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Regulation of Surface Proteins Assembly on the Wall of Gram-Positive Bacteria

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REGULATION OF SURFACE PROTEINS ASSEMBLY ON THE WALL OF GRAM-POSITIVE BACTERIA

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REGULATION OF SURFACE PROTEINS ASSEMBLY ON THE WALL OF GRAM-POSITIVE BACTERIA

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Cell wall peptidoglycan-anchored surface proteins are essential virulence factors in many Gram-positive bacteria. Attachment of these proteins to the peptidoglycan is achieved through a transpeptidation reaction, whereby sortase cleaves a conserved C-terminal LPXTG motif and attaches the protein to the peptidoglycan precursor lipid II. This thesis deals with the spatial regulation of the sorting reaction, and the role sortase localization plays in the correct distribution of surface proteins. We have introduced a new immunofluorescence procedure to study the distribution of sub-surface antigens in *Streptococcus pyogenes*, which utilizes the phage lysin PlyC to permeabilize the cell wall of to antibodies. We found that sortase localizes within distinct membranal foci, the majority of which are associated with the division septum and colocalize with areas of active M-protein anchoring.

Protein anchoring takes place at two distinct cellular locations, the division septum and the poles. Anchoring of M-protein at the septum is a rapid process that occurs in concert with septal peptidoglycan synthesis. The localized secretion of M-protein, as well as the prevalence of sortase, lipid II, and PBPs at this location, promote efficient protein anchoring. Sortase localization and M-protein anchoring occurs simultaneously at the septum and the mature equatorial rings, pointing to the possibility that cell division

begins in the daughter cells before the conclusion of the previous division cycle. Anchoring of SfbI at the poles is a much slower and more diffuse process, and therefore requires less sortase. The protein sorting reaction is therefore a dynamic, and a highly regulated process.

In the absence of sortase A, surface proteins accumulate at the membrane-wall interface. We found that stalled surface proteins can be released from the secretion channel by an LPXTG-specific enzyme other than sortase A, whose identity is still unknown. Nonetheless, accumulation of missorted surface proteins has deleterious effects on the cell, resulting in selective pressure to repress surface protein expression. Inhibition of sortase may therefore not only prevent surface protein anchoring but also exert direct pressure on the cell's secretion and folding pathways. A better understanding of the mechanisms controlling the biogenesis of surface molecules, aided by the ability to study sub-surface antigens through immunofluorescence, may yield promising new candidates for the development of new anti-infecting agents.

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1 Introduction

1.1 Introduction outlay

This thesis centers on the study of cellular processes the common human pathogen *S. pyogenes* employs to export and attach a variety of virulence factors to its surface. This chapter will begin with a brief introduction to streptococcal disease and its various manifestations, and will thereafter focus on the molecular mechanisms enabling this bacterium to colonize, invade, survive, and spread within the human host. *S. pyogenes* produces a wide array of virulence factors, which are crucial for its *in vivo* survival. These can be roughly divided into two classes, namely, ones that are associated with the surface of the organisms, and ones that are secreted into the surrounding milieu. Many of the surface associated virulence factors are covalently anchored to the cell wall through their C-terminal anchor domain containing a conserved LPXTGase motif. This process, termed protein sorting, will be discussed in some detail, followed by a discussion of some of the key wall-anchored virulence factors, and their role in pathogenesis. I will then discuss the biogenesis and function of pili, a special class of surface-anchored virulence factors, which extend 500nm from the bacterial surface and play a critical role in adhesion and invasion of this organism. I will also discuss LPXTGase, a peptidase specific for the LPXTG motif found in the C-terminal cell-wall anchoring domain of surface protein, but whose exact role in this process is still under study. The last part of the introduction will

discuss the mechanism of protein secretion, and the role played by some of the secreted virulence factors.

1.2 *S. pyogenes* and streptococcal disease

S. pyogenes is a major human pathogen, responsible for about 500,000 deaths per year worldwide (Carapetis *et al.*, 2005). The most common infection caused by *S. pyogenes* is a self-limiting pharyngitis, estimated at around 500 million cases per year (Carapetis *et al.*, 2005), however infection of the skin is also common. Most infection cases are limited in scope, but complication of the disease may lead to more severe disease including streptococcal toxic shock syndrome, scarlet fever, septicemia, pneumonia, meningitis, and necrotizing fasciitis or "flesh-eating disease", which may lead to a fatal outcome. In addition, infection with *S. pyogenes* may lead to sequelae, which include rheumatic fever, acute glomerulonephritis, reactive arthritis, and Tourette's syndrome (Carapetis, 2007, Cunningham, 2000).

1.2.1 Pharyngitis, scarlet fever, and rheumatic heart disease

S. pyogenes is a common colonizing organism of the human pharyngeal mucosa, a condition that may lead to pharyngitis or tonsillitis. While most such infections are self-limiting and are generally treatable with antibiotics, complication of pharyngeal infection may lead to scarlet fever. Scarlet fever typically results from the secretion of one or more of the streptococcal pyrogenic exotoxins A, B, and C, (carried on the genome of prophage) which leads to uncontrolled inflammation (Weeks & Ferretti, 1984, Bohach *et al.*, 1990), manifested as a rash,

strawberry tongue, and desquamation of the skin. Even a mild infection with *S. pyogenes*, may result in rheumatic fever, a post-streptococcal infection sequelae that occurs in ~2% of individuals, when infection is left untreated. Acute rheumatic fever is an autoimmune disease where antibodies directed against the streptococci cross-react with self antigens and cause irreversible damage to the heart valves. It is the most serious post streptococcal infection sequelae and results in disability and death in children as well as adults worldwide. Rheumatic heart disease has resurged in the United Stated in the past two decades in several locations, with a particularly severe outbreak in Salt Lake City Utah. Streptococcal serotypes associated with the new outbreaks of rheumatic fever were M types 1, 3, 5, 6, and 18 (Kaplan *et al.*, 1989).

1.2.2 Streptococcal skin infection and invasive streptococcal disease

S. pyogenes is a common organism colonizing the skin asymptomatically, particularly in tropical climates. If streptococci gain access through abrasions of the skin however, the organism may establish an infection (impetigo), which is usually suppurative and limited to the epidermis. In more severe cases where the streptococci become more invasive, the infection may lead to the development of streptococcal toxic shock syndrome, which is characterized by hypotension and multiple organ failure (Cone *et al.*, 1987, Stevens *et al.*, 1989). While no single streptococcal product has been implicated in the disease, these symptoms result from the non-specific induction of the immune system by the streptococcal extracellular pyrogenic exotoxins A, B, and C, and F, as well as the streptococcal superantigens SSA, SpeG, SpeH, SpeJ, SmeZ, and SmeZ-2 (Mollick *et al.*, 1993, Norrby-Teglund *et al.*, 1994a,

Norrby-Teglund *et al.*, 1994b, Kamezawa *et al.*, 1997, Proft *et al.*, 1999). Interaction of these superantigens with major histocompatibility complex (MHC) class II molecules and T-lymphocyte receptor results in the nonspecific activation of a massive numbers of T cells, resulting in the release of cytokines, interleukins, tumor necrosis factor and gamma interferon. (Fast *et al.*, 1989, Hackett & Stevens, 1992, Norrby-Teglund et al., 1994b). Streptococcal skin infection is caused by different strains than those causing infection of the throat (Wannamaker, 1970). Strains of M-types 1, 3, 11, 12, and 28 are the ones usually associated with streptococcal toxic shock syndrome, with M1 and M3 being the most common.

Necrotizing fasciitis, or "flash eating disease" may accompany the streptococcal toxic shock syndrome (Breiman *et al.*, 1993). In this case bacteria invade and destroy soft tissues, resulting in permanent damage that may extend to entire limbs. This results in an estimated 1500 deaths annually in the United States (O'Loughlin *et al.*, 2007). Sequelae associated with streptococcal skin infection are different than those associated with throat infection and include acute glomerulonephritis (AGN), and reactive arthritis. Similar to rheumatic heart disease, reactive arthritis stems from the creation of self-reacting antibodies, in this case specific to antigens in the joints, whereas AGN appears to be the result of a secreted toxin (Cunningham, 2000, Cunningham, 2008).

1.2.3 Identification of streptococci – Lancefield groups

Pathogenic streptococci were initially grouped by Rebecca Lancefield based on the immunological differences in their cell wall polysaccharides (groups A, B, C, F, and G) or lipoteichoic acids (group D) (Koneman *et al.*, 1997). The group A carbohydrate antigen is composed of N-acetyl-β-D-glucosamine linked to a polymeric rhamnose backbone. Group A streptococci were further divided into serotypes based on their immunological reactivity with different surface M-protein molecules. This was achieved through using highly absorbed rabbit sera raised against specific streptococcal strains, reacting particularly with the hypervariable N-terminal region of the M molecule (Lancefield, 1928). The use of this method developed by Rebecca Lancefield, led to the identification of over 80 serotypes of *S. pyogenes*.

M-protein molecules from different strains are typically divided into two classes. Class I molecules react with the 10B6 monoclonal antibody, directed at the C repeat region of the M molecule (Bessen *et al.*, 1989, Bessen & Fischetti, 1990b). Strains of this class, such as M types 1, 3, 5, 6, 14, 18, 19, and 24, are usually opacity factor negative, and are often associated with pharyngeal infection, and rheumatic fever occurrence (Bessen *et al.*, 1995). Class II molecules do not react with 10B6 (Bessen et al., 1989, Bessen & Fischetti, 1990b). Strains of this class, such as M types 2, 49, 57, 59, 60, and 61, are usually opacity factor positive, and are associated with skin infection. An additional method of typing was developed based on sera specific to the trypsin resistant antigen (or T-antigen) (Lancefield & Dole, 1946, Schneewind *et al.*, 1990, Jones *et al.*, 1991). T- types and M types are in many cases associated (Beall *et al.*, 1998a, Beall *et al.*, 1998b).

1.3 Covalent anchoring of proteins to the cell wall of Gram-positive bacteria

Wall-anchored virulence factors play a vital role in the pathogenesis of *S. pyogenes*. This chapter will start with a brief introduction of the cell wall synthesis mechanism, and then describe the properties of cell wall anchored proteins and the mechanism of their covalent attachment to the wall. A more detailed discussion of the secretion mechanism responsible for membrane translocation of these proteins is presented in chapter 1.7.

1.3.1 The cell wall composition and assembly

Gram-positive bacteria possess a single cell membrane surrounded by a cell wall composed of a single cross-linked macromolecule, about 20-100 nm thick. In addition to the peptidoglycan, the cell wall contains a number of secondary cell wall polymers such as wall teichoic acids (WTA), which are covalently attached to the cell wall, and lipoteichoic acids (LTA), which are anchored to the membrane (Araki & Ito, 2008). The cell wall serves both as an extracellular skeleton, protecting the bacteria from physical damage, and as a selective barrier, preserving cation homeostasis and importing nutrients (Neuhaus & Baddiley, 2003).

S. pyogenes cell wall is composed of chains with repeating subunits of MurNAc-[L-Ala - D-isoGln - L-Lys(L-ala$_2$) - D-Ala - D-Ala] - β(1-4)-GlcNAc)]. It is synthesized in the cytoplasm in a multiple step reaction in which, a soluble intermediate, Park's nucleotide (UDP-MurNAc - L-Ala - D-isoGln - L-Lys - D-Ala - D-Ala) (Chatterjee & Park, 1964), is synthesized from its various components and linked to a bactoprenol carrier through phosphodiester linkage, yielding lipid I (C55-

PP-MurNAc - L-Ala - D-isoGln - L-Lys - D-Ala - D-Ala) (Chatterjee & Park, 1964, Matsuhashi *et al.*, 1967) . This membrane bound intermediate is further modified with *N*-acetylglucosamine (GlcNAc), and cross bridge (L-ala – L-ala in *S. pyogenes*) at the ε-amino group of L-Lys, yielding lipid II [C55-PP-MurNAc - [L-Ala - D-isoGln - L-Lys(L-ala$_2$) - D-Ala - D-Ala] - β(1-4)-GlcNAc)]. Lipid II, is then translocated across the membrane (Nakagawa *et al.*, 1984), where it serve as the basic building block for peptidoglycan synthesis.

Cell wall precursors are polymerized into a complex cell wall structure through the action of penicillin binding proteins (PBPs), which possess transglycosylation and transpeptidation activities (Goffin & Ghuysen, 1998). Transglycosylation polymerizes MurNAc-GlcNAc subunits into glycan strands (Tipper & Strominger, 1965). Transpeptidation involves the cleavage of the bond between the D-ala – D-ala moieties of the pentapeptide precursor, and the formation of an amide bond between the D-alanine at position four and the amino group of the cross-bridge, pentaglycine in the case of *Staphylococcus aureus* (Izaki *et al.*, 1966), and di-L-alanine in the case of *S. pyogenes.*

S. pyogenes has four high molecular weight (HMW) PBPs, of which three are class A PBPs, possessing both glycosyl transferase activity (for the synthesis of the glycan strands) and transpeptidase activity (for the cross-linking of the peptidoglycan), and one is a HMW class B PBP, which possess only a transpeptidase activity. In addition, *S. pyogenes* possess between two and three (depending on the serotype) low molecular weight (LMW) PBPs, which usually have carboxypeptidase or endopeptidase activity and are needed for preservation of the correct shape of the

sacculus, and control the amount of cross-linking (Zapun *et al.*, 2008). The structure and function of the cell wall is discussed in more detail in the following reviews (Beveridge, 2000, Neuhaus & Baddiley, 2003).

1.3.2 The sorting reaction – an overview

Wall-anchored proteins typically possess two motifs that play important roles in their biogenesis, an N-terminal signal peptide responsible for directing the protein for secretion through the SecYEG channel, and a C-terminal sorting signal, responsible for cell wall anchoring. The sorting signal is composed of an LPXTG motif, followed by a hydrophobic region, and a few positively charged amino acids at the polypeptide C-terminus (Schneewind *et al.*, 1992). During protein export, the C-terminal anchor domain is stalled in the secretion channel, leaving the LPXTG motif exposed at the outer leaflet of the membrane (Navarre & Schneewind, 1994). The transpeptidase sortase then cleaves this motif between the threonine and glycine residues (Mazmanian *et al.*, 1999), and attaches the freed threonine to the peptidoglycan precursor, Lipid II (Perry *et al.*, 2002). Lipid II then serves as substrate for peptidoglycan synthesis, leading to the covalent attachment of the protein to the cell wall.

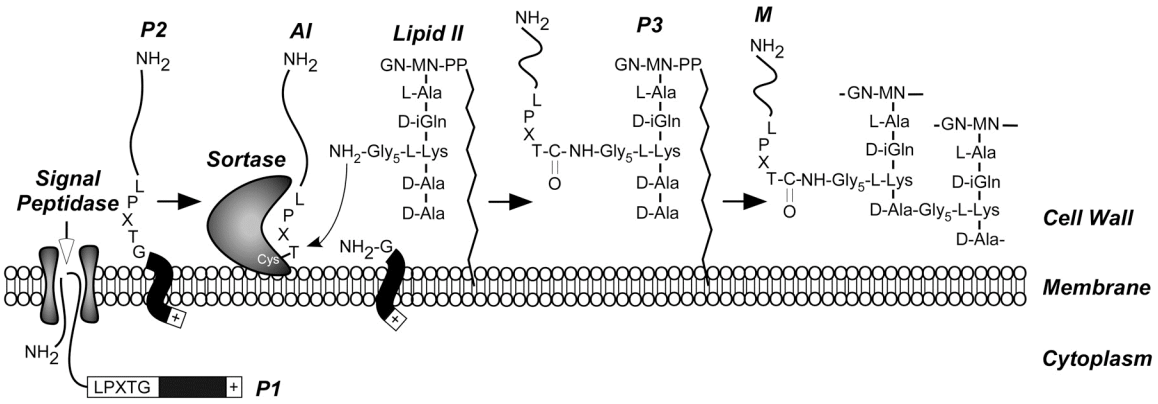


Figure 1.1 Schematic representation of the sorting reaction

Surface proteins are synthesized in the cytosol, and translocated through the Sec system. Following cleavage of the N-terminal signal sequence, surface proteins are stalled in the secretory pathway by the C-terminal hydrophobic domain (black box) and positively charged tail (+), leaving the LPXTG sequence exposed at the outer leaflet of the membrane. Sortase then cleaves the LPXTG motif between the threonine and glycine, and forms an acyl-enzyme intermediate with the threonine residue of the surface protein. The cross-bridge amino group in lipid II attacks the acyl-enzyme intermediate, linking the surface protein to this peptidoglycan precursor through its threonine residue. Through the action of penicillin binding proteins, the lipid II - surface protein complex is incorporated into the cell wall. Adopted from (Marraffini *et al.*, 2006).

1.3.3 The Cell Wall Sorting Signal (CWSS)

The cell wall sorting signal (CWSS) is about 35 amino acid long, and is comprised of three components: an LPXTG motif followed by a hydrophobic region and a few positively charged amino acids at the C-terminus (Fischetti *et al.*, 1990, Schneewind et al., 1992, Schneewind *et al.*, 1993). All three components are important for correct anchoring of surface proteins. Removal of the hydrophobic domain or the positively charged C-terminal residues results in secretion of the protein into the medium. Removal or mutation of the LPXTG motif on the other hand results in missorting of the protein to the medium, cell wall, membrane and cytoplasm (Schneewind et al., 1992).

Fusion of a CWSS to the C-terminus of a secreted protein such as PhoB is sufficient for anchoring to the cell wall (Schneewind et al., 1992), and can even function when the CWSS is placed in the middle of the molecule (Navarre & Schneewind, 1994). CWSS are ubiquitous in Gram-positive bacteria, and various CWSS from streptococcal, enterococcal, and listerial origin could facilitate the anchoring of truncated protein A to the cell wall of *S. aureus*, although the distance between the hydrophobic domain and the LPXTG motif was sometime needed to be altered for efficient sorting (Schneewind et al., 1993). During anchoring, the LPXTG motif is cleaved between the threonine and glycine moieties of the LPXTG motif (Navarre & Schneewind, 1994) and anchored to the cross bridge of the cell wall (pentaglycine for *S. aureus*) (Schneewind *et al.*, 1995).

The nature of the attachment point between the surface protein and the peptidoglycan was studied in *S. aureus* using an elegant system comprised of Seb

(secreted staphylococcal exotoxin) fused to the CWSS of *S. aureus* protein A. In this system, a methionine followed by a hexahistidine tag was inserted N-terminally to the LPXTG motif. Following anchoring of this construct to the cell wall, the SEB construct was released from the cell wall through the use of muralytic enzymes, and subsequently purified and cleaved at the methionine moiety using cyanogen bromide (CnBr). The cleaved anchor was then purified using metal ion affinity chromatography and characterized by mass spectrometry. Using this system and various cell wall hydrolases, each breaking a specific bond in the cell wall of *S. aureus*, wall proteins were shown to be anchored to the pentaglycine cross-bridge of this organism (Ton-That *et al.*, 1997) (Navarre *et al.*, 1998).

1.3.4 Sortase

Through the screening of a chemically mutagenized *S. aureus* library, a mutant defective in the anchoring of a reporter protein to the wall was identified. Complementation of this mutant with a plasmid library containing *S. aureus* genomic DNA led to the discovery of sortase, the enzyme responsible for the cleavage of the LPXTG motif and its covalent attachment to the cell wall (Mazmanian et al., 1999). This enzyme was subsequently cloned and produced recombinantly as a his-tagged molecule lacking its N-terminal transmembrane anchor, and was shown to cleave the LPXTG motif *in vitro* (Ton-That *et al.*, 1999). Deletion of the sortase A gene from the genome of *S. aureus* abolished the anchoring of LPXTG surface protein to the cell wall (Mazmanian *et al.*, 2000)**.**

Sortase is a membrane bound enzyme with an N-terminal transmembrane domain. NMR studies (Ilangovan *et al.*, 2001) and X-ray crystallography (Zong *et al.*, 2004) determined that *S. aureus* SrtA folds into an eight-stranded β-barrel. The active site sulfhydryl of *S. aureus* sortase is cysteine-184. Calcium binding near the active site stimulates catalysis, possibly by altering the surface loop that recognizes the newly translocated peptide (Naik *et al.*, 2006).

Sortase catalyses a transpeptidation reaction in which a conserved cysteine residue in the enzyme active site performs nucleophilic attack on the scissile peptide bond between threonine and glycine of the LPXTG motif and form an acyl-enzyme intermediate (Huang *et al.*, 2003, Ton-That et al., 1999). Subsequently, the amino group of a pentaglycine crossbridge serves as a nucleophile and attacks this bond, resulting in the covalent attachment of the protein to the cell wall.

The catalytic activity of recombinant SrtA, lacking its N-terminal membrane anchor, was initially measured using a fluorescence resonance emission transmission (FRET) substrate composed of an LPETG peptide with fluorophore/quencher pair attached on its sides (Ton-That et al., 1999). Cleavage of this peptide by sortase separates the fluorophore from the quencher and creates a measurable fluorescent signal. This reaction is induced by the presence of a nucleophile such as hydroxylamine, glycine, and oligo-glycine chains. When a nucleophile such as hydroxylamine is added to the staphylococcal culture medium, surface proteins are released into the medium during the anchoring process due to competition between the hydroxylamine and the pentaglycine crossbridge as a nucleophile in the sorting reaction (Ton-That et al., 1999).

The substrate specificity of purified recombinant *S. aureus* sortase was tested *in vitro* using a peptide library replacing each of the amino acids in the LPXTG motif with all other possible amino acids. Consistent with the conservation of the LPXTG motif in wall-anchored proteins, only leucine, proline, threonine and glycine were tolerated in their respective positions whereas any amino acid could be tolerated in the third position (Kruger *et al.*, 2004).

The nucleophile specificity has also been studied in some detail. While the staphylococcal pentaglycine crossbridge serve as better substrates than shorter glycine chains (Ton-That et al., 1999), gly, gly2, and gly3 can be used as nucleophiles by sortase *in vitro*, and are linked to the threonine of the LPXTG peptide. Diglycine-alanine, diglycine-leucine, glycine-alanine, and glycine-valine can also be used with reduced efficiency, however alanine-glycine and valine-alanine cannot be used, indicating that glycine is required at the attacking position (Huang et al., 2003). These results are in agreement with the anchoring efficiency of mutants in the *femA*, *femB*, and *femX* genes, responsible for cross-bridge synthesis. While the pentaglycine cross-bridge is the preferred *in vivo* nucleophile serving to anchor LPXTG proteins, tri-glycine cross-bridge and even a cross bridge consisting of a single glycine moiety could serve as nucleophile, however the ε-amino group of the pentapeptide lysine group could not serve as nucleophile in the absence of a cross bridge (Ton-That *et al.*, 1998).

Sortase is inhibited by agents that bind the active site cysteine, such as [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) and *p*-hydroxymercuri-

benzoic acid, which also inhibit the sorting reaction *in vivo* (Ton-That & Schneewind, 1999).

