathogen in invasive diseases such as septicemia and meningitis. Invasive pneumococcal disease is preceded by initial colonization of the nasopharynx, but the detailed mechanisms of adhesion are still not well understood.

Most bacterial pathogens have long filamentous structures known as pili or fimbriae extending from their surface. These structures are often involved in the initial adhesion of the bacteria to host cells and

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tissues, as well as to other bacteria, during colonization. Pilus expression mediates host cell responses that depend on the host receptor being recognized and may also increase the host inflammatory responses.[3,4] Until recently, pili had only been recognized as an attribute of Gram-negative bacteria in which they have been well characterized from the genetic, biochemical, and structural perspectives. During the last few years, some major human Gram-positive bacterial pathogens have also been shown to encode pili, and their biogenesis, structure, and function are now being investigated.[3,5–9]

Multiple-drug resistant *S. pneumoniae* strains have emerged over the last 30 years, requiring rapid development of novel preventive and therapeutic approaches including the development of protein-based vaccines.[10] We have previously demonstrated that globally spreading penicillin nonsusceptible pneumococci belong to clonal lineages that frequently carry pili, encoded by the *rlrA* pathogenicity islet, suggesting that pili contribute to the successful spread of these clones.[11] Pili enhance initial adhesion as well as the subsequent capacity of the bacteria to cause pneumonia and bacteremia.[3] The *rlrA* pilus islet in *S. pneumoniae* encodes three surface proteins, RrgA, RrgB, and RrgC, which form the pneumococcal pilus as well as three pilus-associated sortases, SrtB, SrtC, and SrtD.[3,12,13] The stalk of the pneumococcal pilus is composed of repeating units of RrgB to which RrgA and RrgC are covalently associated. The RrgA protein also occurs associated to the cell wall.[3] RrgC is frequently present at the tip of pili but may also appear along the pilus stalk and in proximity of RrgA.[12] RrgA has been shown to be the major pilus-associated adhesin of pneumococcal pili, but RrgC may also have some binding functions.[13]

In general, sortases can be described as membrane-associated transpeptidases that transfer and covalently link surface proteins to the peptidoglycan cell wall.[14,15] The linkage mechanism is initiated by the cleavage between the threonine and glycine residues of the conserved LPXTG motif (where *X* represents any amino acid) in substrate proteins.[15,16] The C-terminal threonine of the surface protein is concomitantly linked to the sortase catalytic cysteine, after which it is incorporated to the cell wall through the formation of an amide bond between the C-terminal threonine and the stem peptide of the peptidoglycan.[14] In contrast to these so-called housekeeping sortases, pilus-associated sortases covalently link the different subunits of the pilus to each other, resulting in the assembly of mature pili.[5,8,17]

The relative contributions of pneumococcal-associated sortases, SrtB, SrtC, and SrtD, have recently been established.[12] While SrtD did not polymerize any of the Rrg subunits, SrtB and SrtC were redundant in terms of their activity, as both were able to polymerize RrgB in order to form the structural backbone of the pilus as well as link RrgA to this backbone. However, in contrast to SrtB, SrtC cannot incorporate RrgC to the pilus.[12] SrtC also differs from SrtB and SrtD in that the absence of

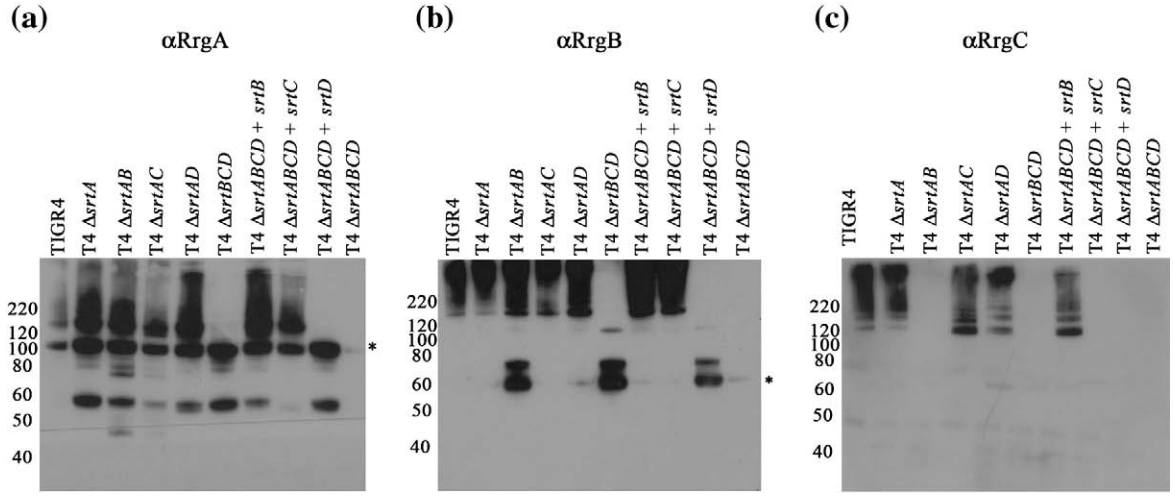

Fig. 1. Role of individual sortases in pilus biogenesis and cell wall anchoring. The production of polymeric high-molecular-mass cell wall-associated pili in isogenic T4 mutants was evaluated by immunoblotting for RrgA (a), RrgB (b), and RrgC (c). In all cases, cell wall proteins were separated by gradient SDS-PAGE, transferred to polyvinylidene fluoride, and probed. Approximate molecular masses are indicated in kilodaltons to the left. The three images show immunoblotting for RrgA (a), RrgB (b), and RrgC (c) in the wild-type TIGR4 ('T4'); the single sortase mutant strain T4Δ*srtA* ('Δ*srtA*'); the double-mutant strains T4Δ*srtAB* ('Δ*srtAB*'), T4Δ*srtAC* ('Δ*srtAC*'), and T4Δ*srtAD* ('Δ*srtAD*'); the triple-mutant strain T4Δ*srtBCD* ('Δ*srtBCD*') that expresses only SrtA; the *trans*-complemented *srtABCD* quadruple-mutant strains T4Δ*srABCD+lacE*::*srtB* ('Δ*srtABCD+srtB*'), T4Δ*srABCD+lacE*::*srtC* ('Δ*srtABCD+srtC*'), and T4Δ*srABCD+lacE*::*srtD* ('Δ*srtABCD+srtD*'); and the quadruple-mutant strain T4Δ*srABCD*. The predicted molecular masses for the RrgA (90 kDa) and RrgB (67 kDa) monomers are indicated by an asterisk. All four sortases are redundant in their capacity to transfer pili and/or pilin monomers to the cell wall. SrtD shows an *in vivo* activity. SrtB and SrtC differ only in their capacity to incorporate RrgC into the pilus.

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