1.3.5 Lipid II serves as the sorting reaction nucleophile *in vivo*

There are several lines of evidence suggesting that proteins are anchored to the peptidoglycan precursor lipid II *in vivo*, rather than directly to mature cell wall. First, *in vivo* the sorting reaction is inhibited by vancomycin and moenomycin, but not penicillin G (Ton-That & Schneewind, 1999). Vancomycin binds lipid II and inhibits both the transpeptidation and transglycosylation reactions catalyzed by PBPs, while moenomycin is a lipid II analogue that interferes with the transglycosylation reaction. Penicillin G on the other hand binds PBPs directly and does not interact with lipid II in any way. It is likely therefore that vancomycin and moenomycin inhibit the sorting reaction by direct interaction with lipid II rather than by inhibition of peptidoglycan synthesis. This notion is further supported by the observation that in spheroplasts, from which the cell wall has been removed enzymatically, the LPXTG motif of surface proteins is cleaved at a rate similar to that of intact cells (Ton-That & Schneewind, 1999). Attachment of an LPXTG peptide to lipid II is catalyzed by sortase *in vitro*, and this reaction is inhibited by the addition of vancomycin (Ruzin *et al.*, 2002). More direct evidence was obtained by purifying sorting intermediates from cells labeled with ^{32}P phosphoric acid, which is incorporated into lipid II. Such ^{32}P-labeled intermediates proved to be hydrophobic when separated by thin layer chromatography (TLC), and bound nisin, an antibiotic that specifically interacts with lipid II (Perry et al., 2002).

1.3.6 Sortase A is required for pathogenesis

Due to the importance of surface proteins for pathogenesis, mutation of sortase, which prevent their anchoring to the cell wall, results in a marked reduction in pathogenesis. This phenomenon has been observed in several animal model studies including staphylococcal intraperitoneal injection, and an organ abscess murine model (Mazmanian et al., 2000), a septic arthritis model (Jonsson *et al.*, 2002, Jonsson *et al.*, 2003), and a staphylococcal endocarditis model (Weiss *et al.*, 2004).

1.3.7 *S. pyogenes* sortase

The *S. pyogenes* gene encoding sortase A was found through the screening of a transposon mutagenesis library for mutants that did not clump in the presence of anti-SfbI (PrtF1) IgM monoclonal antibody. The sortase gene encodes a protein of 249 amino acids containing a predicted N-terminal trans-membranal segment of 39 amino acids and a TLXTC motif, characteristic of sortases (Barnett & Scott, 2002). Deletion of this gene abolished the anchoring of M-protein, SfbI (PrtF1), ScpA, and GRAB to the cell wall but did not affect anchoring of the T-antigen, which is anchored by SrtB (see below).

1.4 Cell wall anchored virulence factors

Wall anchored proteins are essential for survival in the human host. They have various functions including resistance to phagocytosis, binding of plasma proteins and immunoglobulins, adhesion and invasion to human cells, stimulation of host response such as cytokine production, and more. Genomic analysis shows that *S. pyogenes* M1 has 13 potential surface-anchored proteins (Ferretti *et al.*, 2001), although the number and type of surface proteins varies slightly between different serotypes of *S. pyogenes* (Banks *et al.*, 2004, Green *et al.*, 2005, Nakagawa *et al.*, 2003, Smoot *et al.*, 2002, Sumby *et al.*, 2005). This chapter will describe the major cell wall anchored virulence factors, and the mechanism responsible for their attachment to the cell wall.

1.4.1 M-protein

M-protein is among the best studied of the *S. pyogenes* wall-anchored proteins (Fischetti, 1989). It forms an alpha-helical coiled-coil dimer that extends 60 nm from the cell surface (Manjula & Fischetti, 1980, Manjula & Fischetti, 1986, Manjula *et al.*, 1985, Fischetti, 1989, Nilson *et al.*, 1995), and appears as surface fibrils when studied by electron microscopy (Phillips *et al.*, 1981, Swanson *et al.*, 1969). These fibrils may be an aid to cell aggregation, as secondary structure analysis indicate that the N-terminus of two M-protein molecules can interact in an anti-parallel fashion and stabilize each other (Frick *et al.*, 2000), suggesting a Velcro-like mechanism that binds cells together.

The molecule contains an N-terminal signal peptide, followed by four repeat regions termed A to D. At the C-terminus of the molecule there is an area rich in proline and glycine residues, which is embedded in the cell wall, followed by a call wall sorting signal with an LPXTG motif (Hollingshead *et al.*, 1986). The repeat regions (A-D) have different levels of variability ranging from hyper-variable for the A repeats, semi-variable for the B repeats, and conserved for the C and D repeats. These repeat regions bind various host factors, which differs from serotype to serotype.

M-protein is vital for the virulence of *S. pyogenes*, and function in numerous roles, which vary slightly between serotypes. One of the most prominent roles M-proteins play is in conferring the bacterium resistance to phagocytosis (Lancefield, 1962). Absence of the *emm* gene allows rapid phagocytosis of the bacteria (Scott *et al.*, 1985) while re-introduction of the gene on a plasmid restores resistance to phagocytosis (Perez-Casal *et al.*, 1992, Scott *et al.*, 1986). Resistance to phagocytosis is achieved through the binding of the serum protein factor H, which inhibits the deposition of the complement C3b factor, and thus prevents opsonization (Horstmann *et al.*, 1988, Perez-Casal *et al.*, 1995, Okada *et al.*, 1995). Binding fibrinogen was also suggested as a mechanism by which M-protein inhibits the deposition of C3b, leading to escape from phagocytosis (Whitnack & Beachey, 1982, Whitnack & Beachey, 1985, Whitnack *et al.*, 1984). Yet another mechanism to avoid phagocytosis involves the binding of human C4b-binding protein (C4BP), a plasma protein that inhibits complement activation (Andre *et al.*, 2006).

M1 serotype M-protein has also been shown to play an important role in the invasion of host cells (Cue *et al.*, 1998). This is achieved by binding fibronectin, which in turn interacts with integrin $\alpha_5\beta_1$. This results in major cytoskeletal rearrangements, which require the activation of phosphatidylinositol 3-kinase (Purushothaman *et al.*, 2003). Uptake through an M-dependent mechanism shows a zipper-like morphology, and is carried through the classic endocytic pathway, leading to the phagolysosomes (Dombek *et al.*, 1999).

M-protein was shown to bind human kininogen. Kininogen plays a double role, initially inhibiting the activity of host cyteine proteases thus protecting the streptococcal surface proteins. At a later stage, kininogen is cleaved by SpeB, to release bradykinin, a vasoactive and proinflammatory peptide, into the plasma (Ben Nasr *et al.*, 1995, Herwald *et al.*, 1996, Ben Nasr *et al.*, 1997). M-protein has been implicated in the binding of several additional host proteins such as the membrane cofactor protein CD46, plasminogen, IgA, IgG, and human serum albumin (Giannakis *et al.*, 2002, Okada et al., 1995, McArthur & Walker, 2006).

The importance of M-protein in the pathogenesis of streptococci has been demonstrated in several models, among which are murine pharyngeal colonization (Hollingshead *et al.*, 1993), and skin abscess models (Ashbaugh *et al.*, 1998), and a primate model (Ashbaugh *et al.*, 2000). M-protein is also a promising vaccine target, as antibodies against the C repeat region protected animals against mucosal challenge and colonization with multiple serotypes of *S. pyogenes* (Bessen & Fischetti, 1990a). This observation led to several studies on the possible use of M-protein as a vaccine (Dale *et al.*, 1993, Schulze *et al.*, 2006).

1.4.2 M-like proteins

Certain *S. pyogenes* serotypes contain a number of genes that share homology with the M-protein. In serotype 49, deletion of either M-protein (*emm-49*) or one of the two M-like proteins *enn-49*, and *mrp-49* resulted in reduced resistance to phagocytosis in human blood and purified polymorphonuclear leukocytes (PMNs) (Podbielski *et al.*, 1996a, Ji *et al.*, 1998). Mrp of serotype M4 *S. pyogenes* was shown to induce resistance to phagocytosis, and to bind fibrinogen (Courtney *et al.*, 2006). M-related proteins were also shown to bind the Fc portion of immunoglobulins, and other plasma proteins, such as albumin, factor H, fibrinogen, and plasminogen (Heath & Cleary, 1987, Podbielski *et al.*, 1994, Pack & Boyle, 1995, Podbielski *et al.*, 1995).

1.4.3 SfbI

SfbI, also known as protein F (PrtF1), is a wall-anchored protein, which is the major fibronectin binding protein in certain *S. pyogenes* strains (Hanski & Caparon, 1992). Fibronectin binding is mediated through a C-terminal domain containing four repeats of 37 aa residues each, which contain the conserved motif ED(T/S)(X,7-10)GG(X,4)(I/V)(D/E)(F/I/T). In addition, the upstream spacer region has also been shown to bind fibronectin when expressed as a separate polypeptide (Talay *et al.*, 2000). The N-terminal aromatic region of the molecule is highly variable, and as a result a large number of different alleles of the molecule are present in different *S. pyogenes* serotypes (Towers *et al.*, 2003).

The binding of fibronectin to SfbI promotes attachment of the bacterium to the ECM and adhesion to host cells (Hanski & Caparon, 1992, Hanski *et al.*, 1992). SfbI

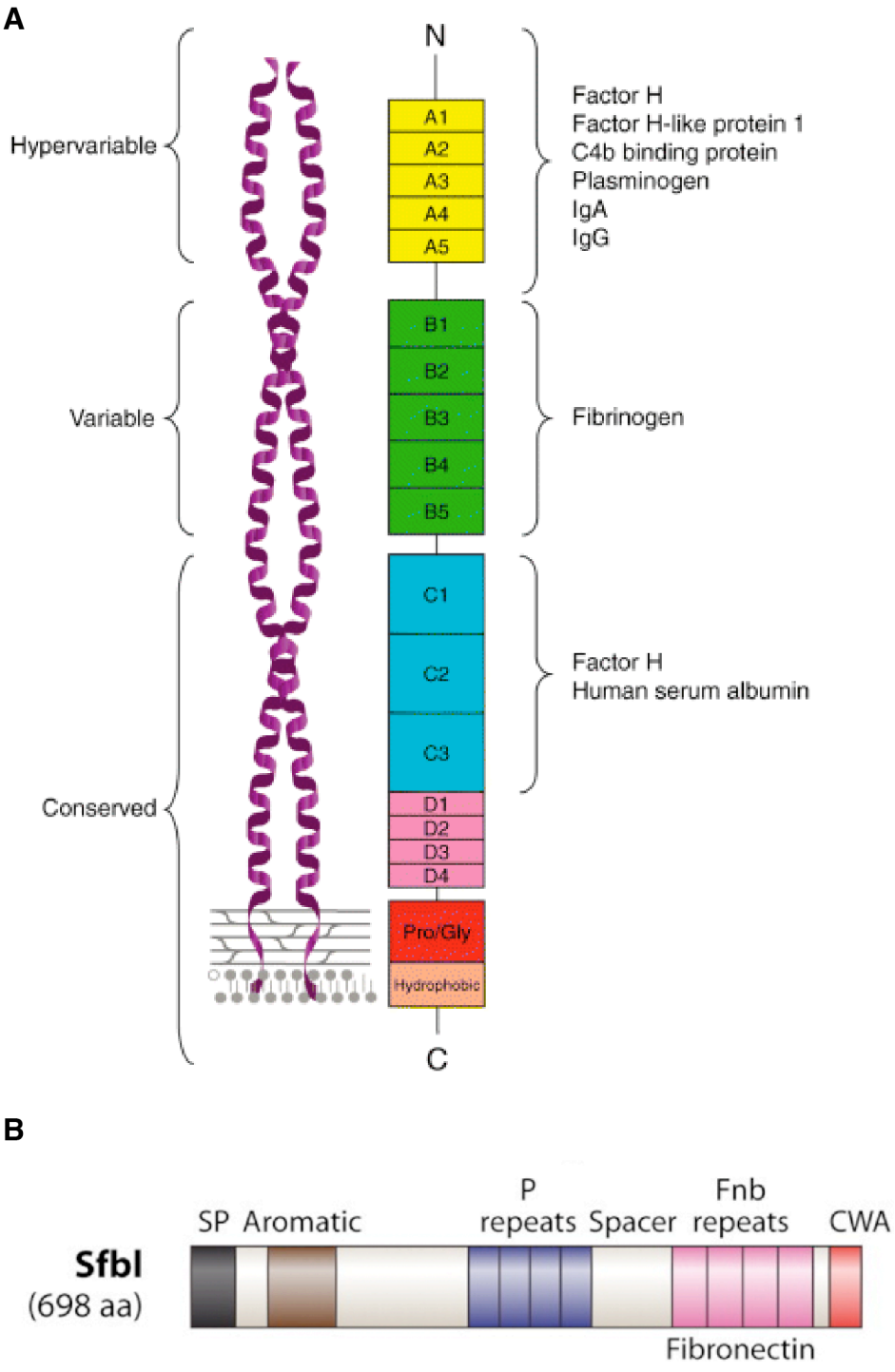
is also of major importance in invasion to host cells (Okada *et al.*, 1994, Molinari *et al.*, 1997, Jadoun *et al.*, 1998, Kreikemeyer *et al.*, 2004). Invasion is mediated through the binding of SfbI-bound fibronectin to integrin $\alpha_5\beta_1$ via its RGD motif. Integrin clustering results in the formation of focal complexes and focal adhesions, and the triggering of two independent host signaling pathways. One pathway is mediated through the phosphorylation of a tyrosine moiety in focal adhesion kinase (FAK) and paxillin, which leads to cytoskeletal rearrangement through the action of Src kinases, Src, Yes, and Fyn. In the second pathway the GTPases Rac and Cdc42 are recruited and activated. Through the combined action of both pathways, caveolae are formed beneath the attached bacteria, and subsequently fuse to form a large invagination through which the bacteria are internalized (Ozeri *et al.*, 1998, Ozeri *et al.*, 2001).

Intranasal vaccination of animals with SfbI mediates protective immunity in the serum and lungs of animals, protecting them against homologous and heterologous serotypes of *S. pyogenes* (Guzman *et al.*, 1999, Schulze et al., 2006).

Figure 1.2 Domain structure of M-protein and SfbI

(A) M-protein is a coiled-coil molecule anchored to the cell wall peptidoglycan via its C-terminal cell wall sorting signal. The capacity of the various repeat-regions to bind host factors is indicated on the right. Adapted from (Fischetti, 2006). (B) Domain representation of SfbI, Adapted from Nobbs et al. (Nobbs *et al.*, 2009).

Figure 1.2


A
N
A1
A2
A3
A4
A5
Factor H
Factor H-like protein 1
C4b binding protein
Plasminogen
IgA
IgG
Hypervariable
B1
B2
B3
B4
B5
Variable
Fibrinogen
C1
C2
C3
Factor H
Human serum albumin
Conserved
D1
D2
D3
D4
Pro/Gly
Hydrophobic
C
B
SP
Aromatic
P
repeats
Spacer
Fnb
repeats
CWA
SfbI
(698 aa)
Fibronectin

1.4.4 Serum opacity factor (SOF) / SfbII

Serum opacity factor is a wall-anchored aspartic apoproteinase, specifically degrading apoprotein AI of HDL (Martinez *et al.*, 1978, Saravani & Martin, 1990b) (Saravani & Martin, 1990a). This activity results in the induction of opacity in human serum (Rakonjac *et al.*, 1995). Independently, the protein was isolated and found to bind fibronectin and was therefore named SfbII (Kreikemeyer *et al.*, 1995). At a later date, the two were found to be the same protein (Kreikemeyer *et al.*, 1999). The binding of fibronectin to SOF was later confirmed, and the protein was shown to be important for virulence in a murine model (Courtney *et al.*, 1999).

1.4.5 Protein G-related α2 macroglobulin (GRAB)

GRAB is a cell anchored protein that specifically binds the active form of the broad spectrum protease inhibitor α_2M, which is present in relatively high amounts in human plasma (Rasmussen *et al.*, 1999). Binding of α_2M to the cell surface protects surface proteins such as M-protein from degradation by both host and streptococcal proteases, such as SpeB. GRAB was found to be important for the pathogenicity of *S. pyogenes* in a murine intraperitoneal challenge (Rasmussen et al., 1999).

1.4.6 C5a peptidase (ScpA)

The C5a peptidase is a surface bound endopeptidase highly specific for the complement-derived chemotaxin C5a, cleaving it at its PMN-binding site (Chen & Cleary, 1990, Cleary *et al.*, 1992). Cleavage of C5a inhibits the recruitment of phagocytic cells to the site of infection (Ji *et al.*, 1996, Wexler *et al.*, 1985).

Mutations in the *scpA* gene increase clearance of streptococci from subdermal sites of infection and from the nasopharyngeal mucosa of intranasally infected mice. Intranasal immunization of mice against ScpA produced a vigorous serum and secretory antibody response that enhanced clearance of the bacteria from the oral mucosa (Ji et al., 1996, Ji *et al.*, 1997). The C5a peptidase therefore presents a second line of defense against the complement system, in addition to the activity of M-protein.

1.4.7 Streptococcal Inhibitor of Complement (SIC)

SIC is a cell-anchored protein that inhibits the formation of complement membrane attack complex (MAC) (Akesson *et al.*, 1996, Fernie-King *et al.*, 2001). The role SIC plays *in vivo* is not absolutely clear since the MAC complex is not believed to be effective against Gram-positive pathogens owing to their thick cell wall. SIC displays an extreme level of polymorphism between different strains, suggesting that the level of host selective pressure acting on this protein is high (Mejia *et al.*, 1997). The preservation of this protein despite such selective pressure demonstrates its importance for survival *in vivo*.

1.4.8 Collagen like proteins

Scl1 and Scl2 are collagen-like proteins, which function as adhesins (Lukomski *et al.*, 2000, Lukomski *et al.*, 2001, Rasmussen *et al.*, 2000, Rasmussen & Bjorck, 2001), (Whatmore, 2001). These proteins interact with human integrins and induce intracellular signaling pathways (Humtsoe *et al.*, 2005). In addition, they have been implicated in the binding of factor H (Caswell *et al.*, 2008).

1.4.9 Additional surface anchored proteins

Various *S. pyogenes* strains contain additional characterized and putative wall-anchored proteins. Among these are Cpa (Kreikemeyer *et al.*, 2005), Vitronectin binding protein (important for adhesion) (Valentin-Weigand *et al.*, 1988), Streptococcal protective antigen (Spa) (McLellan *et al.*, 2001), and various Ig binding proteins such as protein H (Frick *et al.*, 1995), the IgA receptor protein (Arp) (Husmann *et al.*, 1995), the streptococcal Ig receptor (Sir) (Stenberg *et al.*, 1994), Lzp (Okamoto *et al.*, 2008). For further information on surface anchored proteins and their role in pathogenesis refer to the following reviews (Cunningham, 2000, Cunningham, 2008, Olsen *et al.*, 2009, Scott & Barnett, 2006).

1.5 Pili

1.5.1 Discovery and assembly mechanism

Pili or fimbriae (the two terms are equivalent) are long appendages that play an important role in bacterial adherence and invasion to their human host. Pili of Gram positive pathogens were first observed in *Corynebacterium renale* by electron microscopy (Yanagawa *et al.*, 1968, Yanagawa & Otsuki, 1970), and have since been identified in *Actinomyces naeslundii* (Cisar *et al.*, 1988, Yeung & Ragsdale, 1997) (Yeung *et al.*, 1998, Mishra *et al.*, 2007), *Corynebacterium diphtheriae* (Ton-That & Schneewind, 2003, Gaspar & Ton-That, 2006) *S. pyogenes* (Mora *et al.*, 2005), Group B Streptococci (GBS) (Lauer *et al.*, 2005, Rosini *et al.*, 2006) *Streptococcus pneumoniae* (Barocchi *et al.*, 2006, LeMieux *et al.*, 2006),*Enterococcus faecalis* (Nallapareddy *et al.*, 2006), *Bacillus cereus* (Budzik *et al.*, 2007, Budzik *et al.*, 2008a, Budzik *et al.*, 2009), and *Bacillus anthracis* (Budzik *et al.*, 2008b). Unlike Gram-negative pili, which are stabilized by non-covalent interactions, Gram-positive pili are composed of one covalently bound molecule, polymerized by specialized sortases to form a large pilus.

Maria Yeung, John Cisar, and colleagues first suggested the involvement of a sortase gene in the formation of *A. naeslundii* pili. In this organism, the pilus subunit genes *orf1* and *fimP*, which encode proteins containing a cell wall sorting signal, and the sortase homologue *orf4* are located in an operon. Deletion of the sortase gene results in the abolishment of pilus formation, and the accumulation of *orf1* and *fimP* subunits (Yeung & Ragsdale, 1997, Yeung et al., 1998), suggesting the involvement of sortase in pilus assembly.

Ton-That et al. studied the formation of *Corynebacterium diphtheriae* pili, and addressed the role of sortase in their polymerization in more detail *(Ton-That & Schneewind, 2003)*. Corynebacterial pili are composed of 3 subunits, SpaA, SpaB, and SpaC, all containing an LPXTG motif. Immuno-electron microscopy studies reveal that SpaA subunits are found throughout the length of the pilus, SpaB subunits decorate the pilus at spaced intervals, and SpaC is only found at the tip. SrtA, the corynebacterial pili-specific sortase (not to be confused with the house keeping sortase), polymerizes SpaA subunits into a covalently linked pilus shaft, and is needed for the decoration of the pili with the ancillary pilus proteins SpaB and SpaC. All the pilus subunits as well as SrtA are genetically linked in an operon, a phenomenon common to many bacterial species.

Each pilus subunit, in addition to the signal sequence, and the C-terminal sorting signal, also contains the conserved amino acid sequence WxxxVxVYPK termed "pilin motif". The conserved lysine residues in the pilin motif serves as a nucleophile, which attacks the scissile bond between the threonine and glycine of the LPXTG motif of the subsequent pilin subunit, thereby covalently-linking the two. This process is then repeated several times to produce a chain of linked subunits that form the pilus shaft. SpaC does not contain a pilin motif and therefore cannot facilitate polymerization, but only serves as the first subunit in the pilus, positioning it at the pilus tip (Ton-That & Schneewind, 2003). SpaA contains an addition conserved amino acid motif YxLxETxAPxGY termed the E box. Mutation of the conserved glutamic acid in this motif abolished the decoration of the SpaA pili with SpaB and SpaC subunits.

The elongation of the pilus is terminated when lipid II, instead of a pilus subunit, serves as a nucleophile in the sorting reaction. Through this process the pilus is tethered to the cell wall peptidoglycan (Ton-That & Schneewind, 2003). Anchoring of the polymerized pilus to the peptidoglycan is carried out by the house keeping sortase, SrtF in the case of *C. diphtheriae*, and in its absence pili are polymerized but are not anchored to the cell wall, and can therefore be solubilized by boiling in SDS (Ton-That & Schneewind, 2003, Ton-That *et al.*, 2004, Swaminathan *et al.*, 2007). Attachment of the polymerized pilus to the cell wall is facilitated through the incorporation of the minor pilin subunit SpaB to the base of the pilus, and its subsequent attachment to the cell wall peptidoglycan (Mandlik *et al.*, 2008a). Control of pilus length trough the introduction of a specific pilus subunit that terminates polymerization and facilitate attachment is a mechanism found in GBS as well (Nobbs *et al.*, 2008).

1.5.2 The pili of *S. pyogenes*

In *S. pyogenes*, the pilus subunit genes and pilus-related sortases are located at a genetic locus called the FCT region. The FCT region, which stands for fibronectin binding, collagen binding, and T-antigen, is a highly variable 11kb pathogenicity island, flanked by the highly conserved genes spy0123 and spy0136. The genomic organization of this region suggests that it had been acquired through horizontal gene transfer. (Bessen & Kalia, 2002, Kreikemeyer et al., 2004, Kratovac *et al.*, 2007). Six classes of FCT regions have been described on the basis of gene content and organization (Kratovac et al., 2007). FCT-2, FCT-3, and FCT-4 are present in strains

of the serotypes most commonly associated with disease in the western world, serotypes M1, M3, M5, and M18 (Kratovac et al., 2007). A given strain of *S. pyogenes* encodes only a single FCT locus, and therefore produces only a single type of pilus. Each of the FCT regions contains between one and two sortase genes, and between 3 and 5 genes containing a cell wall sorting signal, which include the pilus subunits as well as other wall-anchored proteins (Mora et al., 2005). Many of these wall-anchored proteins are associated with the binding of host cellular and ECM molecules, and have been implicated in the adhesion an invasion to host cells.

Two sortase genes are responsible for the polymerization of *S. pyogenes* pili, *srtB* and *srtC1*/*srtC2*. The *srtB* gene was found through a bioinformatic search of the *S. pyogenes* M1 genome for sortase A homologs (Barnett & Scott, 2002). The gene encodes a sortase with an N-terminal signal peptide and a C-terminal membrane anchor, which is specific for T-antigen in the M6 genome. The other pili-related sortase of *S. pyogenes* has two alleles (*srtC1* and *srtC2*), which are mutually exclusive in *S. pyogenes* genomes (Barnett *et al.*, 2004). *SrtC1*, present in the M1 strain SF370, and *srtC2*, present in the M3, M5, and M18 serotypes (Barnett et al., 2004). *SrtC2* from the M3 serotype strain was shown to be necessary for the anchoring of SpyM3_100, which contains a QVPTGV sorting motif. Replacement of this sequence with an LPXTG sorting motif abrogated the anchoring of the protein (Barnett et al., 2004). Both SrtB and SrtC1/SrtC2 are important for the polymerization of the T-antigen pilus shaft, as well as for the attachment of pilus ancillary subunits, in the respective strains where they are found (Mora et al., 2005).

T-antigen (Schneewind et al., 1990) composes the *S. pyogenes* pilus shaft (Mora et al., 2005). This protein was used for a long time in the stereotyping of *S. pyogenes* strains (Lancefield & Dole, 1946), along side with M-protein (Lancefield, 1928). Recently immuno-EM studies of cells labeled with the T antigen serum revealed that the T-antigen forms a pilus extending 500nm from the bacterial surface (Mora et al., 2005). The T6 pilus of the serotype M6 strain has an LPXTG motif followed by a serine, and is polymerized by SrtB (Barnett & Scott, 2002). Other *S. pyogenes* serotypes have T-antigen shaft proteins containing QVPTG or EVPTG sequences, which are polymerized by SrtC1 or SrtC2. T-antigen lacks the pilin motif and E-box, characteristic of pili in other Gram-positive organisms, suggesting that these pili may be polymerized through a slightly different mechanism. Structural studies of the T1 pilus revealed two intramolecular isopeptide bonds (Kang *et al.*, 2007), each formed between the ε-amino group of a lysine residue and the carboxyl group of an asparagines residue. Site-specific mutagenesis demonstrated that a glutamate residue located near each of the intramolecular bonds is required for the formation of the bonds in a reaction that appears to be spontaneous. These intramolecular bonds may make the pilus more resistant to the shearing forces it encounters in nature, but are not important for pilus polymerization (Quigley *et al.*, 2009). Intramolecular bonds were also shown to be present in the shaft of *B. anthracis* pili (Budzik et al., 2009).

In addition to the pilus shaft, one or two ancillary pilus subunits are found in the FTC region. Polymerization of the pilus shaft is not dependent on the presence of the ancillary subunits, however the ancillary subunits cannot be polymerized in the

absence of the pilus shaft gene (Mora et al., 2005). In the strain M1 *orf128* encodes the T-antigen forming the pilus shaft, whereas *orf130* and *cpa* encodes ancillary subunits; all three are polymerized by SrtC1 (Mora et al., 2005). Cpa is a virulence factor with the ability to bind host collagen type I, leading to adhesion and internalization of streptococci (Kreikemeyer et al., 2005). The M1 Cpa has a VVPTG sorting signal, and is a substrate of SrtC1 (Mora et al., 2005). In strains containing SrtC2, such as in the case of the T3 pilus, the anchoring sequence of Cpa is VPPTG (Barnett et al., 2004). Replacement of this motif with LPXTG abolishes the attachment of Cpa to the pilus. Cpa is attached through its VPPTG motif to a ε-amino group of a lysin residue of the T3 shaft. Since Cpa itself does not contain a lysin residue that could facilitate polymerization, it can only serve as the first molecule in the chain, placing it at the tip. Localization at the tip of the pilus is indeed observed by immuno-EM (Quigley et al., 2009). Serotype M1 contains in addition to Cpa another ancillary protein, referred to as *orfB*. This subunit was found by immunogold electron microscopy (EM) to be associated with the pilus structure (Mora *et al.*, 2005). How this pilus subunit is added to the pilus is not known at this time.

The SipA2 gene, present in FCT-2, FCT-3, and FCT-4 (Mora et al., 2005), encodes a signal peptidase, an enzyme that cleaves the N-terminal signal peptide of secreted proteins. M3-SipA2 was found to be required for the polymerization of T3 protein and for addition of Cpa to this polymer. The signal peptidase activity of SipA2 however was not required for this role in pilus biogenesis and it is therefore believed to play a role as a pilus chaperone (Zahner & Scott, 2007). SipA2 was also found to be required for the biogenesis of the pilus of the M49 strain (Nakata *et al.*, 2009), as

well as for certain *S. pneumoniae* strains (Bagnoli *et al.*, 2008), however the requirement of SipA2 for pilus biogenesis is not a general phenomenon in most other Gram-positive bacteria.

The importance of pili and the FCT-encoded ECM-binding proteins for the pathogenesis of *S. pyogenes* was demonstrated in a number of studies. *S. pyogenes* pili mediate attachment to primary human keratinocytes and to human tonsillar tissue, as well as to several tissue culture cell lines (Abbot *et al.*, 2007, Manetti *et al.*, 2007) indicating that they are critical for attachment of *S. pyogenes* to host tissue. They have also been implicated in formation of biofilms, which may be important for disease development (Manetti et al., 2007). Additionally, in both *S. pyogenes* (Mora et al., 2005), and *Streptococcus agalactiae* (Lauer et al., 2005, Maione *et al.*, 2005) vaccination with pilus components was shown to be protective in a murine model of infection indicating that pili may be promising candidates for vaccine development. For more information regarding the biogenesis and role of pili refer to the following reviews (Scott & Zahner, 2006, Telford *et al.*, 2006, Mandlik *et al.*, 2008b).

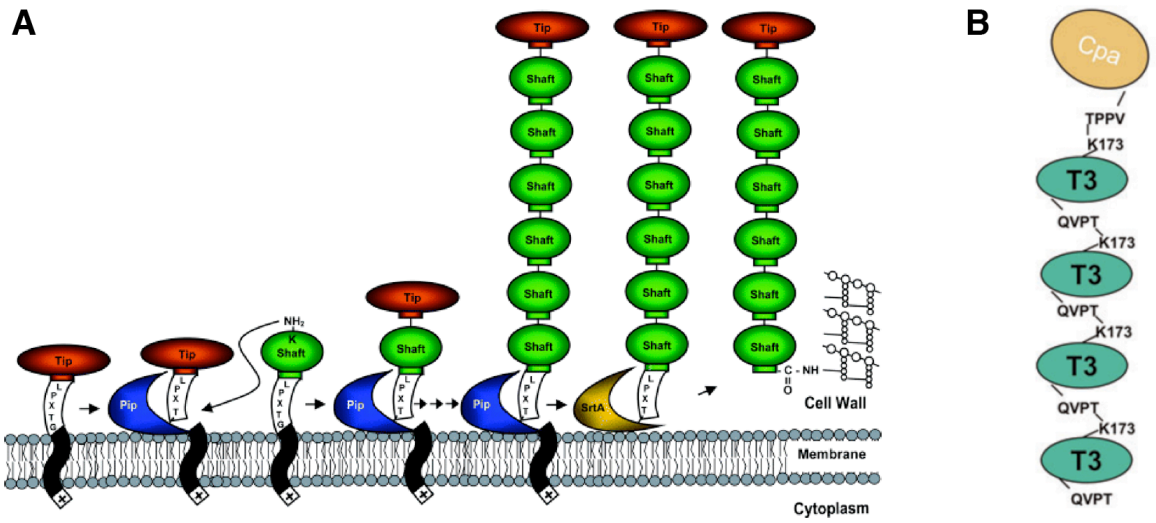


Figure 1.3 Polymerization and structure of *S. pyogenes* pili

(A) A model for the polymerization of *S. pyogenes* pili. Pilus assembly is initiated when a pilus-specific sortase forms an acyl-enzyme bond with the ancillary pilus subunits Cpa (leading to its placement at the pilus tip). This acyl-enzyme bond is resolved through a nucleophilic attack of the ε-amino group of a lysin residue in the pilus shaft subunit. Subsequently (or previously) a pilus specific sortase forms an acyl-enzyme bond with the threonine of the LPXTG (or QVPTG) motif in the pilus shaft subunit, now bound to Cpa. Elongation of the pilus follows, through the addition of additional shaft subunits following the same mechanism. Pilus elongation is terminated when the pilus is transferred to SrtA, the house keeping sortase, which covalently attaches it to lipid II, resulting in its incorporation into the cell wall. This model is likely to contain variations between different organisms. Adopted from (Scott & Zahner, 2006). (B) Structure of the M3 T3 pilus. Adopted from (Quigley et al., 2009).

1.6 LPXTGase

1.6.1 LPXTGase discovery

In addition to sortase, *S. pyogenes* possess a unique membranal enzyme that specifically cleaves the LPXTG motif found in the C-terminus of surface proteins, called LPXTGase. LPXTGase was first discovered through the screening of a fractionated *S. pyogenes* lysate for LPXTG cleavage activity, measured by the release of radioactive material from bead-bound ^{125}I-labeled KRQLPSTGETANPFY peptide (the N-terminal of the peptide is bead-bound while the tyrosine is ^{125}I-labeled). While no such activity could be detected in the crude fractions, following ultrafiltration of the membrane extract through a 3kDa membrane a small molecular weight inhibitor was removed, unmasking the enzyme activity.

LPXTGase was found to elute at the 14kDa range on a G50 gel filtration column, although mass spectrometry analysis on the purified material revealed a major peak at the 8kDa range, raising the possibility that the native form of the enzyme may be a dimer. The enzyme displayed no UV adsorption at 280nm, indicating the absence of aromatic amino acids. When either radioactively-, or FITC-labeled enzyme was analyzed by SDS PAGE, the majority of labeled substance remained in the stacking gel suggesting that the enzyme forms large aggregates in the presence of SDS.

1.6.2 LPXTGase composition

The composition of LPXTGase is very unusual. The enzyme is highly glycosylated with various N-linked complex sugar structures, at least some of which are longer then 3 moieties each (Lee *et al.*, 2002). Carbohydrate moieties constitute 30% of the enzyme's dry weight and are critical for both enzyme activity and solubility in aqueous environment.

The amino acid composition is also unique. *S. pyogenes* LPXTGase is composed of 61 known amino acids, as well a number of unknown amino acids, which comprise about 30% of the peptide backbone weight (Lee *et al.*, 2002). The 61 known amino acids belong to only 11 amino acid species, of which none is aromatic, and only a few are hydrophobic. The enzyme contains 24 alanines, accounting for 39% of the known amino acids in the enzyme, of these 6-7 are in a D-conformation (Lee & Fischetti, 2003). Non-canonical amino acids were found to compose about 3000 Dalton or about 30% of the peptide backbone, and appear to confer the hydrophobicity to the enzyme. Despite repeated attempts, automatic amino acid sequencing of LPXTGase was unsuccessful. The sequence of a de-glycosylated tryptic fragment of LPXTGase could be obtained by manual sequencing, however of the 7 amino acids obtained in this manner, 4 were non canonical. All these unique features suggest that LPXTGase may be synthesized non-ribosomally, and if so this would be the first and only enzyme known to date to be synthesized in this manner (Lee et al., 2002).

A similar enzyme was isolated from a *S. aureus* membrane extract, displaying a molecular mass of about 14kDa by gel filtration and 7807.4 Dalton by mass spectrometry (Lee & Fischetti, 2006). LPXTGase produced from *S. aureus* has many similar characteristics with the *S. pyogenes* enzyme. Both enzymes exhibit a similar pH range, and a similar inhibition pattern when treated with various salts. Additionally, both enzymes contain abundant N-linked carbohydrate, lack aromatic amino acids, and contain both D-conformation, and unknown amino acids. Interestingly while the *S. pyogenes* enzyme is extremely rich in alanines, the staphylococcal enzyme is enriched in glycine moieties. This difference in amino acid composition between LPXTGase from the two organisms corresponds to a similar difference in the amino acid composition of their peptidoglycan, mostly attributable to the difference in their cross-bridge. This fact, in addition to the presence of alanine moieties in the D-conformation (which are also found in the cell wall) suggest that cell wall synthesizing enzymes may play a role in the assembly of LPXTGase (Lee & Fischetti, 2006).

1.6.3 LPXTGase catalytic activity and cleavage specificity compared to sortase.

The catalytic activity of LPXTGase is quite distinct from that of sortase. While sortase cleaves the LPXTG motif strictly between the threonine and glycine residues, LPXTGase cleavage appears to be more promiscuous. When the KRQLPSTGETANPFY peptide, part of the *S. pyogenes* M-protein anchor domain was used as substrate, both *S. aureus* and *S. pyogenes* LPXTGase cleaved at two

locations: between the serine and threonine, and following the glutamic acid residue, resulting in TANPFY, and TGETANPFY radioactive fragments (Lee et al., 2002, Lee & Fischetti, 2006). When the AQALPETGEENPFY peptide, part of the *S. aureus* protein A anchor domain was used however, both enzymes cleaved between the threonine and glycine moieties (similar to sortase), while the *S. pyogenes* enzyme also cleaved the peptide following the first glutamic acid, resulting in the cleavage fragments GEENPFY, and ENPFY (Lee & Fischetti, 2006). The cleavage activity of LPXTGase is specific for the LPXTG sequence since reversing or scrambling this motif abolished cleavage activity (Lee et al., 2002). Additionally the enzyme had no detectable cleavage activity when native bovine serum albumin (BSA) was used as substrate (Lee et al., 2002).

1.7 Secretion

1.7.1 Introduction

Protein translocation across the cytoplasmic membrane is needed not only for the correct processing of secreted proteins but also to allow membranal and wall-anchored proteins to reach their correct position in the cell. Secreted and surface exposed proteins are responsible for the majority of interactions between pathogens and their human host, emphasizing the importance of the proper function of the secretion apparatus for pathogenesis. Bacterial secretion has been most thoroughly studied in *Escherichia coli and Bacillus subtilis*. While the basic mechanism is highly conserved, there are also several differences between the two organisms, which would be discussed below. The Sec apparatus is the most common pathway for protein translocation, and this mechanism is ubiquitous in all sequenced genomes (Cao & Saier, 2003). Some organisms possess an additional secretion apparatus termed the Tat pathway (Yen *et al.*, 2002, Palmer *et al.*, 2005), however since components of this pathway are not found in sequenced *S. pyogenes* genomes (Dilks *et al.*, 2003), the Tat pathway will not be discussed further.

1.7.2 The signal sequence

Secretory proteins possess a 14-25 amino acid long N-terminal signal sequence, which targets them to the Sec apparatus. The signal sequence is composed of an N-terminal region encompassing one to three positively charged amino acid residues (the N domain), a hydrophobic core region consisting of 10–15 residues (the H domain), and a more polar C terminus, which constitutes the signal peptidase

cleavage site (C domain). The cleavage site usually has an Ala-X-Ala cleavage motif, where the alanines are found at the –1 and –3 positions relative to the cleaved bond (von Heijne & Abrahmsen, 1989). Following secretion, the signal peptide is cleaved by signal peptidase, releasing the mature protein for secretion (von Heijne, 1990). Proteins targeted for membrane integration, on the other hand, do not possess a signal sequence but instead have a transmembrane segment, which serves both to target the protein for translocation and to subsequently anchor it to the membrane.

1.7.3 The secretion apparatus

The SecYEG complex forms the protein conducting channel, whose general architecture is conserved among bacteria, archaea and eukaryotes (Pohlschroder *et al.*, 1997). It is composed of three integral membrane proteins, termed SecY, SecE, and SecG, that together form a stable complex (Brundage *et al.*, 1990). An additional complex, SecDFYajC, composed of SecD and SecF and YajC, is loosely associated with SecYEG to form a supramolecular translocase complex (Duong & Wickner, 1997). In *B. subtilis* and some other organisms, SecD and SecF are fused into a single membrane protein of 571 amino acids that is predicted to span the membrane 12 times (Bolhuis *et al*., 1998). The SecDFYajC complex is not essential for secretion, and its deletion only causes a mild secretion defect when secreted proteins are over-expressed (Bolhuis et al., 1998).

Because SecYEG does not generate a motive force on its own, it must associate with cellular components that provide the driving force necessary for polypeptide translocation or membrane insertion. The motor force for the

translocation is provided either by the ribosome itself or the associated ATPase motor SecA. Translocation is also assisted by proton motive force (PMF) (Prinz *et al*., 2000, Driessen, 1992).

1.7.4 SecA

SecA is an ATP-dependent motor protein, which plays an important role in protein translocation (Kourtz & Oliver, 2000). It is a soluble protein that localizes both to the cytosol and the cytoplasmic membrane (Cabelli *et al*., 1991). Its association with the membrane occurs via low-affinity interactions with anionic phospholipids (Lill *et al*., 1990), and high-affinity interactions with the SecYEG complex (Hartl *et al*., 1990). Most organisms contain a single copy of *secA*, however some organisms, among which are *Listeria monocytogenes, Mycobacterium tuberculosis*, and *S. aureus*, possess an additional copy, termed *secA2*, which drives the translocation of a specific subset of proteins (Rigel & Braunstein, 2008); *S. pyogenes* however does not possess a *secA2* (Caparon *et al.*, 2007).

1.7.5 SecB-dependent versus SRP-dependent translocation

In contrast to eukaryotes, the translation and translocation of secreted proteins in *E. coli* are uncoupled events (Randall, 1983). Nascent secreted proteins are recognized by the cytoplasmic chaperone SecB, which maintains them in a stable unfolded state, and subsequently targets them for secretion through its interaction with translocase-bound SecA (Fekkes *et al.*, 1997) (Fig 1.4A). Most integral membrane proteins on the other hand, are targeted for secretion through interaction of

their transmembrane segment with the signal recognition particle (SRP) (Valent *et al.*, 1998, Fekkes et al., 1997). The *E. coli* SRP is composed of a 4.5S RNA and a 48kDa GTPase called P48 or Ffh (for Fifty-Four Homolog) (Poritz *et al.*, 1990). While in eukaryotes interaction between the ribosome and the SRP results in translation arrest, this phenomenon has not been observed in *E. coli*. Following the binding of SRP to the nascent peptide on the ribosome, the complex binds the SRP receptor FtsY, which targets it to the secretion apparatus (Bernstein *et al.*, 1989). FtsY binds the membrane through its interaction with anionic phospholipids (de Leeuw *et al.*, 2000), but it also interacts directly with the SecYEG apparatus (Angelini *et al.*, 2005). Upon interaction between the Ffh part of the SRP and FtsY, and subsequent binding and hydrolysis of GTP, the complex dissociates, and the released ribosome-bound peptide is transferred to the SecYEG translocase, which then binds the ribosome directly (Prinz et al., 2000).

The Gram-positive model organism *B. subtilis* presents a slightly different picture of protein translocation. The most prominent difference between the two organisms is that *B. subtilis* lacks the secretion related chaperone SecB, which is central for SRP-independent protein translocation in *E. coli*. It is possible that secreted proteins are kept in a stable unfolded state in the cytoplasm of *B. subtilis* through interactions with general chaperones such as GroEL, DnaJ, and DnaK, or a secretion specific chaperone such as CsaA (Muller *et al.*, 2000). Another possibility is that the SRP pathway in this organism plays a much more prominent role in protein secretion, and compensates for the lack of a SecB-dependent pathway (Fig 1.4B). In contrast to *E. coli*, *B. subtilis* Ffh binds SecA directly, and with high affinity (Bunai,

1999), in a fashion reminiscent of the SecA binding to the precursor–SecB complex in *E. coli* (Fekkes *et al.*, 1998). These observations support a prominent role for SRP in *B. subtilis* protein secretion, in contrast to its role in *E. coli,* where it mainly functions in the targeting of integral membrane proteins (Ulbrandt *et al.*, 1997, Seluanov, 1997).

In streptococci, in contrast to *E. coli* and *B. subtilis*, Ffh (and therefore the SRP pathway as a whole) is not essential (Crowley *et al.*, 2004, Hasona *et al.*, 2005, Rosch *et al.*, 2008, Hirose *et al.*, 2000, Phillips & Silhavy, 1992). Deletion of *S. pyogenes* Ffh inhibited the export of SPN and SLO, however secretion and anchoring of M-protein was unchanged, and secretion of SpeB was increased, although its processing and maturation was delayed (Rosch et al., 2008). Although Ffh mutants are viable, deletion of Ffh greatly reduced the pathogenicity of the strain in a zebrafish model of necrotic myositis, and in a murein model of subcutaneous infection (Rosch et al., 2008), suggesting that some virulence factors require this pathway for correct processing.

The situation in streptococci is therefore somewhat perplexing. These organisms on the one hand do not have a SecB homologue, which is key to the correct processing of most *E. coli* secreted proteins, but on the other hand deletion of Ffh affects only a small subset of secreted proteins, ruling out the possibility that, as in *B. subtilis*, the SRP pathway could compensate for the absence of SecB. As mentioned earlier, the genome of *S. pyogenes* does not possess components of the Tat pathway for protein secretion (Dilks *et al.*, 2003), ruling out the possibility that this

pathway plays a role. How M-protein and SpeB are secreted in the absence of both the SecB and SRP pathways is not clear at the moment.

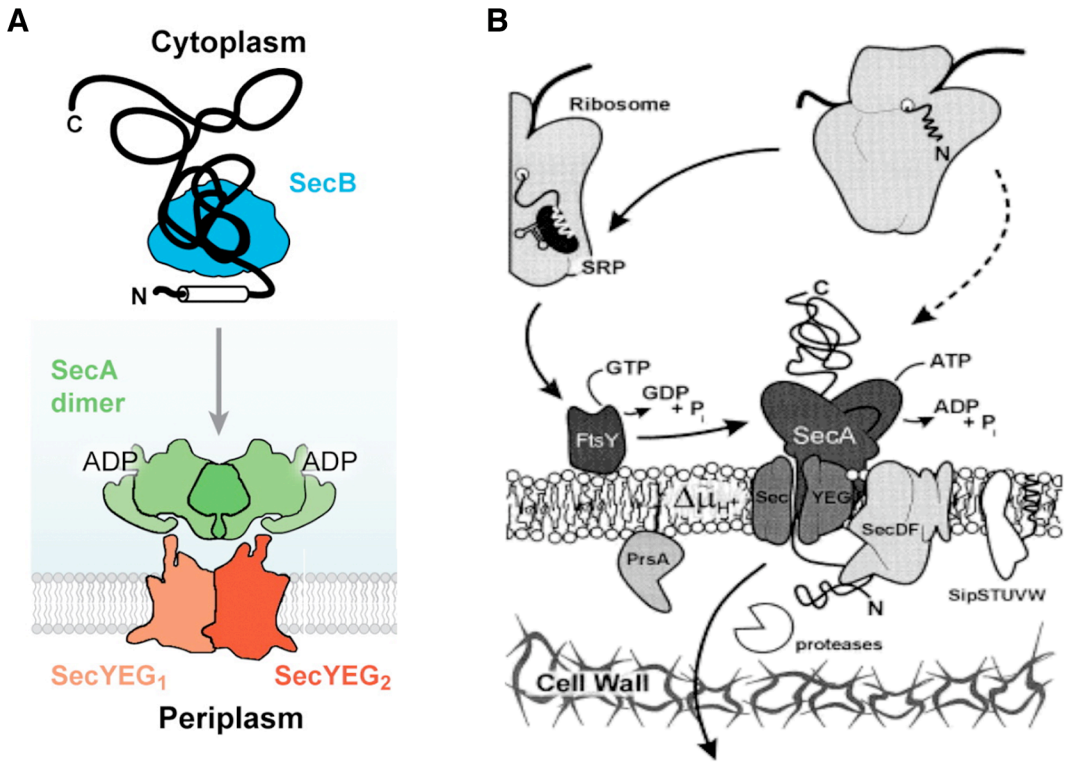


Figure 1.4 A schematic representation of different secretion pathways

(A) SecB dependent secretion in *E. coli*. During translation, a nascent polypeptide is bound by the cytoplasmic chaperone SecB, which preserves it in a stable unfolded state and targets it for SecA assisted translocation. Adopted from (Driessen & Nouwen, 2008). (B) Secretion through an SRP dependent mechanism in *B. subtilis*. The signal recognition particle (SRP) binds a nascent polypeptide on the ribosome, and subsequently binds its membranal receptor FtsY, which targets it to the Sec

channel. The dashed line represents a possible SRP-independent pathway. Adopted from (van Wely *et al.*, 2001).

1.7.6 Considerations regarding Gram-positive secretion

In most biological systems, proteins are not secreted directly into the extracellular milieu, but instead are secreted into dedicated organelles, which aid in their correct folding. In eukaryotes, secretory proteins are folded in the ER (Skach, 2007), and in Gram-negative bacteria the periplasmic space plays this role (Dautin & Bernstein, 2007). Gram-positive bacteria on the other hand were believed for a long time not to possess such a dedicated organelle, until recent cryo-EM data revealed the presence of periplasmic-like space between the membrane and cell wall of *B. subtilis* (Matias & Beveridge, 2005), *S. aureus* (Matias & Beveridge, 2006), *Enterococcus gallinarum*, and *Streptococcus gordonii* (Zuber *et al.*, 2006). Furthermore, many Gram-positive homologues of Gram-negative bacterial periplasmic proteins are lipid modified, which would target them to this "periplasmic" region (Sarvas *et al.*, 2004).

Another factor that must be taken into account is that the thick cell wall of Gram-positive pathogens has limited permeability, which has been shown to be a rate limiting factor in the secretion of *L. monocytogenes* proteins (Snyder & Marquis, 2003). *E. coli* cell wall is permeable to molecules of about 25-50 kDa, but larger molecules cannot diffuse through the cell wall (Demchick & Koch, 1996). Given the thickness and complexity of the Gram-positive cell wall, the cutoff limit is likely to be even lower. How secreted proteins traverse the cell wall is unclear at the moment, however since passive diffusion through the cell wall is not likely, this process is likely to be regulated in some manner.

1.7.7 Asymmetric localization of the secretion apparatus

In studies on the localization of the Sec apparatus, several of the Sec components were localized in helical arrays in the rod-shaped bacteria *E. coli* (Shiomi *et al.*, 2006) and *B. subtilis* (Campo *et al.*, 2004). In *B. subtilis* SecA and SecY GFP fusion proteins were found to co-localize in foci found at poles, septa, and intermediate positions. SecA-GFP formed two spiral-like structures around the cell, while SecY-GFP formed arcs, rather then continuous helices. While the SecA localization pattern remained visible in all growth phases, in stationary phase SecY became delocalized and was found in the cytoplasm. The observed "Sec coil" does not colocalize with a similar coil formed by the actin-like cytoskeletal protein MreB. Conflicting results however suggest that the Sec system is evenly distributed within the cytoplasmic membrane of *E. coli* with no apparent pattern (Brandon *et al.*, 2003).

In *S. pyogenes* the only component of the secretion apparatus whose localization has been studied is SecA, the soluble ATPase that promotes protein translocation. Localization of SecA was carried out using immuno-EM and yielded conflicting results. Certain studies have shown that SecA localizes to one microdomain on the *S. pyogenes* membrane, called the ExPortal (Rosch & Caparon, 2004). In this domain SecA was shown to be co-localized with the secreted cysteine protease SpeB (Rosch & Caparon, 2004), and the protease/chaperone HtrA, required for SpeB maturation (Rosch & Caparon, 2005). Localization of SpeB secretion to a single microdomain has also been demonstrated through the use of a quenched substrate, which becomes fluorescent upon cleavage by SpeB (Rosch & Caparon, 2004). The ExPortal was found to be enriched in anionic lipids (see below) (Rosch *et*

al., 2007), and recently, SrtA was found to be colocalized with SecA at the ExPortal in *Streptococcus mutans* (Ping *et al.*, 2008) and *E. faecalis* (Kline *et al.*, 2009). The sum of these data suggest that the location of protein secretion as well as the location of protein anchoring in the cell is dictated by the localization of all the relevant factors to one location in the cell, namely the ExPortal, and no other information is needed for the localization of secreted substrates.

In contrast to this view, several lines of evidence suggest that at least as far as surface proteins are concerned, secretion to only one location in the cell does not fit with the observed data. It has long been established that *S. pyogenes* M-protein is anchored solely at the septum (Cole & Hahn, 1962, Swanson et al., 1969, Carlsson *et al.*, 2006). More recently SfbI was shown to be anchored at the poles (Ozeri et al., 1998). Anchoring at these two locations is controlled by specific cues found on the signal sequence of these proteins (Carlsson et al., 2006). Switching the signal sequence between M-protein and SfbI resulted in an equivalent change in the location of their secretion and cell wall anchoring (Carlsson et al., 2006). Additionally, localization studies showed that SecA is distributed randomly on the *S. pyogenes* membrane (Carlsson *et al.*, 2006) rather than in a single ExPortal (Rosch & Caparon, 2004). The reason for this difference between the two studies is not clear since the two groups used the same protocol (namely Immuno-EM) and the same reagents.

It was noted that the signal sequence of M-protein contain a YSIRK/GS motif while that of SfbI does not (Carlsson et al., 2006). The YSIRK/GS motif has initially been implicated in optimal secretion of certain staphylococcal proteins (Rosenstein & Gotz, 2000, Bae & Schneewind, 2003). Of the 21 surface proteins present in

sequenced *S. aureus* genomes, 13 contain a YSIRK/GS motif while the others don't. The relative distribution of 4 representative proteins from each category has recently been studied (DeDent *et al.*, 2008). Proteins containing a YSIRK/GS motif in their signal sequence were distributed to 2-4 foci located within a ring associated with the septal area. Proteins not containing this motif on the other hand were localized to foci primarily associated with the periphery or displayed a hemispherical localization. Co-localization experiments showed that the proteins of the two types were not anchored at the same location. Similar to *S. pyogenes* M-protein and SfbI, switching the signal sequence between two such staphylococcal proteins resulted in a distribution similar to that of the protein from which the signal sequence originated. Despite its apparent importance, mutations in the YSIRK/GS motif itself of both *S. pyogenes* and *S. aureus* surface proteins did not affect their distribution, suggesting that this motif is not needed *per se* for the correct localization of secretion, but is useful in distinguishing between the two types of signal sequences (Carlsson et al., 2006, DeDent et al., 2008).

1.7.8 Anionic lipids play a role in secretion

Anionic lipids play an important role in the secretion of proteins across the membrane (de Vrije *et al.*, 1988) and determining the topology of membrane proteins. In *E. coli*, non bi-layer lipid contribute to the efficiency of protein translocation (Rietveld *et al.*, 1995). In *B. subtilis*, anionic phospholipids are essential for protein translocation and SecA ATPase activity (van der Does *et al.*, 2000). An increase in the amount of anionic phospholipids in the membrane improves the translocation

efficiency (Suzuki *et al.*, 1999). A reconstituted *B. subtilis* translocase functions optimally in a lipid environment that mimics the polar head group composition of the native *Bacillus* membrane, i.e., about 30% phosphatidylethanolamine and 70% phosphatidylglycerol (van der Does et al., 2000).

The fluorescent membrane Dye 10-N-Nonyl Acridine Orange (NAO), which preferentially binds anionic lipids, fluoresces in the green spectrum when bound to Phosphatidylglycerol (PG), and in the red spectrum when bound to cardiolipin (CL) (Mileykovskaya & Dowhan, 2000, Mileykovskaya *et al.*, 2001). When labeled with NAO, both *E. coli* and *B. subtilis*, display enrichment of cardiolipin (CL) at the division site (Mileykovskaya & Dowhan, 2000, Kawai *et al.*, 2004). In *B. subtilis* a dotty helical pattern is visible as well at some stages of the growth cycle. Phosphatidylethanolamine (PE) and several enzymes responsible for lipid synthesis were subsequently found to localize at the *B. subtilis* septum (Nishibori *et al.*, 2005). Anionic phospholipids are required for the correct localization of SecA in *B. subtilis*, and in their absence SecA is randomly distributed in the membrane (Campo et al., 2004). Analysis of *E. coli* mini-cell membranes (which contain a much greater percentage of polar to cylindrical membrane compared to normal cells) are enriched in cardiolipin (Koppelman *et al.*, 2001), supporting the fluorescent labeling data.

Lipid analysis of the *S. pyogenes* membrane indicates that the membrane is composed mainly of glycolipids, while two acidic phospholipids, PG and CL, are also present (Trombe *et al.*, 1979). *S. pyogenes* cells dyed with NAO display a single micro-domain which fluoresces at the green channel indicating enrichment in PG, but do not show clear fluorescence in the red channel, suggesting that very little CL is

present (Rosch *et al.*, 2007). Genomic analysis suggests that *S. pyogenes* has the genetic pathway for the synthesis of both PG and CL. Deletion of *cls*, which resulted in the abolishment of CL synthesis, did not alter the NAO fluorescence pattern or effected secretion significantly. This indicates that PG is the major anionic lipid labeled by NAO, and that PG is not needed for efficient secretion. Deletion of the gene responsible for PG synthesis could not be obtained. When NAO labeled cells were incubated with quenched SpeB fluorescent substrate, sites of SpeB secretion colocalized with the NAO-labeled anionic lipid microdomain (Rosch *et al.*, 2007).

1.8 Secreted factors

1.8.1 Pyrogenic exotoxins SpeA and SpeC

The pyrogenic exotoxins SpeA (Weeks & Ferretti, 1984, Weeks & Ferretti, 1986), and SpeC (Goshorn & Schlievert, 1988) are encoded by a lysogenized bacteriophage and function as superantigens (Goshorn & Schlievert, 1989). Superantigens bind a large number of T-cells non-specifically (Kappler *et al.*, 1989, Kotzin, 1994), and at the same time bind the MHC class II molecule expressed on B cells, monocytes, and dendritic cells. (Fast et al., 1989, Herman *et al.*, 1991, Kotb, 1995). This results in uncontrolled T-cell proliferation and release of excessive amounts of inflammatory cytokines, largely responsible for the toxic shock syndrome (TSSS) typical of infection with some *S. pyogenes* strains.

1.8.2 SpeB

SpeB is a cysteine protease, active on a large number of substrates (Hauser & Schlievert, 1990). It is secreted as a 40-kDa zymogen which is cleaved to a proteolytically active 28-kDa mature enzyme under reducing conditions (Hauser & Schlievert, 1990, Musser *et al.*, 1996, Elliott, 1945, Liu & Elliott, 1965). Maturation of SpeB into its active form is aided by M-protein (Collin & Olsen, 2000), and the membrane bound chaperone/protease HtrA (Spiess *et al.*, 1999, Poquet *et al.*, 2000) (Lyon & Caparon, 2004). The active form of SpeB is in part secreted into the medium, and in part associated with the cell wall of *S. pyogenes* (Hytonen *et al.*, 2001). SpeB is active on a large number of substrates, whose degradation either

facilitates spread of the bacterium through the human host, or induces the unregulated activation of the host immune system.

Spread through the human host is mediated either by direct cleavage of host tissue proteins such as vitronectin, fibrin and fibronectin (Elliott, 1945, Kapur *et al.*, 1993b) (Schmidtchen *et al.*, 2001), or the activation of host proteases such as the 66-kDa human endothelial cell matrix metalloprotease (Burns *et al.*, 1996). Furthermore, SpeB was shown to cleave human immunoglobulins (Collin & Olsen, 2001b, Collin & Olsen, 2001a). Modulation of the immune system is achieved through conversion of an IL-1β precursor into active IL-1β, which acts as an inflammatory cytokine (Kapur *et al.*, 1993a). SpeB also cleaves the monocytic cell urokinase receptor and releases an active fragment of the receptor from the cell surface (Wolf *et al.*, 1994). Additionally, SpeB releases biologically active kinins from their precursors (Herwald et al., 1996), and releases proapoptotic factors, which promote further tissue damage (Tamura *et al.*, 2004). All these properties make SpeB particularly important in the facilitation of tissue destruction in necrotizing fasciitis.

1.8.3 Streptolysin O (SLO)

SLO is a cytolysin, which can form pores in the host cell membrane, through which secreted factors can be translocated into the cells (Ghosh & Caparon, 2006). Through the use of this mechanism, streptococci can evade internalization into the lysosome, and thus promote their intracellular survival (Hakansson *et al.*, 2005). Internalization of streptococci may in turn contribute to long-term persistence of the bacteria, as well as to antibiotic resistance.

1.8.4 Streptokinase

Streptokinase is a 46-kDa protein with four compact domains (Damaschun *et al.*, 1992, Huang *et al.*, 1989, Malke, 1993). Streptokinase cleaves host plasminogen and releases active plasmin, a potent protease, which is part of the fibrinolytic system. Plasminogen is recruited to the bacterial surface by several cellular factors, and its activation by streptokinase allows the bacterium to dissolve fibrin cloths, which are a common mechanism used by the host to contain pathogens (Kuusela *et al.*, 1992, Lottenberg *et al.*, 1992, McArthur *et al.*, 2008).

2 Materials and Methods

2.1 Bacterial strains

The *E. coli* strains DH5α and BL21 were used for molecular cloning and recombinant protein expression respectively. The *S. pyogenes* M6 serotype strain D471 was from the Rockefeller University collection. SF370 is the *S. pyogenes* M1 serotype strain whose genome was sequenced (Ferretti et al., 2001).

2.2 Culture conditions

E. coli strains were grown in Luria-Bertani (LB) medium, and *S. pyogenes* were grown in Todd-Hewitt medium (Difco) supplemented with 1% yeast extract (Fisher) at 37°C. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; erythromycin, 200 μg/ml for *E. coli*, and 15 μg/ml for *S. pyogenes*; spectinomycin, 20 μg/ml for *E. coli*, and 120 μg/ml for *S. pyogenes*. When applicable, 0.05% trypsin was added to the media. When trypsin and pronase were added in concert to the medium, 0.35 mg/ml trypsin and 0.04 mg/ml pronase were used.

2.3 Reagents and antibodies

The 10B6 monoclonal antibody (Jones *et al.*, 1985) was used at a 1:30,000 dilution for Western blot, and 1:2000 for immunofluorescence. The 3B8 monoclonal

(Jones & Fischetti, 1988), was FITC-labeled according to manufacturer instructions (PIERCE) and used at 1:10. Affinity purified rabbit anti-GAPDH serum (Pancholi & Fischetti, 1992) was used at 1:3,000 for Western blots and 1:2,000 for immunofluorescence. Affinity purified mouse anti-sortase A antibodies were used at 1:50 for Western blotting and 1:10 for immunofluorescence. SfbI-specific rabbit serum was a generous gift from Susanne Talay, and was used at a 1:1000 dilution. Goat anti-mouse IgG, Rhodamine Red (Jackson ImmunoResearch), and Alexa Fluor 647 (Invitrogen) conjugates, were used at 1:500-1:1000. Goat anti-rabbit IgG, FITC conjugate (Sigma) was used at 1:2,000. WGA Marina Blue and Alexa Fluor 594 conjugates (Invitrogen) were used at 5μg/ml. Vancomycin BODIPY FL, and NAO (Invitrogen) were used at 1μg/ml, and 10μM respectively. PlyC was purified as described elsewhere (Nelson *et al.*, 2006), and a stock with a final concentration of 6000 U/ml was stored in aliquots at -80^{o} until use. All other reagents were purchased from Sigma unless otherwise noted.

2.4 DNA manipulation

Standard protocols were used for DNA manipulation and for *E. coli* transformation (Sambrook *et al.*, 1989). Plasmid DNA was isolated from *E. coli* using QiaQuick spin columns (Qiagen), or by alkaline lysis (Sambrook *et al.*, 1989). PCR amplification procedures were performed using Taq DNA polymerase (Invitrogen), Vent DNA polymerase (NEB), or Phusion DNA polymerase (NEB). Oligonucleotides were from Eurofins. Electroporation procedure for the transformation of *S. pyogenes* is described elsewhere (Perez-Casal *et al.*, 1991).

Table 2.1 Primers used in this work

Name	Sequence 5' to 3'
61_5_srtA_SalI	gtggtcgacatggtaaaaaaacaaaagcgtcg
62_3_srtA_NotI	gtagcggccgcctaggtagatacttggttataagaatgattaaaag
63_5_srtA_aa34_on_SalI	gaggtcgacaacaaacctatccgaaatacattaattgctc
64_5_srtAKOupst_SalI	cccgtcgacgaagaagaagatgttttgattaccttgtc
65_3_srtAKOupst_XhoI	cccctcgagtttttactccttattatgattaattatctttatttttg
66_5_srtAKOdown_NcoI	cccccatggatatatcttaaatataatatttaaaaagtaggaccatagagaaaatttatc
67_3srtAKOdown_SmaI	ccccccgggtacagcaggaaccactaataaaacc
71_3_srtA_BglII	gtaagatctctaggtagatacttggttataagaatgattaaaag
73_5_srtA_BamHI	gtgggatccatggtaaaaaaacaaaagcgtcg
74_5_srtA_BamHI_utr	gcgggatcctcgcacaaatgttgcaaatatttc
75_prGAPDH_F1	aattcttatttgcatttttttctgaaatagttatataatagttctgttgaaaggttgttacagatgactgtaagt
76_prGAPDH_F2	taatcttttcacaatagggtagggagcattccctctaaataatattcttttgattttcataaggaggaaatcactag
77_prGAPDH_R1	tcaacagaactattatataactatttcagaaaaaatgcaaataag
78_prGAPDH_R2	ctccctacctattgtgaaaagattaacttacagtcatctgtaacaacctt
79_prGAPDH_R3	gatcctagtgatttcctccttatgaaaatcaaaagaatattatttagagggaatg
89_5_Mp_UTR_EcoRI	cgcgaattcacagcctagccgcagaaactc
95_3_Mp_Npart_BglII	cgcagatctagtttccttcattggtgctttgttttg
97_5_Mp_Cpart_3C_H6_BglII	cgcagatctctggaagttctgttccaggggccccatcatcatcatcatcataagagacagttaccatcaacaggtgaaac
105_5_Mp_Cpart_3C_EGTSPL_BglII	gcagatctctggaagttctgttccaggggccccatcatcatcatcatcataagagacaggaaggtacatcaccattaacagctaacccattcttc
106_5_Mp_Cpart_3C_TEPGSL_BglII	gcagatctctggaagttctgttccaggggccccatcatcatcatcatcataagagacagacagaaccaggttcattaacagctaacccattcttc
135_5_new_MCS_plz-spec	aattccccaagcttcccagatctaaaccgcggaaacagctgaaaccatggaaagcatg
136_3_new_MCS_ plz-spec	ctttccatggtttcagctgtttccgcggtttagatctgggaagcttgggg
137_3_Mp_Cpart_SphI	cgcgcatgcttagttttcttctttgcgttttacaac
138_5_Mp_Cpart_SacII	tcaccgcggcagcccttactgttatgg
139_5_M_mid_HindIII	aaaaagctttagaagaagcaaacagcaaattag
140_3_M_LtoA_SacII	ctgccgcggtgaagaatgggttagctgtttcacctgttgatggtgcctgtctcttag
141_3_M_PtoA_SacII	ctgccgcggtgaagaatgggttagctgtttcacctgttgatgctaactgtctc
142_3_M_StoA_SacII	ctgccgcggtgaagaatgggttagctgtttcacctgttgctggtaactgtc
143_3_M_TtoA_SacII	ctgccgcggtgaagaatgggttagctgtttcacctgctgatggtaactg
144_3_M_GtoA_SacII	ctgccgcggtgaagaatgggttagctgtttcagctgttgatgg
145_3_M_EtoA_SacII	ctgccgcggtgaagaatgggttagctgttgcacctgttgatgg
146_3_M_EGTSPL_SacII	ctgccgcggtgaagaatgggttagctgttaatggtgatgtaccttcctgtctcttagtttccttcattggtgc
147_3_M_TEPGSL_SacII	ctgccgcggtgaagaatgggttagctgttaatgaacctggttctgtctgtctcttagtttccttcattggtgc
148_3_M_LPXTGE_SacII	ctgccgcggtgaagaatgggttag
151_3_M_PtoN_SacII	ctgccgcggtgaagaatgggttagctgtttcacctgttgagtttaactgtctc

Table 2.2 Plasmids used in this work

Plasmid	Template	5'-primer	3'-primer	Parent plasmid	CWSS	Reference
pFW15						(Podbielski *et al.*, 1996)
pLZ12-Spec						(Husmann *et al.*, 1995)
pJRS42.13						(Fischetti *et al.*, 1984)
pCM18						(Hansen *et al.*, 2001)
pAR86	D471	62_3-srtA_notI	63_5_srtA_aa34_on_salI	pET21a (modified)	-	This work
pAR94	D471	64_5_srtAKOupst_SalI	65_3_srtAKOupst_XhoI	pFW15	-	This work
pAR95	D471	66_5_srtAKOdown_NcoI	67_3srtAKOdown_SmaI	pAR94	-	This work
pAR93	SF370	64_5_srtAKOupst_SalI	65_3_srtAKOupst_XhoI	pFW15	-	This work
pAR96	SF370	66_5_srtAKOdown_NcoI	67_3srtAKOdown_SmaI	pAR93	-	This work
pAR102	-	GAPDH promoter primers 75-79	GAPDH promoter primers 75-79		-	This work
pAR107	D471	73_5_srtA_BamHI	71_3_srtA_BglII	pAR102	-	This work
pAR161	-	135_5_new_MCS_pLZ12-Spec	136_3_new_MCS_ pLZ12-Spec	pLZ12-Spec	-	This work
pAR163	pJRS42.13	89_5_Mp_UTR_EcoRI	95_3_Mp_Npart_BglII	pAR161	-	This work
pAR164	pJRS42.13	138_5_Mp_Cpart_SacII	137_3_Mp_Cpart_SphI	pAR163	-	This work
pAR166	pJRS42.13	97_5_Mp_Cpart_3C_H6_BglII	137_3_Mp_Cpart_SphI	pAR163	3C-H6-LPSTGE	This work
pAR169	pJRS42.13	105_5_Mp_Cpart_3C_EGTSPL_BglII	137_3_Mp_Cpart_SphI	pAR163	3C-H6-EGTSPL	This work
pAR170	pJRS42.13	106_5_Mp_Cpart_3C_TEPGSL_BglII	137_3_Mp_Cpart_SphI	pAR163	3C-H6-TEPGSL	This work
pAR178	pJRS42.13	139_5_M_mid_HindIII	140_3_M_LtoA_SacII	pAR164	APSTGE	This work
pAR179	pJRS42.13	139_5_M_mid_HindIII	141_3_M_PtoA_SacII	pAR164	LASTGE	This work
pAR180	pJRS42.13	139_5_M_mid_HindIII	142_3_M_StoA_SacII	pAR164	LPATGE	This work
pAR181	pJRS42.13	139_5_M_mid_HindIII	143_3_M_TtoA_SacII	pAR164	LPSAGE	This work
pAR182	pJRS42.13	139_5_M_mid_HindIII	144_3_M_GtoA_SacII	pAR164	LPSTAE	This work
pAR183	pJRS42.13	139_5_M_mid_HindIII	145_3_M_EtoA_SacII	pAR164	LPSTGA	This work
pAR184	pJRS42.13	139_5_M_mid_HindIII	146_3_M_EGTSPL_SacII	pAR164	EGTSPL	This work
pAR185	pJRS42.13	139_5_M_mid_HindIII	147_3_M_TEPGSL_SacII	pAR164	TEPGSL	This work
pAR186	pJRS42.13	139_5_M_mid_HindIII	148_3_M_LPSTGE_SacII	pAR164	LPSTGE	This work
pAR187	pJRS42.13	139_5_M_mid_HindIII	151_3_M_PtoN_SacII	pAR164	LNSTGE	This work

2.5 Cloning of sortase A from *S. pyogenes*

Soluble and catalytically active sortase is routinely produced as a hexahistidine-tagged recombinant protein, lacking its N-terminal transmembrane domain (Ton-That et al., 1999). To determine the boundaries of the *S. pyogenes* SrtA N-terminal transmembrane domain, we subjected the amino acid sequence of the protein to bioinformatic analysis using the DAS software (Cserzo *et al.*, 1997). The soluble portion of SrtA was PCR amplified from D471 genomic DNA using primers 62_3-srtA_notI and 63_5_srtA_aa34_on_salI, and a hexahistidine-tagged version of the protein (H6-SrtAΔN) was obtained by the insertion of this fragment between the SalI and NotI sites of a modified pET21a vector (a generous gift from Erec Stebbins) (Hsu *et al.*, 2008), yielding pAR86.

2.6 Purification of H6-SrtAΔN

An overnight culture of *E. coli* BL21/pAR86 was diluted 1:100 into 1L of LB medium containing ampicillin, and placed in an environmental shaker. Upon reaching OD_{600} 0.5, the expression of H6-SrtAΔN was induced with α-lactose at a final concentration of 0.2% for 4h. The cells were harvested and resuspended in 60ml MCAC buffer (30mM tris pH 7.4, 0.5M NaCl, 10% glycerol, 1mM DTT), homogenized, and sonicated briefly to shear DNA. Cell debris was removed by centrifugation, and the supernatant was filtered through a 0.22μm filter (Millipore). The cleared lysate was loaded on a NiNTA column equilibrated with MCAC buffer, followed by washes with MCAC containing 20mM imidazole, and elution with MCAC containing 100mM imidazole. The eluted fraction was concentrated using an

amicon ultrafiltration device, fitted with a 3k membrane, and applied to a G-100 gel filtration column, equilibrated with 30mM tris pH 7.5, 150mM NaCl, 1mM DTT. The final product is shown in figure 3.1A.

2.7 Production and affinity purification of antibodies specific for sortase A

For immunization of mice to produce antibodies, H6-SrtAΔN was further purified by separation on 16 10% SDS polyacrylamide gels. The gels were dyed with Gel-Code blue (Pierce), and the H6-SrtAΔN band was excised, and placed into a 3kD dialysis bag containing 15ml 50mM tris-glycine buffer pH 8.3, 0.04% SDS. The bag was then placed in a mini-protean transfer device (Bio-Rad) containing the same buffer. The protein was eluted from the gel by the application of 50V current for 8 hours, and the liquid fraction was transferred to a new dialysis bag, which was subsequently dialyzed against 100 volumes of PBS overnight. It was then concentrated to 2ml using a Centricon (Amicon), resulting in a final protein concentration of 1mg/ml, as determined by BCA assay.

Three BALB/C female mice (Jackson Laboratories) were each immunized with 300μg of purified H6-SrtAΔN in complete Freund's adjuvant, and boosted twice with 100μg H6-SrtAΔN in incomplete Freund's adjuvant, at one-month intervals. A week following the last injection, blood was obtained by heart puncture, and serum was separated by centrifugation.

For the affinity purification of sortase-specific antibodies, a NiNTA column was loaded with purified H6-SrtAΔN and equilibrated with TSA (10mM tris pH 8.0, 0.14M NaCl, 0.2% NaN_3). Serum from one of the mice was applied to the column,

and non-specific antibodies were removed by washes with TSA and 50mM tris pH 8.8, 0.5M NaCl. Sortase specific antibodies were eluted with 50mM diethanolamine pH 11.5, 0.15mM NaCl and the pH was adjusted to 7.5.

2.8 Creation of the sortase knockout (KO) derivates of D471 and SF370

The plasmid pFW15 was used for gene replacement as described by Podbielski et al. (Podbielski *et al.*, 1996b). For the deletion of *srtA* in D471 a 1kb DNA fragment upstream of the *srtA* gene was amplified from D471 genomic DNA using primers 64_5_srtAKOupst_SalI, 65_3_srtAKOupst_XhoI, and inserted between the SalI and XhoI sites of pFW15, yielding pAR94. Primers 66_5_srtAKOdown_NcoI and 67_3srtAKOdown_SmaI were used to amplify a 1kb region downstream of the srtA gene, and this fragment was inserted into the NcoI and SmaI sites of pAR94, yielding pAR95. Plasmid pAR96 for the deletion of *srtA* in SF370 was produced through the same procedure but using genomic DNA from the M1 strain SF370 as template. A 1kb DNA fragment upstream of the *srtA* gene was amplified from SF370 genomic DNA using primers 64_5_srtAKOupst_SalI, 65_3_srtAKOupst_XhoI, and inserted between the SalI and XhoI sites of pFW15, yielding pAR93. Primers 66_5_srtAKOdown_NcoI and 67_3srtAKOdown_SmaI were used to amplify a 1kb region downstream of the srtA gene from SF370 genomic DNA, and this fragment was inserted into the NcoI and SmaI sites of pAR93, yielding pAR96. pAR95 and pAR96 were transformed into D471 and SF370 respectively, and erythromycin resistant colonies were screened for a double crossover event by PCR, represented schematically in figure 3.2A. Primers 61_5_srtA_SalI and

62_3_srtA_NotI amplify the sortase gene (figure 3.2A, white arrows); absence of product reflects the deletion of the sortase gene (figure 3.2B). Primers 74_5_srtA_BamHI_utr and 67_3srtAKOdown_SmaI are located on regions flanking *srtA* on the genome (figure 3.2A, black arrows); replacement of the gene with an erythromycin cassette results in an increase of 300bp in the size of PCR product amplified by these primers (figure 3.2C). The resulting Δ*srtA* strains were termed AR01 (D471Δ*srtA*) and AR02 (SF370Δ*srtA*).

2.9 Construction of pAR107, a sortase complementation plasmid

A 150bp region upstream to the gene encoding GAPDH was constructed by the ligation of primers 75_prGAPDH_F1, 76_prGAPDH_F2, 77_prGAPDH_R1, 78_prGAPDH_R2, and 79_prGAPDH_R3. This DNA fragment, which harbors promoter activity, was inserted into the EcoRI and BamHI sites of pLZ12-Spec (Husmann *et al.*, 1995) yielding pAR102. The sortase reading frame was amplified using primers 73_5_srtA_BamHI and 71_3_srtA_BglII, and inserted into the BamHI and BglII sites of pAR102, yielding pAR107. This plasmid was transformed into both *srtA* KO strains AR01 and AR02.

2.10 Production of M-protein constructs containing defined mutations in the LPXTG motif (pAR178-pAR187)

As a first step, part of the multi-cloning site (MCS) of pLZ12-Spec was replaced with a new MCS. This was done by heating the complementary primers 135_5_new_MCS_pLZ-Spec and 136_3_new_MCS_pLZ12-Spec to 70^{o}, and then

slowly cooling them to room temperature allowing them to anneal. The resulting double stranded DNA fragment containing the new MCS EcoRI-HinDIII-BglII-SacII-PvuII-NcoI-SphI was inserted into the EcoRI and SphI sites of pLZ12-Spec, yielding pAR161.

Into pAR161 the M-protein gene was inserted in 3 fragments. First, a PCR product containing the M-protein UTR, as well as a large N-terminal fragment was amplified using primers 89_5_Mp_UTR_EcoRI and 95_3_Mp_Npart_BglII, and using plasmid pJRS42.13 (Fischetti *et al.*, 1984) as template. This fragment was inserted into the EcoRI and BglII sites of pAR161, yielding pAR163. Next a small C-terminal fragment was amplified using primers 138_5_Mp_Cpart_SacII and 137_3_Mp_Cpart_SphI, and inserted into the SacII and SphI sites of pAR163, yielding pAR164. The SacII site was placed in such a manner that the resulting amino acid sequence would not be altered by the introduction of this site. Into the HinDIII (found naturally in the sequence of M-protein) and SacII sites of pAR164, 10 different PCR products were inserted, each carrying a defined mutation in the LPSTGE sequence, yielding pAR178-pAR187. These are elaborated in table 2.2.

2.11 Production of M-protein 3C-hexahistidine constructs

The C-terminal part of M-protein was amplified as a PCR product containing a LPSTGE motif (for the creation of pAR166), a reversed EGTSPL motif (for the creation of pAR169), or a scrambled TEPGSL motif (for the creation of pAR170), and inserted into pAR163 (Tables 2.1 and 2.2). The 3C-cleavage site, as well as the

hexahistidine tag was encoded on the upstream primer of these PCR products (Tables 2.1 and 2.2).

2.12 Light Microscopy

Light microscopy procedures were adjusted for *S. pyogenes* from Levin et al. (Levin, 2002). Paraformaldehyde and glutaraldehyde were added to the culture medium to final concentrations of 2.6% and 0.012% respectively, and phosphate buffer pH 7.4 was added to 30mM. The cells were incubated for 15 minutes at room temperature, and 30 minutes on ice, washed with PBS, and attached to poly-lysine coated cover slips. The slides were washed with PBS, dipped in ice-cold methanol for 10 to 60 seconds, and dipped in PBS. The cells were then treated with 3U/ml PlyC in PBS for 10 minutes at room temperature, washed, and blocked for 15 minutes with normal goat serum (Zymed) supplemented with 1% gelatin from cold-water fish skin (Sigma). Antibodies and dyes were diluted in PBS containing 2% BSA and 1% gelatin, and incubated with the cells in a moist chamber for one hour at room temperature. Between incubation steps the cells were washed thoroughly with PBS. To reduce bleaching of the fluorochromes, the slides were mounted in 50% glycerol and 0.1% p-phenylenediamine in PBS pH 8. Images were obtained using a DeltaVision image restoration microscope (Applied Precision / Olympus) equipped with CoolSnap QE cooled CCD camera (Photometrics). An Olympus 100X/1.40 NA, UPLS Apo oil immersion objective was used in conjunction with a 1.5X optovar. Z-stacks were taken at 0.1μm intervals. Images were deconvolved using the SoftWoRx software (Applied Precision / DeltaVision), and corrected for chromatic aberrations.

ImageJ (http://rsb.info.nih.gov/ij/) was used to analyze the fluorescence distribution profiles, and raw data was transferred to Microsoft Excel for the creation of average distribution plots. Adobe photoshop version 7 was used for the preparation of the figures.

2.13 Scanning Electron Microscopy

For morphology studies, slides were prepared as described for light microscopy. Following PlyC treatment, the cells were fixed in 2.5% glutaraldehyde at 4^{o}C overnight. The cells were then treated with 1% osmium tetroxide in 0.1M cacodylate buffer pH 7.4 for an hour, dehydrated using graded ethanol solutions, and critical point dried. The slides were coated with a thin gold-palladium layer using a Desk IV coater (Denton Vacuum). Images were obtained using a LEO 1550 scanning electron microscope, with field-emission electron gun.

2.14 Transmission Electron Microscopy

For transmission electron microscopy cells were fixed for 24 hours at 4^{o}C in 0.1M cacodylate buffer containing 2.5% glutaraldehyde. The cells were centrifuged into a pellet and washed thoroughly with 0.1 M cacodylate buffer (pH 7.4). After post-fixation treatment with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at 4°C, and 1 hour of *en bloc* staining with 0.5 % uranyl acetate, the pellets were dehydrated in graded ethanol solutions, and embedded in Spurr's low-viscosity resin (Electron Microscopy Sciences). Blocks were cut with a diamond knife on a Leica UltraCut E Microtome. Ultra-thin (~70nm) sections were collected

on coated 200mesh grids and stained with Uranium and Lead. Grids were viewed with a Tecnai Spirit BT Transmission Electron Microscope (From FEI) at 80 KV, and pictures were taken with Gatan 895 ULTRASCAN Digital Camera.

2.15 Fractionation of *S. pyogenes* cells – sup, wall, spheroplasts

Overnight *S. pyogenes* cultures were diluted 1:100 into 5ml TH+Y supplemented with spectinomycin when appropriate. Cells were grown to OD_{600} 0.5, at which time 1ml was harvested by centrifugation. The cell pellet was washed with 1ml PBS/30% raffinose, resuspended in 200µl of the same buffer containing 300U/ml PlyC (Nelson *et al.*, 2006), and incubated for 15 minutes at room temperature. The resulting spheroplasts were harvested by centrifugation, washed once with PBS/30% raffinose, resuspended in 60µl Laemmli buffer, and sonicated briefly ("Spheroplasts" fraction). The culture supernatant ("supernatant" fraction) and the PlyC digestion supernatant ("Wall" fraction) were supplemented with 5% trichloroacetic acid (TCA), and incubated on ice for an hour. Protein precipitates were collected by a 10 minutes centrifugation at 20800g in a cooled Eppendorf 5417R centrifuge, incubated with 1ml acetone for an hour with agitation, centrifuged, and resuspended in 60µl Laemmli buffer.

2.16 Fractionation of *S. pyogenes* cells – SDS boiling protocol

This protocol is used to distinguish between proteins that are covalently linked to the bacterial cell wall, and proteins that are associated with, but are not covalently linked to the cell wall. 1ml of log phase culture was harvested and washed with

30mM tris pH6.3. The bacterial pellet was suspended in 50µl 2% SDS, boiled for 10 minutes and centrifuged. The supernatant, containing non-covalently bound proteins, was supplemented with 12µl 5X SDS loading buffer. The cell pellet was washed with 200µl deionized water and suspended in 50µl 30mM tris pH6.3 containing 300U/ml PlyC for 15 minutes. The reaction was quenched with the addition of 12µl 5X SDS loading buffer. This fraction contains proteins covalently attached to the cell wall. A washed cell pellet similar to the starting material (not boiled in SDS) was treated with PlyC as described above. This fraction represents the total protein in the cell (both covalently, and non-covalently linked).

2.17 Western blot analysis

Samples were boiled for 10 minutes and separated by electrophoresis on a 10% SDS polyacrylamide gel (Laemmli, 1970). Blotting to polyvinylidene difluoride membranes was according to Matsudaira (Matsudaira, 1987). PBS supplemented with 5% non-fat dry milk, and 0.1% Tween 20 (PBS-DT) was used to block the membranes as well as for antibodies and HRP-conjugates dilution. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (PIERCE).

2.18 Semi-quantitative dot-blot analysis

One milliliter of cell culture was harvested and kept frozen until all samples were ready; the supernatant was kept separately. Each cell pellet was resuspended in 500µl 30mM tris pH 6.3 containing 9U/ml PlyC and incubated at room temperature

for 30 minutes. Serial dilutions were prepared, using 25µl of the lysate, or 50µl of supernatant (equivalent amounts of starting material) for the first well. The diluted samples were applied to a nitrocellulose membrane using a dot-blot vacuum apparatus. The blot was dried briefly and blocked with PBS-DT for 2 hours at room temperature with shaking. The blots were washed with TBS-T and incubated overnight in 10ml PBS-DT containing the appropriate dilution of primary antibody at 4^{o}, with shaking. The blots were washed with TBS-T, and incubated with a horseradish peroxidase conjugate diluted in PBS-DT for 2 hours. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (PIERCE).

2.19 Measurement of LPXTG cleavage activity

LPXTGase and sortase activity was measured through the release rate of radioactive material from bead-bound ^{125}I-labeled KRQLPSTGETANPFY peptide (the N-terminal of the peptide is bead-bound while the tyrosine is ^{125}I-labeled) as previously described (Lee et al., 2002). Briefly, LPXTGase or sortase were incubated with 10µl peptide-conjugated beads in 20mM HEPES, 5mM $CaCl_2$ pH 7.6 (sortase) or 30mM tris 0.1% Brij 35 pH7.6 (LPXTGase), at a final reaction volume of 50µl. The samples were shaken vigorously for an hour at 37^{o}, at which point 100µl of deionized water were added, the samples were vortexed and centrifuged, and 100µl of the supernatant were removed for analysis in a γ-counter.

2.20 Isolation of RNA from *S. pyogenes*

All the procedures described herein were conducted using RNAse free reagents. Mini-centrifuge spins were done at 12000 RCF, 4°, for 10 minutes. Fifteen milliliters of log phase or five milliliter of stationary phase cells were harvested and suspended in 100µl TE 0.1X (1mM tris, 0.1mM EDTA, pH 8.0), and immediately flash frozen in an ethanol / dry ice bath. Each sample was thawed separately in a 55° water bath, and as soon as the sample thawed and reached approximately room temperature, 7µl of concentrated PlyC (6000U/ml) were added. As soon as the sample cleared (approximately one minute) 125µl homogenization solution (4M guanidinium thiocyanate, 25mM Na_3-citrate, 0.5% sodium sarkosyl, 0.1M β-mercaptoethanol, pH 7.0), and 400ul phenol were added and vortexed. After all the samples were processed in this fashion 500µl chloroform and 150µl sodium acetate 2M were added, and the samples were vortexed vigorously and left on ice for 5 minutes. The samples were then centrifuged and the upper phase was transferred to a new tube. 500µl chloroform were added and the samples were vortexed and left at room temperature for five minutes, centrifuged, and the upper phase was transferred to a new tube. An equal volume of isopropanol was added, the samples were vortexed, and left at room temperature for 15 minutes. The samples were then centrifuged again and the RNA pellet was washed with 70% ethanol for a prolonged period (typically between an hour and overnight), centrifuged, and resuspended in 30µl TE 0.1X.

2.21 Northern blot

Electrophoresis was carried out in RNA running buffer (20mM MOPS, 5mM Na-acetate, 0.1mM EDTA), using 1.2% agarose gel (1.2% agarose, 1X RNA running buffer, 2.2M formaldehyde). Samples were adjusted to 10μg in 5μl, to which 20μl denaturation buffer (prepared through the mixing of 80μl 10X RNA running buffer, 140μl formaldehyde 37%, and 400μl formamide) were added. The samples were heated to 65^{o}C in a water bath for 15 minutes, at which point 5μl loading buffer was added, and the samples were loaded on the gel. Following electrophoresis, the gel was rinsed five times in deionized water for a total of 20 minutes, soaked for 15 minutes in RNA processing buffer (50mM NaOH, 10mM NaCl, 1.5μg/ml ethidium bromide), and neutralized in 0.5M tris, 1.5M NaCl pH 7.4 for 30 min, at which point a UV image of the gel was taken. The gel was then soaked in 10X SSC (150mM NaCl, 15mM Na_3-Citrate pH 7.0, for 1X) for 30 minutes and transferred to a nylon membrane overnight using 10X SSC as transfer buffer. The RNA was UV cross-linked to the membrane using STRATALINK, and the M-protein transcript was detected using ECL direct nucleic acid labeling and detection system (Amersham Biosciences) according to the manufacturer instruction. A PCR product encompassing the M-protein gene was used as probe.

3 Localization of Sortase A in *S. pyogenes*

Due to importance of surface proteins for the survival of bacteria in the host, much research has been directed at understanding the sorting reaction. While much is known about the biochemical aspects of this reaction, the spatial regulation of protein sorting is far less well understood. This chapter discusses the development of an immunofluorescence method suitable for the localization of sub-surface antigens in *S. pyogenes*, and its utilization for the detection of sortase, and other cellular components related to the sorting reaction.

3.1 The catalytic activity of *S. pyogenes* sortase A is enhanced in the presence of hydroxylamine, and L-ala – L-ala

Sortase A was cloned from the genome of the *S. pyogenes* M6 strain D471 as a fusion protein, in which the N-terminal transmembrane domain has been replaced with a hexahistidine tag (H6-SrtAΔN), as described in the methods section. The protein was purified as described in the methods section, and the final product is presented in figure 3.1A.

The catalytic activity of *S. aureus* sortase A is enhanced in the presence of a nucleophile, such as hydroxylamine and the staphylococcal cross bridge pentaglycine (Ton-That *et al.*, 2000, Ton-That et al., 1999). To test the catalytic activity of recombinant *S. pyogenes* sortase (H6-SrtAΔN) we used the method of Lee et al. whereby cleavage activity is measured by the release of radioactive material from bead-bound ^{125}I-labeled KRQLPSTGETANPFY peptide, which represent a fragment of the M-protein C-terminal anchor domain (Lee *et al.*, 2002).

A fixed amount of H6-SrtAΔN was incubated with ^{125}I-labeled bead-bound peptide containing activity of 150,000 CPM for one hour at 37^{o}C with vigorous shaking. The reaction mixture contained 20mM HEPES, 5mM $CaCl_2$ pH 7.6, in a total volume of 50μl (black bars). Addition of 400mM of the streptococcal cross-bridge L-ala – L-ala, or 50mM hydroxylamine significantly increased the cleavage activity of sortase, in agreement with the result observed with the staphylococcal enzyme. Addition of L-ala – L-ala or hydroxylamine in the absence of sortase (white bars) had no effect. The experiment was done in triplicates.

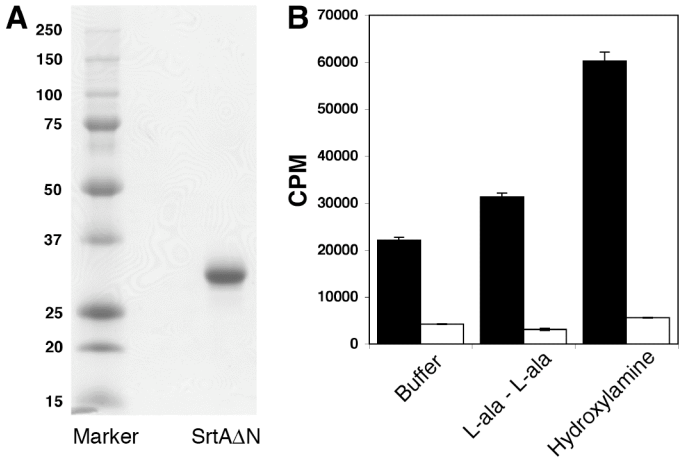


Figure 3.1 Purified H6-SrtAΔN is active in the cleavage of LPXTG peptide

(A) Purified H6-SrtAΔN (right lane) was separated by 10% SDS-PAGE, and dyed with Coommassie brilliant blue. Molecular marker was run on the left lane. (B) Cleavage of bead-bound ^{125}I-labeled KRQLPSTGETANPFY peptide, in the presence (black bars) or absence (white bars) of sortase. L-ala – L-ala and hydroxylamine were added to the appropriate reactions at 400mM and 50mM respectively. The experiment was done in triplicates; error bars represent one standard deviation.

3.2 The distribution of M-protein, and sortase in D471, SrtA-mutant AR01, and complemented AR01+pAR107

Sortase A has been deleted from the genomes of *S. pyogenes* strains D471 and SF370 by replacing the sortase gene with an erythromycin resistance cassette, as described in the methods section. The D471 *srtA* deletion mutant was termed AR01, and the SF370 *srtA* deletion mutant was termed AR02. Deletion of the sortase A from both genomes was validated by PCR (Fig. 3.2), as described in more detail in the methods section. A plasmid expressing the *srtA* gene under the control of the GAPDH promoter was produced as described in the methods section, and termed pAR107. This plasmid was transformed into AR01 and AR02. The production and affinity purification of mouse anti-SrtA polyclonal serum is described in the methods section.

Deletion of the sortase gene has been shown to result in the missorting of surface proteins (Mazmanian et al., 2000, Barnett & Scott, 2002). We therefore tested the distribution of M-protein in the sortase knockout and complemented strains. While the majority of M-protein was found in the cell wall fraction of WT D471, displaying a characteristic banding pattern resulting from the attachment to differently sized cell wall fragments, the sortase mutant AR01 missorted M-protein to the supernatant, wall, and spheroplast fractions. Complementation of AR01 with the plasmid pAR107, expressing sortase, restored the wild type phenotype (Fig. 3.3A).

We then tested the specificity of the anti-sortase antibodies by Western blot (Fig. 3.3B). These antibodies reacted with a band of the correct size in the wild type strain D471, but not with the sortase mutant AR01. Recombinant sortase expressed from a plasmid restored reactivity to the antibodies. An additional faint band, present

in both D471 and plasmid-complemented AR01, is likely to be a sortase cleavage product, as it is completely absent from AR01.

The amount of sortase displayed by AR01+pAR107 appears to be much smaller than that expressed by the parent strain (Fig 3.3B). To quantify this difference we grew D471, AR01, and AR01+pAR107 to OD_{600} 0.5, at which time 1ml culture was harvested and lysed with PlyC. The resulting lysates were serially diluted and used for semi-quantitative dot blot, as described in the method section. AR01+pAR107 expressed about 20 times less sortase than is present in D471. Surprisingly, this small amount was sufficient for the efficient anchoring of both M-protein (Fig. 3.3A) and SfbI (Fig 4.1). The reason for this apparent excess of sortase in the WT strain is not clear. It is possible that more sortase is required *in vivo* to ensure efficient protein sorting, due to sub-optimal growth conditions in the human host.

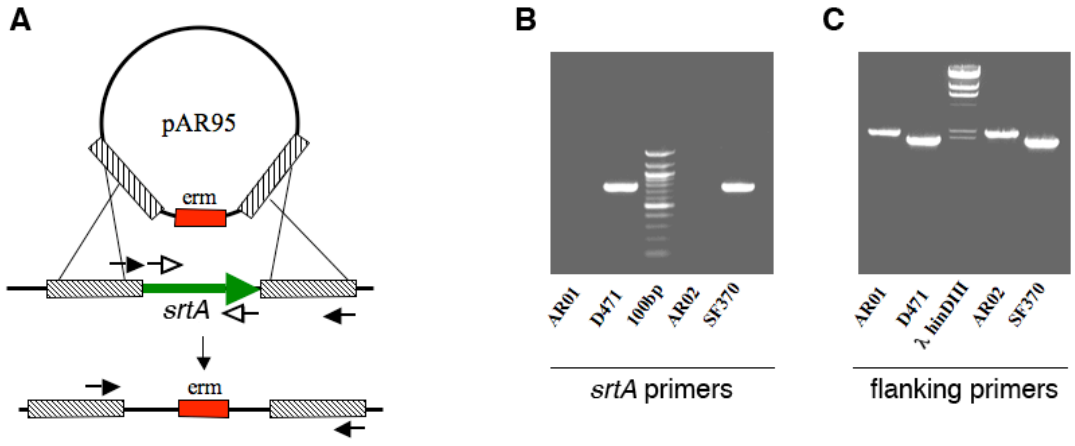


Figure 3.2 Deletion of the *srtA* gene from the genomes of D471 and SF370

(A) Schematic representation of construct used for deletion of *srtA* and primers used for screening; *srtA* primers (61_5_srtA_SalI, and 62_3_srtA_NotI) are depicted with white arrows, and flanking primers (74_5_srtA_BamHI_utr, and 67_3srtAKOdown_SmaI) are depicted with black arrows. Genomic DNA form WT and *srtA* KO derivates AR01 and AR02 was tested by PCR. Deletion of the *srtA* gene results in the absence of a PCR product when using *srtA* primers (B), and an upward band shift when using flanking primers (C).

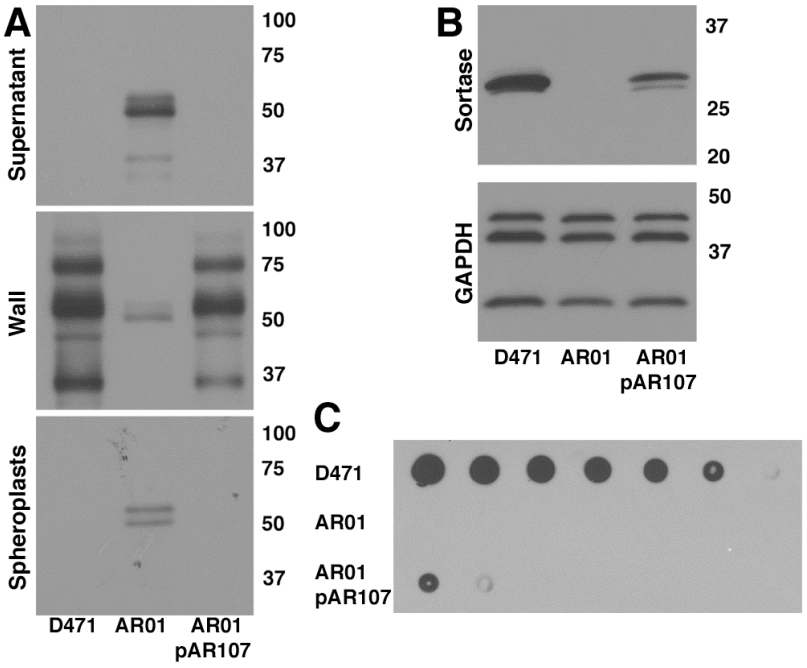


Figure 3.3 Distribution of M-protein and sortase in D471, AR01, and AR01+pAR107

(A) Wild type D471, sortase mutant AR01, and reconstituted AR01+pAR107, were grown in TH+Y to OD_{600} 0.5, and fractionated into supernatant, wall, and spheroplasts. The distribution of M-protein in the different fractions was assessed by Western blot. (B) Cells grown in the same manner were harvested, washed, and lysed. The presence of sortase and cytoplasmic GAPDH (loading control) was assessed by Western blot. (C) D471, AR01, and AR01+pAR107 were grown to OD_{600} 0.5, at which time 1ml culture was harvested and lysed with PlyC. The resulting lysates were serially diluted and used for semi-quantitative dot blot using SrtA-specific antibodies, as described in the method section.

3.3 A method for immunofluorescence in *S. pyogenes*

Our current knowledge regarding the spatial organization of protein sorting in *S. pyogenes* is mainly derived from the study of anchored surface proteins, while the actual distribution of sortase remains unknown. Localization of this protein by immunofluorescence has so far been hindered by the fact that lysozyme, a muralytic enzyme commonly used to permeabilize bacterial cell walls to antibodies, has only a marginal effect on *S. pyogenes* (Krause & McCarty, 1961, Gallis *et al.*, 1976). We therefore sought to develop an alternative method for the permeabilization of the streptococcal cell wall, using the phage lysin PlyC (Fischetti *et al.*, 1971, Nelson et al., 2006) to overcome this problem. Phage lysins are cell wall hydrolyses, produced during the lytic cycle of the phage, to facilitate the release of progeny phage from the bacterial host. Since treatment of Gram-positive bacteria with purified lysins efficiently lyses and destroys them, many such enzymes have been studied as possible treatment for these pathogens (Fischetti, 2005).

In contrast to the use of PlyC to destroy the streptococcal pathogen, we sought to develop a method, in which PlyC could by utilized to permeabilize the streptococcal cells without causing adverse morphological effects, and would thus allow the study of membranal and cytoplasmic antigens by immunofluorescence. *S. pyogenes* cells were first fixed using paraformaldehyde/glutaraldehyde, treated with methanol, and then permeabilized with a low dose of PlyC. Paraformaldehyde and glutaraldehyde are chemical cross-linkers, which prevent the movement and dissociation of proteins and other cellular molecules in subsequent steps. Methanol treatment permeabilizes the membrane and thus equalizes the osmotic pressure within

the cell and the surrounding environment. In the absence of such treatment the higher osmotic pressure of the cell induces membrane bulging through the PlyC-generated holes in the cell wall, and disrupt the cellular morphology.

Wild type D471 cells treated in this manner display only minor morphological alterations compared to untreated cells (Fig. 3.4, compare A, untreated and B, treated). Furthermore, the application of 20X concentrated PlyC, which leads to visible perforation of the cell wall (not used for immunofluorescence), does not result in cell rupture (Fig. 3.4C). Close examination of the perforation pattern displayed by these cells reveals that PlyC is active throughout the cell wall, with somewhat increased activity at the septum, as was previously demonstrated by the binding pattern of fluorescent PlyC (Nelson et al., 2006).

We observed that M-protein, a fibrous coiled coil molecule extending 60 nm from the cell surface (Fischetti, 1989), and readily seen as irregular structures on the wall of WT D471 (Fig. 3.4A), is pivotal in preserving cell wall integrity following PlyC treatment. Even mild PlyC treatment of the isogenic M-protein knockout strain JRS75 causes the removal of the cell wall and the creation of spheroplasts (Fig. 3.4, compare D, untreated and E, treated). Furthermore, removal of M-protein from D471 by trypsin digestion similarly predisposes the cell wall to removal by PlyC, and formation of spheroplasts (Fig. 3.4F). In addition, PlyC treatment of the sortase mutant, AR01, which does not anchor M-protein to the cell wall (Fig. 3.3A), leads to a similar phenotype (Fig. 3.4G), while complementation of AR01 with pAR107 restores near-WT phenotype (Fig. 3.4H). We therefore propose that M-protein may be forming a cross-linked mesh around the cell following fixation, and that this mesh

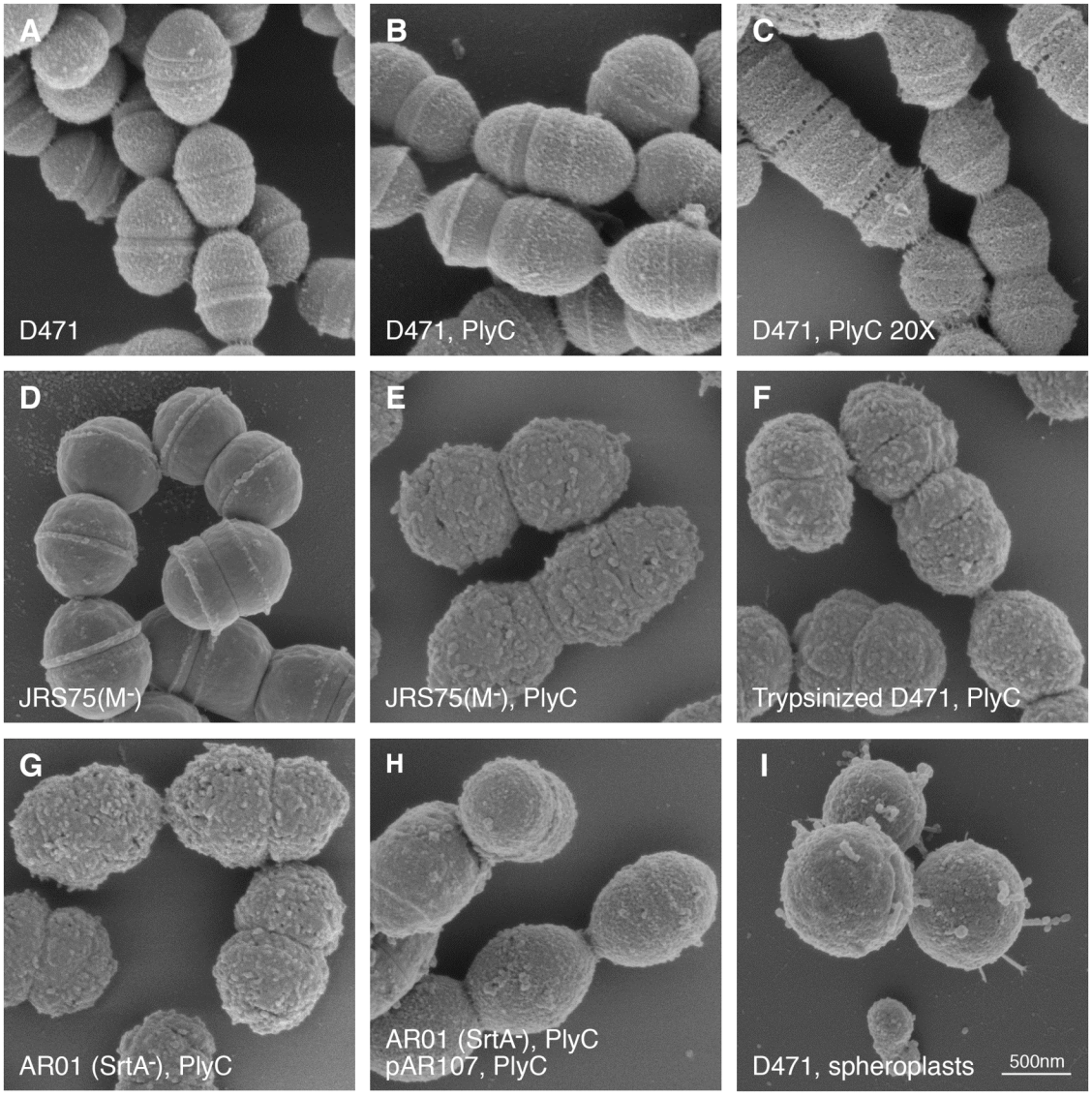
preserves the gross overall integrity of the cell wall following gentle PlyC treatment, despite the partial degradation of the peptidoglycan.

Spheroplasts produced when fixed M-negative cells are treated with PlyC preserve their correct cellular dimensions and chain orientation. They are therefore much better candidates for study by immunofluorescence than spheroplasts produced through traditional methods, where PlyC is applied to unfixed cells suspended in hypertonic solution, which appear swollen and do not remain in chains. (Figure 3.4, compare I – traditional method, to E and F – new procedure).

Figure 3.4 Morphology of *S. pyogenes* following PlyC treatment

Fixed cells were attached to glass slides, dipped in methanol, and washed with PBS. Unless otherwise noted, the cells were treated with 3U/ml PlyC in PBS for 10 minutes at room temperature, before processing for scanning electron microscopy examination. (A) D471 untreated, (B) D471, PlyC treated. (C) D471, 20X PlyC treated (60U/ml). (D) JRS75 untreated. (E) JRS75, PlyC treated. (F) Trypsinized D471, PlyC treated. (G) AR01, PlyC treated. (H) AR01+pAR107, PlyC treated. (I) Spheroplasts produced by suspending unfixed D471 in PBS 30% raffinose containing 3U/ml PlyC.

Figure 3.4


A
D471
B
D471, PlyC
C
D471, PlyC 20X
D
JRS75(M-)
E
JRS75(M-), PlyC
F
Trypsinized D471, PlyC
G
AR01 (SrtA-), PlyC
H
AR01 (SrtA-), PlyC
pAR107, PlyC
I
D471, spheroplasts
500nm

3.4 Localization of sortase in *S. pyogenes*

The application of mild PlyC treatment allowed us to study the localization of sortase A in *S. pyogenes* using deconvolution fluorescence microscopy. The images in figure 3.5 are presented as serial Z stack captures, and represent a typical distribution of sortase in a short chain of streptococcal cells. Sortase was found to localize to a number of foci in D471 cells (Fig. 3.5A), while the sortase mutant AR01 did not react with the antibodies (Fig. 3.5C). Sortase foci were predominantly associated with the division septum, but were not always strictly confined to the division plane. Sortase foci could also be found at the equatorial rings (discussed below), and to a lesser extent, at the poles. In these experiments, wheat germ agglutinin (WGA) was used to visualize the cell wall peptidoglycan and carbohydrate, and GAPDH was used as a cytoplasmic marker.

Cytoplasmic GAPDH labeling was used to rule out the possibility that sortase localization in foci results from incomplete permeabilization of the cell wall. GAPDH is a glycolytic enzyme, which has also been found on the surface of several *S. pyogenes* strains, where it plays a role in binding various host factors (Pancholi & Fischetti, 1992). We found that log phase D471 cells however, expresses very little surface GAPDH in comparison to the vast cytoplasmic pool (not shown). This fact enabled us to use it as a cytoplasmic marker, applying labeling conditions under which surface GAPDH fluorescence is negligible. Effective labeling of cytoplasmic GAPDH demonstrates that antibodies have free access into the cell. In addition, sortase distribution in the M-protein negative strain JRS75, which loses its peripheral cell-wall altogether during PlyC treatment (Fig. 3.4E), is similarly confined to foci

(Fig. 3.5B). Taken together, these results confirm that uneven permeabilization of the cell wall could not account for sortase localization pattern.

While the peripheral cell wall is removed from the M-protein negative JRS75 during PlyC treatment, cell wall labeling with WGA revealed that a small amount of wall material was trapped at the septal regions (Fig. 3.5B, green). This produced a labeling pattern that ranged from faint rings at early stages of the cell division, to strong rings and disks at later stages. A similar WGA labeling pattern was observed in the sortase mutant AR01 (Fig. 3.5C), which does not anchor M-protein to the cell wall (Fig. 3.3A). Our observation that the M-protein negative JRS75 is labeled with the anti-sortase antibodies at least as well as the WT strain D471, suggests that that removal of the cell wall does not account for AR01's lack of reactivity with these antibodies.

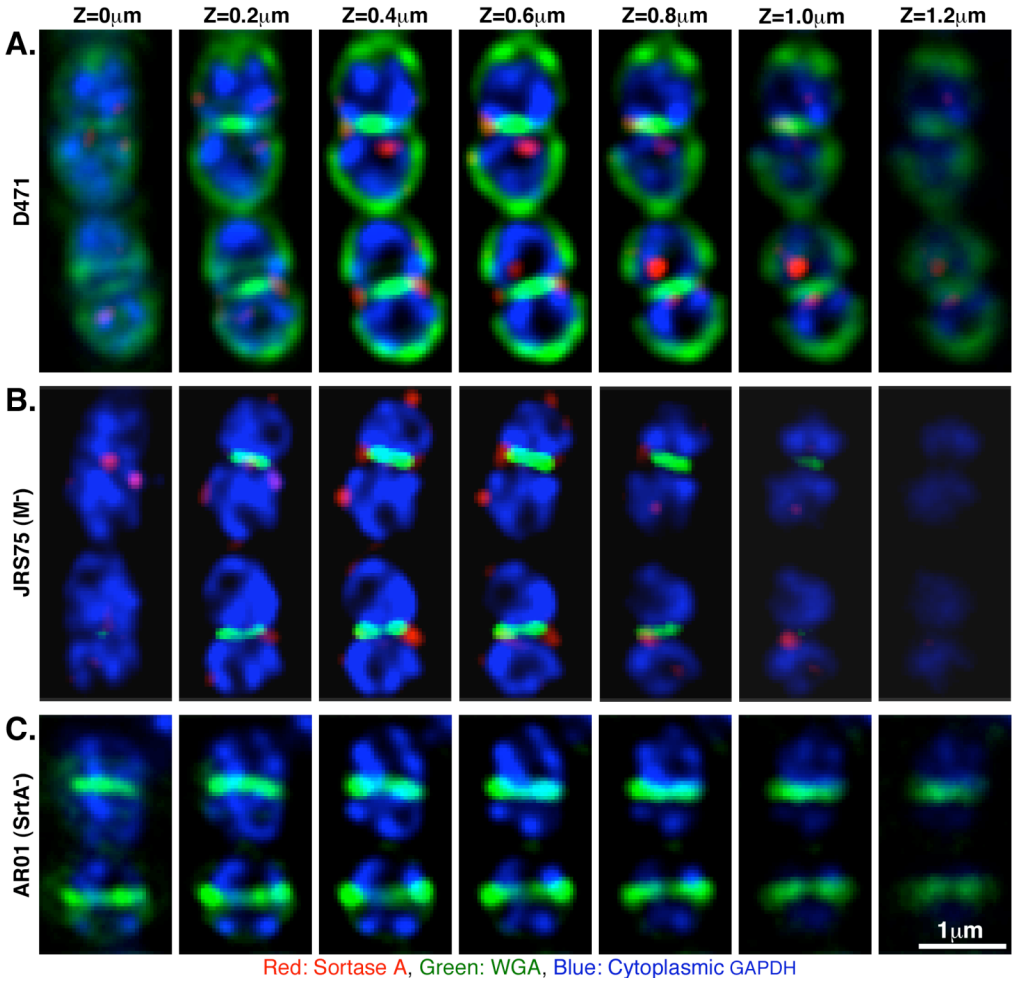


Figure 3.5 Sortase A localizes to distinct foci in *S. pyogenes* cells

D471 (A), JRS75 (B), and AR01 (C) were fixed and permeabilized with PlyC as described in the methods section. The cells were labeled for sortase (red) and cytoplasmic GAPDH (blue) using respective antibodies. Cell wall material was labeled with WGA (green). The data is presented as sequential Z-stacks captures with 0.2μm intervals. To better visualize septal wall material, the WGA channel (green) of panels (B-C) is enhanced as compared to (A).

3.5 Uneven GAPDH cytoplasmic labeling is the result of exclusion from the nucleoid

In our localization experiments we noticed that in many cases GAPDH labeling, which we used as a cytoplasmic marker, was excluded from a large portion of the cytoplasm. To make sure that this is not the result of incomplete permeabilization of the cell to antibodies, we transformed pCM18 (Hansen *et al.*, 2001), a plasmid constitutively expressing soluble cytoplasmic GFP, into D471. We then fixed and permeabilized log phase cells expressing this construct and labeled them using GAPDH-specific antibodies, and DAPI. The results presented in figure 3.6 demonstrate that both GAPDH (red) and GFP (green) were excluded from the nucleoid (Blue), indicating that exclusion of cytoplasmic proteins from the nucleoid is a general feature of *S. pyogenes*, and not the result of a labeling artifact.

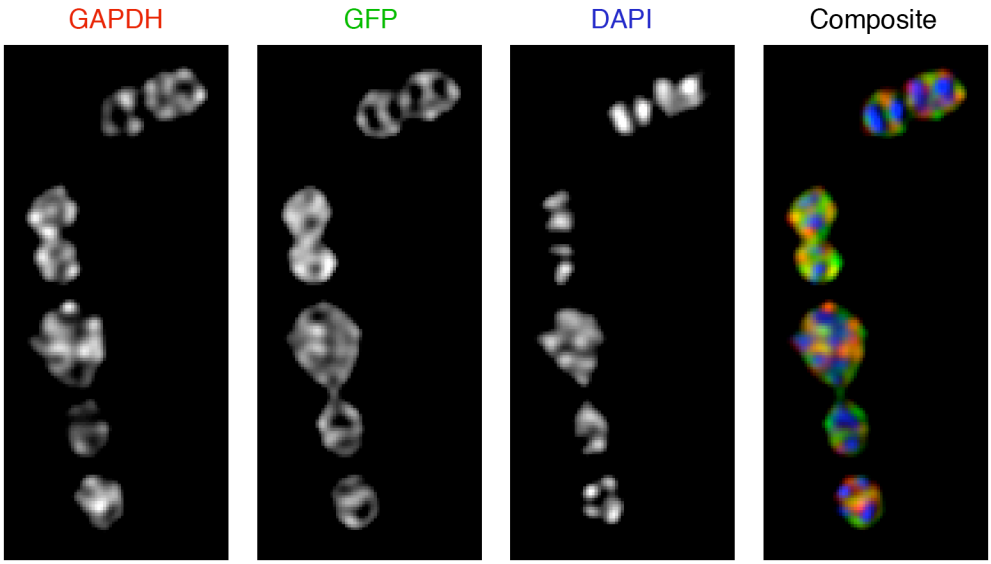


Figure 3.6 Uneven GAPDH cytoplasmic labeling is the result of exclusion from the nucleoid

Log phase D471 cells expressing soluble GFP from the plasmid pCM18 (green) were fixed, permeabilized and labeled for GAPDH (red) using specific antibodies, and DNA using DAPI (blue).

3.6 Sortase foci are associated with the membrane

Sortase A has an N-terminal membrane anchor, and is therefore predicted to be membrane localized. To examine sortase localization in relations to the membrane, we labeled D471 cells with sortase-specific antibodies, the membrane dye nonyl acridine orange (NAO), and the DNA stain DAPI, and determined the distribution of sortase in the middle section of the cells, as the image resolution over the XY plane is better than that of the Z-axis (Fig. 3.7A-G). Sortase foci consistently localized to the membrane and no sortase labeling was observed in the cytoplasm. Control cells (without sortase specific antibodies) showed no sortase labeling (Fig. 3.7H). It is important to note that while NAO labeling shows a membranal micro-domain enriched for anionic lipids in untreated *S. pyogenes* (Rosch et al., 2007), this fine lipid structure is disrupted by the methanol treatment required for membrane permeabilization.

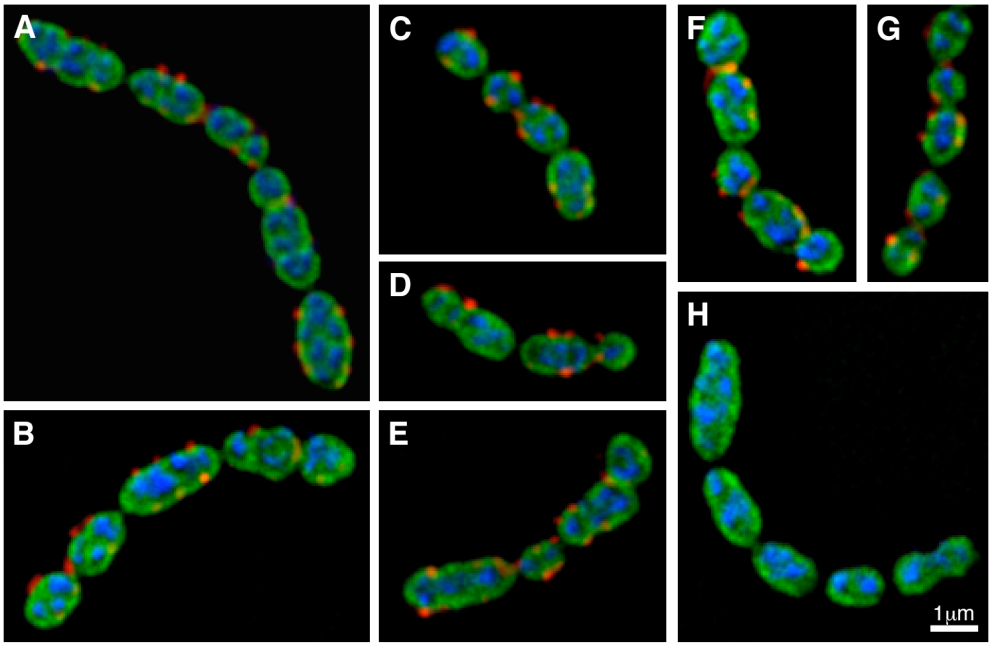


Figure 3.7 Sortase foci are localized to the membrane

(A-G) Log phase D471 cells were fixed, permeabilized, and stained for sortase (Alexa Fluor 647, red), membrane (NAO, green), and DNA (DAPI, blue). (H) Control cells not treated with primary sortase antibodies.

3.7 Localization of sortase as a function of cell-cycle stage

Streptococcal cells grow in chains and divide asynchronously within the chain, a fact that complicates determination of the cell cycle stage of individual cells. We took advantage of the fact that WGA specifically labels the septa of PlyC treated, M-protein negative JRS75, to detect the active division septa. JRS75 cells were stained for sortase (red), septal wall material (green), and cytoplasmic GAPDH (blue). Cells containing a single defined septum were divided into 5 size groups (small to large), representing consecutive stages in the cell cycle. Two-dimensional projections were made from the Z-stack data, and the average fluorescence intensity for each antigen was plotted as a function of the distance from one of the cell poles. In the smaller size groups (Fig. 3.8A-C) the cell orientation in the chain often made it possible to determine which pole is the result of the previous cell division. The fluorescence plots for these cells were aligned so that the younger pole is located to the left. For each size group at least 30 cells were analyzed, and the WGA signal, which regularly gave a sharp peak indicating the septum location, was used to align them to one another for the creation of population distribution plots (Fig. 3.8, right column).

The population distribution plots reveal a clear preference for sortase localization to the septum throughout the cell cycle. Localization to the septum increases gradually following septation (Fig. 3.8A-B) and peaks at mid-division (Fig. 3.8C). In addition to the septum, sortase foci can also be found distributed to other locations in the cell, particularly to the equatorial rings and to a lesser extent, the poles.

The equatorial rings are peptidoglycan features formed following division. They are located on both sides of the septum, at the border between new and old peptidoglycan. Figure 3.9 shows the typical morphology of equatorial rings using transmission electron microscopy in D471 (Fig. 3.9A), and scanning electron microscopy in the isogenic M-negative strain JRS75, where the absence on M-protein makes the equatorial rings particularly apparent (Fig. 3.9B). As the equatorial rings mature, they become the site of the following cell division in the daughter cells (Tomasz, 2000). In many cases peptidoglycan synthesis at the equatorial rings appear to begin before the mother cell has completely matured (Fig 3.9A).

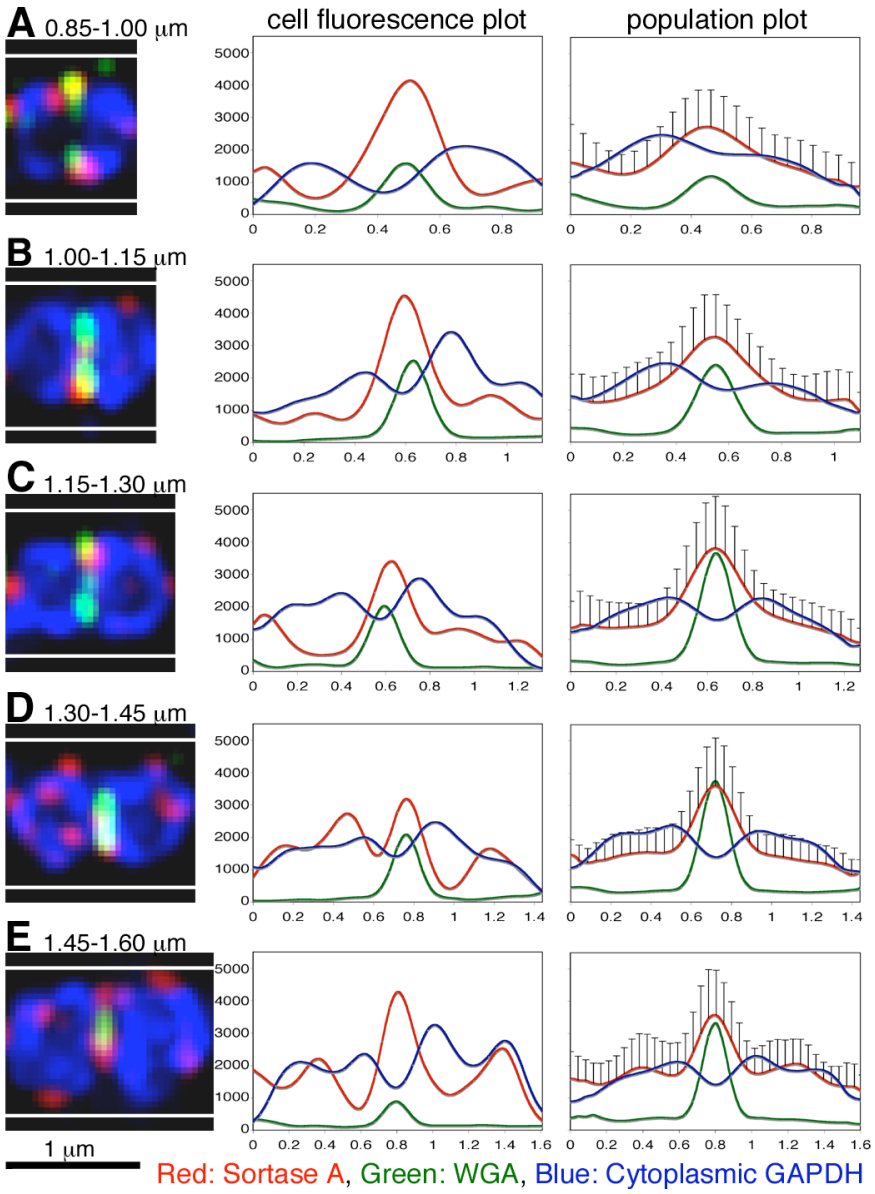
Sortase localization to the equatorial rings increases during late division stages, and is manifested in the population plot as secondary peaks on both sides of the septum (Fig. 3.9E). Assembly of sortase at these sites, before septation in the daughter cells can be detected by WGA staining, illustrates sortase recruitment as an early and gradual event in the division cycle.

Sortase localization to the poles is not uncommon, but is less pronounced. Following conclusion of a division cycle, sortase foci often linger at the closed division septum, seen at the population level as a small peak at the young (left) pole (Fig. 3.8A). Localization to the poles however, is not restricted to very young cells and can be detected on some cells throughout the cell cycle.

Figure 3.8 Localization of sortase A in JRS75 as a function of the cell cycle stage

JRS75 cells were fixed and permeabilized with PlyC as described in the methods section. The cells were labeled for sortase (red) and cytoplasmic GAPDH (blue) using respective antibodies. Septal wall material was labeled with WGA (green). Images of cells in different stages of the cell cycle (A-E) are presented as two-dimensional projections of the 3D data. Fluorescence intensity distribution of the antigens (from left to right, analyzed area is confined by the top and bottom white lines) is presented in a graph by the cells, the X-axis denotes distance from the left pole in μm, and the Y-axis denotes arbitrary fluorescence units. The population plots to the right contain averaged data from at least 30 cells for each division stage; error bars represent one standard deviation.

Figure 3.8


A 0.85-1.00 μm
B 1.00-1.15 μm
C 1.15-1.30 μm
D 1.30-1.45 μm
E 1.45-1.60 μm
cell fluorescence plot
population plot
1 μm
Red: Sortase A, Green: WGA, Blue: Cytoplasmic GAPDH

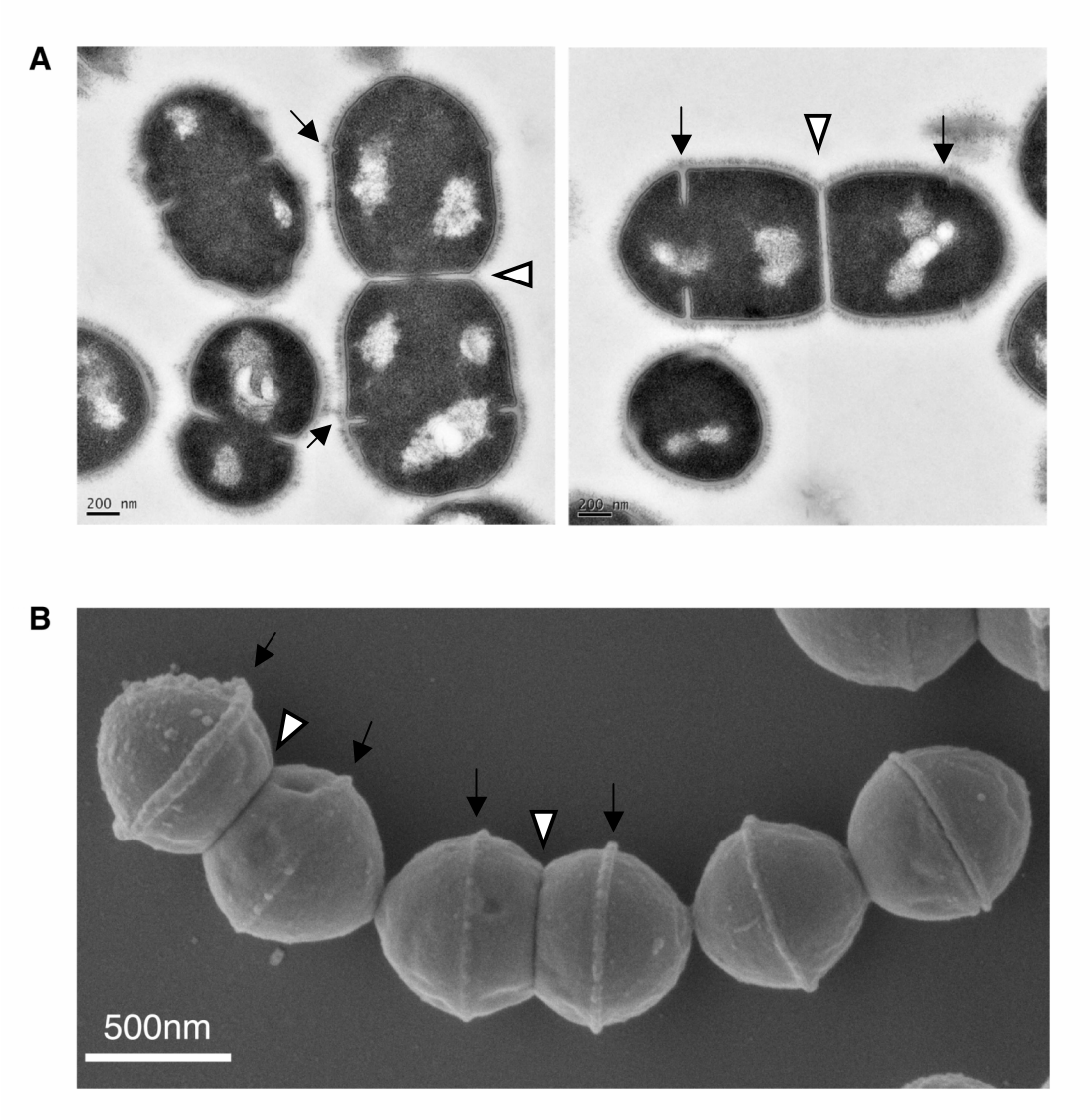


Figure 3.9 Morphology of *S. pyogenes* equatorial rings

(A) Transmission electron microscopy images of D471 cells. (B) Scanning Electron microscopy images of M-negative JRS75. Mother cell septum labeled with a white arrowhead, and equatorial rings labeled with black arrows.

3.8 Sortase foci are predominantly associated with sites of active M-protein anchoring

M-protein, one of the most abundant *S. pyogenes* surface proteins, is anchored exclusively at the division septum (Cole & Hahn, 1962, Swanson et al., 1969). To test the distribution of sortase in relation to areas of active M-protein anchoring, M-protein was removed from D471 cells by trypsin digestion, a treatment that removes surface proteins without harming the cells (Swanson et al., 1969). The cells were then either fixed immediately, or washed and incubated in media without trypsin prior to fixation and permeabilization. Only a small amount of M-protein is detected in cells fixed immediately following trypsin digestion (Fig. 3.10). This small amount was not accessible to trypsin, but could be reached by antibodies after permeabilization with PlyC. To minimize the amount of internal M-protein detected in this manner, we used the 3B8 monoclonal (Jones & Fischetti, 1988), which reacts with a distal epitope in the molecule. Following 15 minutes in media without trypsin, M-protein was regenerated at the septal regions of cells, and sortase foci were regularly seen associated with these areas of newly anchored M-protein. Following 35 minutes incubation, more extensive M-protein anchoring is seen. By this time, septation often began in the daughter cells, and sortase associated with these newly forming septa can be seen at the flanks of areas to which M-protein has been anchored. Note that the integrity of the cell wall during PlyC treatment is dependant on the presence of M-protein. As a result, only areas of the cell wall, to which M-protein has been anchored following trypsinization, remain attached to the cells when PlyC is applied.

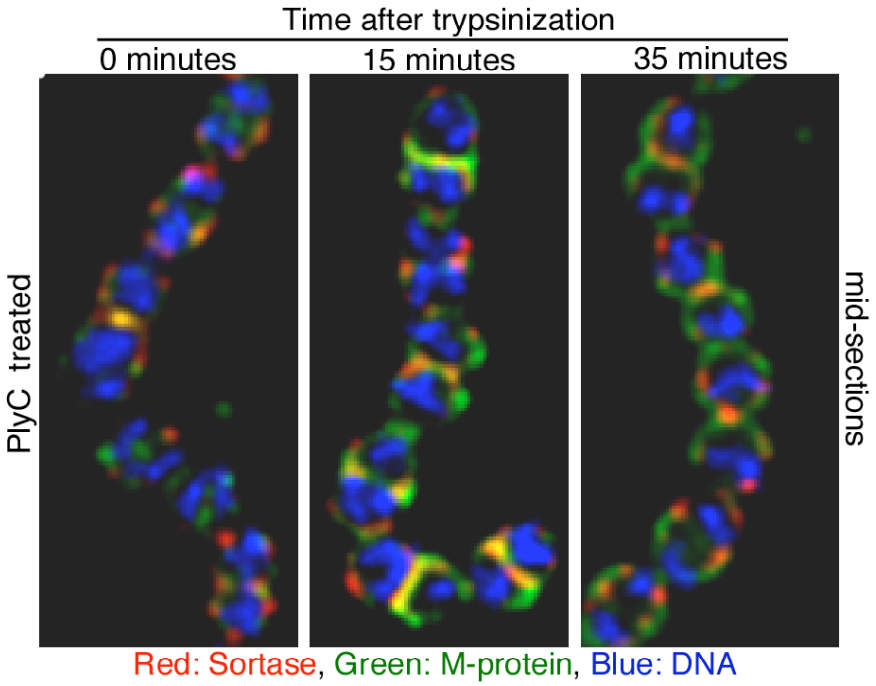


Figure 3.10 Sortase foci preferentially localize to sites of active M-protein anchoring

D471 cells were grown to OD_{600} 0.5 in media containing 0.05% trypsin and either fixed immediately, or washed and incubated in media without trypsin for 15 or 35 minutes prior to fixation. The cells were permeabilized, and stained for sortase (red), and subsequently labeled for M-protein using 3B8-FITC conjugate (green), and DNA (blue).

3.9 M-protein anchoring is localized to sites of lipid II export

Following cleavage of the LPXTG motif in the C-terminus of surface proteins, sortase attaches the protein to the peptidoglycan precursor lipid II (Perry et al., 2002), leading to its covalent linkage to the cell wall. To study the relative localization of active M-protein anchoring and lipid II export, D471 cells were trypsinized as described above, allowed to regenerate M-protein for various time periods, and then fixed but not permeabilized. These cells were labeled with the 10B6 monoclonal (Jones et al., 1985), which binds close to the base of the M-protein molecule and better reflects the location of the actual anchoring sites, and vancomycin-BODIPY, which binds the D-ala – D-ala motif found in Lipid II and newly deposited peptidoglycan (Daniel & Errington, 2003). Vancomycin-BODIPY labeled a strong septal band, and weaker bands at the equatorial rings at some stages of the cell cycle (Fig. 3.11, 5 minutes time point), similar to the pattern observed in *S. pneumoniae* (Daniel & Errington, 2003, Ng *et al.*, 2004). Following 5 minutes of regeneration, newly anchored M-protein initially appeared in foci closely associated with sites of vancomycin-BODIPY labeling. M-protein was often detected simultaneously at the closing primary division septum, and the mature equatorial rings / daughter cell division sites. This observation is in agreement with the assembly of sortase foci at the equatorial rings at the later stages of the cell division (Fig. 3.8). As cells are allowed to regenerate M-protein for longer time periods, M-protein covers larger portions of the cells, and forms continuous patches (Fig. 3.11, 15 and 30 minutes time points). When no trypsin is applied M-protein covers the surface of the organism,

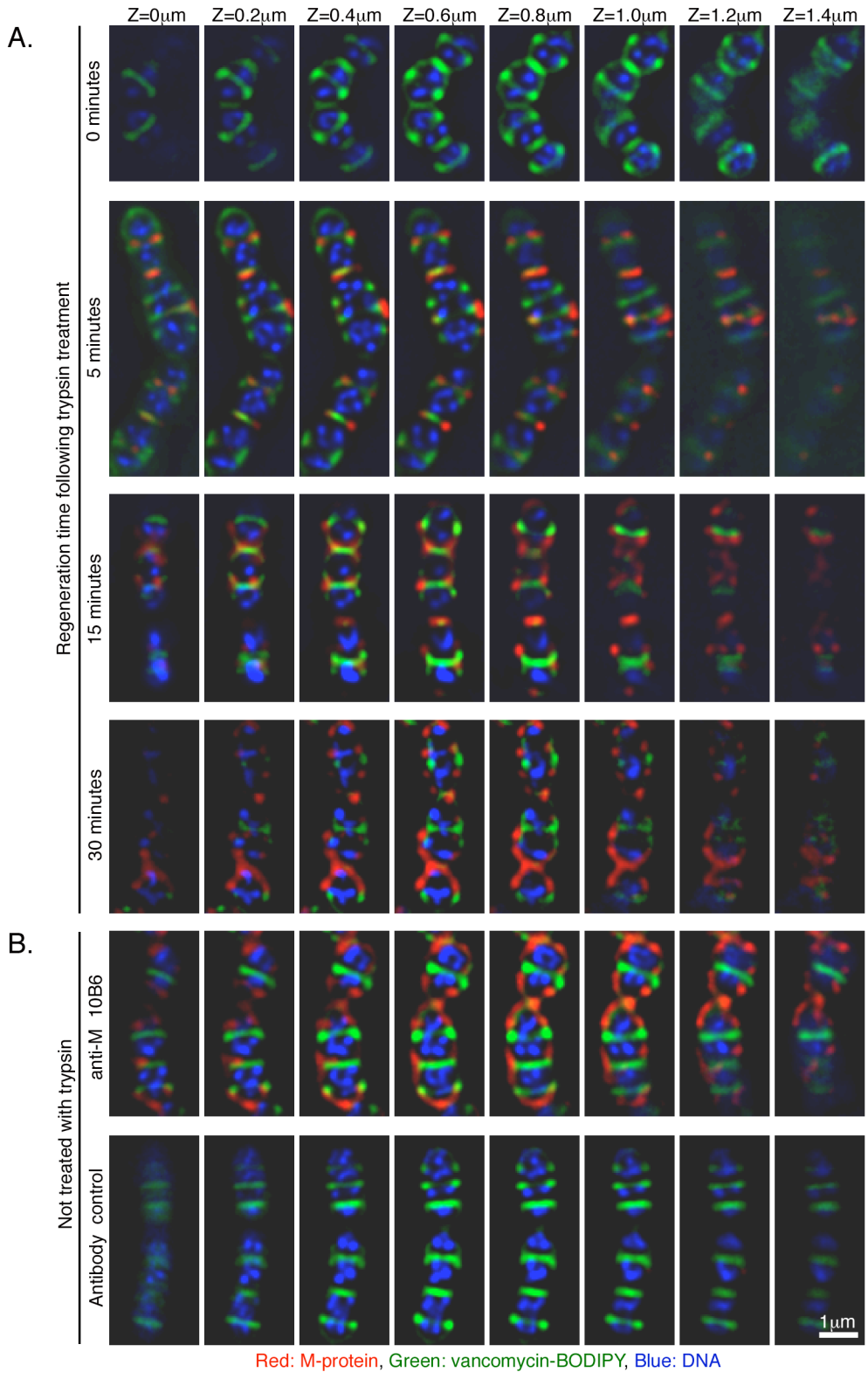
however the distribution of M-protein may not be completely even as some variations in fluorescence intensity are visible (Fig. 3.11B).

Figure 3.11 M-protein is anchored in septum-associated foci, co-localized with lipid II export regions

(A) D471 cells were grown to OD_{600} 0.5 in media containing 0.05% trypsin and either fixed immediately (time 0), or washed and incubated in media without trypsin for 5, 15, or 30 minutes prior to fixation. These cells were not treated with methanol and PlyC. The cells were stained for M-protein (red), using the 10B6 monoclonal and Alexa Fluor 647 conjugate, and DNA (blue). Vancomycin-BODIPY (green) was used to detect Lipid II export regions. Images are presented as sequential Z-stack captures with 0.2μm intervals.

(B) D471 cells, not treated with trypsin, were stained in the same manner. Control cells were not treated with primary M-protein specific antibodies.

Figure 3.11


Z=0μm
Z=0.2μm
Z=0.4μm
Z=0.6μm
Z=0.8μm
Z=1.0μm
Z=1.2μm
Z=1.4μm
A.
Regeneration time following trypsin treatment
0 minutes
5 minutes
15 minutes
30 minutes
B.
Not treated with trypsin
anti-M 10B6
Antibody control
1μm
Red: M-protein, Green: vancomycin-BODIPY, Blue: DNA

4 Differential secretion and anchoring of M-protein and SfbI

4.1 Introduction

Secretion and anchoring of *S. pyogenes* surface proteins is a highly regulated process. In early studies, M-protein was shown to be anchored exclusively at the septal region of the cell (Cole & Hahn, 1962, Swanson et al., 1969). Since cell wall synthesis is believed to be restricted to the septum of *S. pyogenes* (Zapun et al., 2008), anchoring of M-protein at this site leads to the coating of the entire cell wall with this protein. The distribution of M-protein on the surface is not completely even with sites of more intense fluorescence (Fig. 3.11), as is also the case for *S. aureus* protein A (DeDent *et al.*, 2007). Newly anchored M-protein is found in foci that co-localize with areas strongly labeled vancomycin BODIPY, indicating the anchoring occurs in areas of active cell wall synthesis (Fig. 3.11).

In contrast to M-protein, SfbI is anchored to the old poles (Ozeri et al., 2001) indicating that regulation of this protein's secretion and anchoring may be different than that of M-protein. Secretion of these two proteins at their respective positions on the cell wall is governed by differences in their signal sequence (Carlsson *et al.*, 2006). Signal sequences were subsequently found to play a role in directing the localized secretion and anchoring of cell wall proteins in *S. aureus* as well (DeDent et al., 2008). In this organism wall-anchored proteins are distributed either as 2-4 foci associated with the septum, or in a hemispherical pattern (DeDent et al., 2007, DeDent *et al.*, 2008). In both *S. pyogenes* and *S. aureus*, the presence of a YSIRK/GS

motif in the signal sequence is associated with secretion and anchoring at the septum, although mutation of this sequence does not alter the secretion pattern. Signal sequences that do not contain a YSIRK/GS motif direct secretion to the poles (Carlsson et al., 2006, DeDent et al., 2008).

In contrast to the sites of M-protein and SfbI anchoring, SpeB was shown to be secreted at one microdomain on the *S. pyogenes* membrane, termed the ExPortal (Rosch & Caparon, 2004). HtrA, a chaperone required for the maturation of SpeB was also shown to localize to the ExPortal (Rosch & Caparon, 2005). The use of the membrane dye nonyl acridine orange (NAO) revealed that anionic lipids, which in *E. coli* and *B. subtilis* play a role in secretion (de Vrije et al., 1988, Rietveld et al., 1995, Suzuki et al., 1999, van der Does et al., 2000), accumulate in a single microdomain on the *S. pyogenes* membrane. Secretion of SpeB is localized to this microdomain indicating colocalization with the ExPortal (Rosch et al., 2007).

In contrast to our immunofluorescence results showing that *S. pyogenes* sortase localizes to several foci in each cell (Raz & Fischetti, 2008), the localization of *S. mutans* sortase (Ping *et al.*, 2008) and *E. faecalis* sortase (Kline et al., 2009) was studied using Immuno-electron microscopy and was shown to localize to only one focus per cell, co-localized with SecA and by inference, with the ExPortal. Both secretion and anchoring of surface proteins were therefore proposed to take place at the ExPortal.

Several questions therefore remain to be answered. First, there is an inherent contradiction between published data suggesting secretion and anchoring of proteins at both the septum (M-protein) and the poles (SfbI), and data suggesting that proteins

are secreted and anchored by a dedicated organelle, the ExPortal. It would be of interest to determine if either septal or polar protein-secretion is localized to the ExPortal. Secondly, we observed that the majority of sortase foci localize to the septum and much fewer foci localize to the poles. If proteins are anchored at both the septum and the poles, why is sortase predominantly associated with the septum, with only a small amount associated with the poles?

4.2 M-protein and SfbI are not anchored to the cell wall in the *S. pyogenes* sortase mutant

In order to study the relative distribution of M-protein and SfbI, we obtained SfbI specific rabbit serum (a generous gift from Susanne Talay). To validate the specificity of the antibodies, as well as the dependence of both proteins on sortase for cell wall anchoring, we determined their distribution by Western blot, using two fractionation methods. First, the wild type strain D471, a sortase knockout derivate AR01, and the complemented strain AR01+pAR107 were fractionated to supernatant, cell wall and spheroplast, and assayed by Western blot as described in the methods section, using the M-protein specific monoclonal antibody 10B6 and SfbI-specific polyclonal serum (Figure 4.1A). As expected both M-protein and SfbI were primarily associated with the cell wall fraction of WT D471, whereas in the sortase mutant both proteins were missorted to the supernatant, wall and protoplast fractions. Complementation of the sortase knockout with a plasmid expressing sortase restored the WT distribution.

To validate that the proteins found in the wall fraction of the sortase mutant AR01 are indeed only associated with, but not anchored to the cell wall, a different approach was used. A washed cell pellet of the different strains was either lysed directly (L), providing a reference level for the total amount of the relevant protein in the cell, or boiled in 2% SDS, and subsequently centrifuged to separate non-covalently bound proteins that are released into the SDS sup (S), and covalently bound proteins that are not released by this treatment and are found in the pellet (P). The pellet was washed and treated with PlyC to release covalently bound proteins from the cell wall, as described in the methods section.

As expected, both M-protein and SfbI were found to be covalently attached to the cell wall of WT D471, but were completely dissociated from the cell wall of the sortase mutant AR01 following boiling in 2% SDS (Figure 4.1B). Complementation with a plasmid expressing sortase restored anchoring of both proteins to the cell wall. These results display the correct distribution of the two sortase substrates in the strains used in this study, as well as reaffirm the specificity of the antibodies.

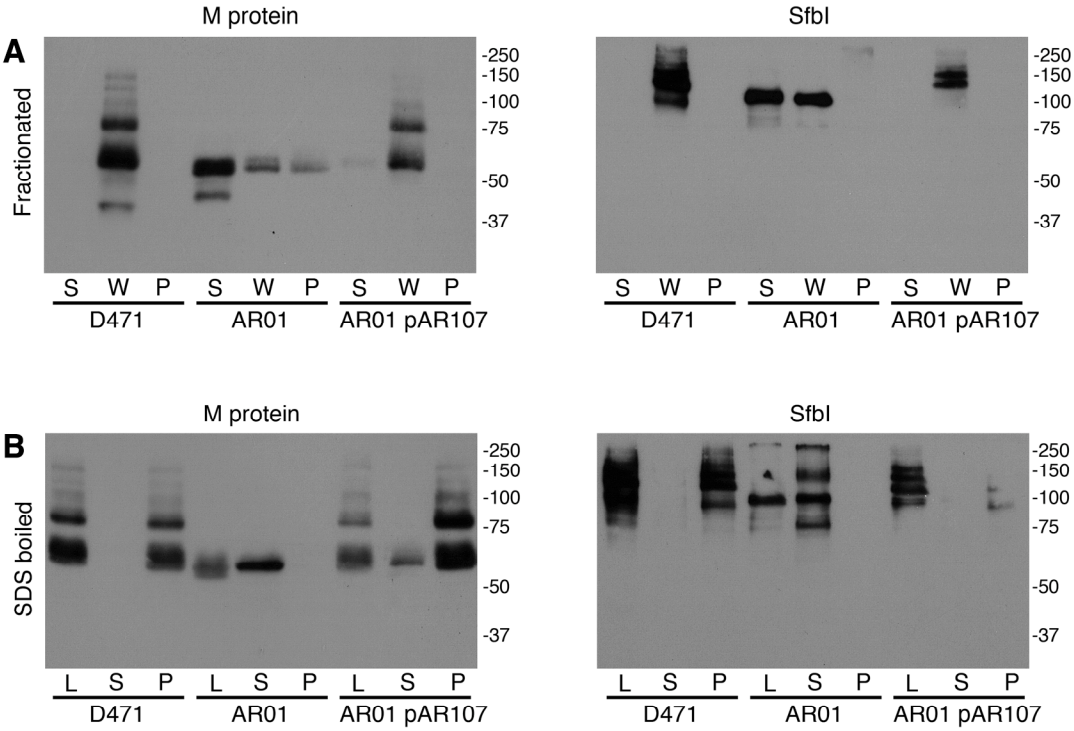


Figure 4.1 Distribution of M-protein and SfbI in D471, AR01, and AR01+pAR107

Cell cultures were grown to OD_{600} 0.5, and fractionated into sup (S), wall (W), and spheroplast pellet (P) as described in the methods section (A). Alternatively, a washed pellet was either lysed directly with PlyC (L), or boiled in 2% SDS and separated in to sup (S) and pellet (P), which was subsequently treated with PlyC to release wall proteins (B). Samples were separated by 10% SDS-PAGE, and examined by western blot using either the M-protein specific monoclonal 10B6, or SfbI-specific polyclonal serum.

4.3 M-protein and SfbI are anchored to different locations on the cell wall of *S. pyogenes*

Previous observations have shown that while M-protein is anchored to the septum, leading to its distribution throughout the cell surface, SfbI is anchored to the old poles (Carlsson et al., 2006). To validate that this distribution is indeed observed using our antibodies and immunofluorescence protocol, we labeled WT cells with the M-specific monoclonal antibody 10B6 (red) and SfbI-reactive polyclonal serum (green). The cell wall was labeled with wheat germ agglutinin (WGA, blue). Consistent with previous observations, M-protein was present on the entire periphery of the cells, although the distribution was not entirely even with some patches of more intense fluorescence, predominantly in the vicinity of the septum. The distribution of SfbI was predominantly confined to the cell poles, however unlike previous observations the fluorescence intensity of SfbI varied greatly between cells. Control cells treated with pre-immune serum showed no fluorescent signal.

To determine the precise location of active proteins anchoring, cells were grown in medium containing trypsin and pronase, which remove surface proteins without affecting sortase or otherwise harming the cells. Cells treated in this manner were either fixed immediately or washed in medium not containing proteases, and allowed to regenerate surface proteins for 5 minutes prior to fixation. Following this short regeneration time, newly anchored M-protein and SfbI could be observed at the septum and old poles of individual cells respectively, demonstrating that the anchoring of both proteins occurs simultaneously, rather than at specific stages of the cell cycle. Simultaneous anchoring of these proteins at different locations within

individual cells suggests that secretion of both proteins through a single ExPortal it is not likely.

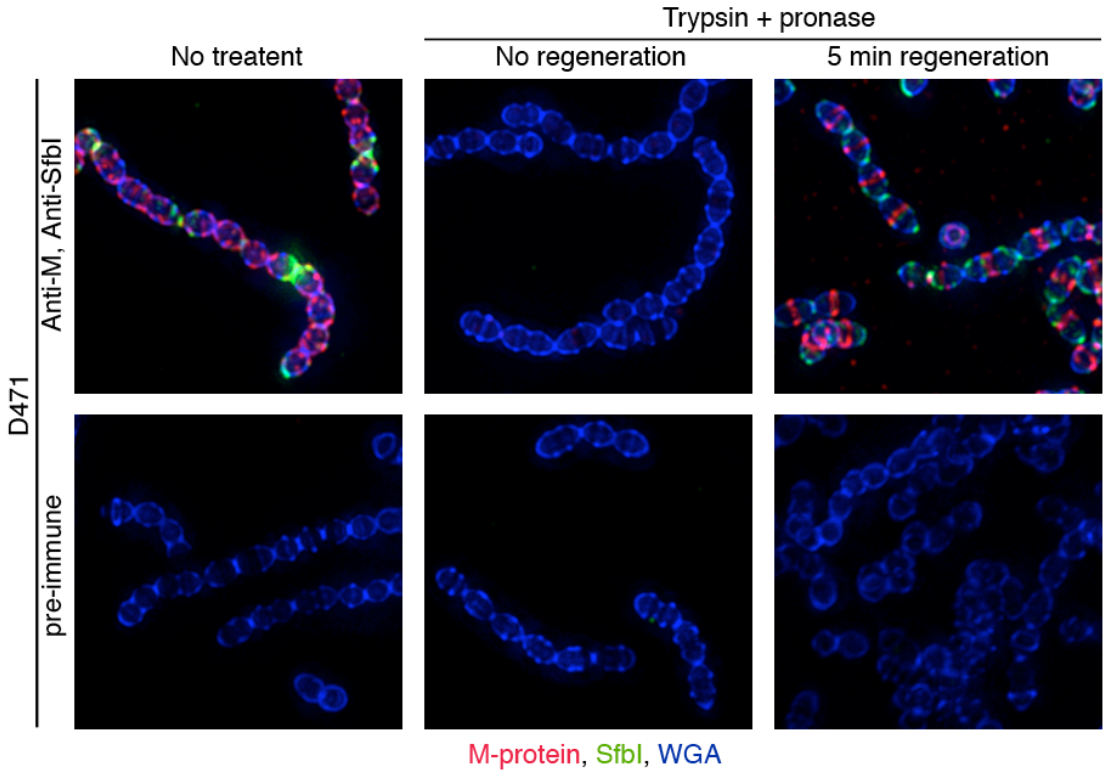


Figure 4.2 Anchoring pattern of M-protein and SfbI in WT D471

Cells were grown to OD_{600} 0.5 in either TH+Y (no treatment) or TH+Y containing 0.35 mg/ml trypsin and 0.04 mg/ml pronase. Protease treated cells were either fixed immediately upon reaching OD_{600} 0.5 (no regeneration) or washed and resuspended in TH+Y for 5 minutes at 37°C prior to fixation (5 min regeneration). The cells were stained for M-protein (red) using the 10B6 monoclonal antibody, and for SfbI (green) using polyclonal serum. The cell wall and carbohydrate were stained with WGA (blue). Pre-immune serum was used as control for the anti-SfbI polyclonal serum.

4.4 Sortase is not required for correct localization of M-protein and SfbI secretion

While studying the distribution of *S. pneumoniae* pili, Falker et al. reported that deletion of either SrtB or SrtD resulted in considerable changes to the distribution of pilus subunits on the surface of the cells. Furthermore deletion of SrtB in particular resulted in distribution of the pili to one or a few foci per cell (Falker *et al.*, 2008). Deletion of *E. faecalis* SrtC also led to the accumulation of pili subunits to one focus per cell (Kline et al., 2009). To test whether deletion of sortase could effect the secretion sites localization of sortase substrates in *S. pyogenes*, we tested the distribution of M-protein and SfbI in AR01. In this strain neither M-protein nor SfbI are anchored covalently to the cell wall (Figure 4.1). These proteins are secreted however, and remain trapped to some extent in the cell wall, where they can be viewed by immunofluorescence. In contrast to WT cells where the distribution pattern reflects a combined result of two processes, namely localized secretion and anchoring, the surface protein distribution observed in AR01 results from secretion alone.

AR01 cells were treated as described above, and stained for M-protein (red), SfbI (green), and cell wall (blue). M-protein could be seen associated with the surface of the cells (Fig 4.3), although the distribution is less even than observed in the WT cells. The fluorescence level of SfbI on the other hand was much weaker, although a small amount could still be detected attached to the surface of AR01. In order to better visualize the relative distribution of SfbI as compared to M-protein in this strain, the SfbI signal (green) was enhanced as compared to WT cells, yet even

following signal enhancement the pre-immune control displayed only a low background signal. When the sites of M-protein and SfbI accumulation on the cell wall were compared, no clear co-localization was observed.

To determine the precise location of M-protein and SfbI secretion we grew the cells first in the presence of trypsin and pronase, and then transferred them to fresh media without the proteases for 5 minutes to allowed them to regenerate new surface proteins as described above. The secretion sites for M-protein and SfbI in the sortase mutant strain AR01 were similar to those observed in the wild type strain D471, namely M-protein was secreted at the septum and SfbI at the poles. Since no anchoring takes place in these cells, the observed distribution reflects the localization of protein secretion. This observation lends further support to the model whereby proteins are secreted at different locations in the cell according to their signal sequence, rather than secreted through a single ExPortal. These results also establish that sortase is not necessary for the correct localization of M-protein or SfbI secretion.

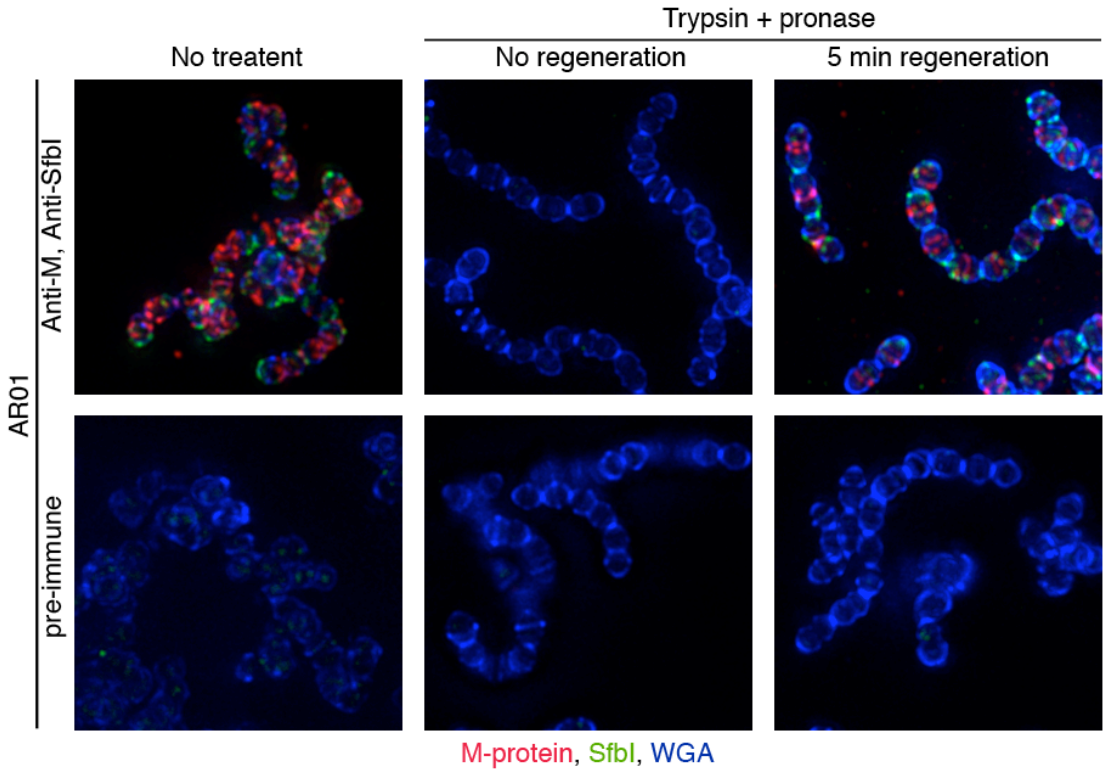


Figure 4.3 Anchoring pattern of M-protein and SfbI in the SrtA KO strain AR01

Cells were grown to OD_{600} 0.5 in either TH+Y (no treatment) or TH+Y containing 0.35 mg/ml trypsin and 0.04 mg/ml pronase. Protease treated cells were either fixed immediately upon reaching OD_{600} 0.5 (no regeneration) or washed and resuspended in TH+Y for 5 minutes at 37^{o}C prior to fixation (5 min regeneration). The cells were stained for M-protein (red) using the 10B6 monoclonal antibody, and for SfbI (green) using polyclonal serum. The cell wall and carbohydrate were stained with WGA (blue). Pre-immune serum was used as control for the anti-SfbI polyclonal serum.

4.5 The export sites of M-protein and SfbI do not co-localize with the ExPortal

While our immunofluorescence data suggest that *S. pyogenes* sortase localizes to a number of foci in each cell (Raz & Fischetti, 2008), *S. mutans* sortase (Ping et al., 2008), and more recently *E. faecalis* sortase (Kline et al., 2009), were found through the use of immuno-EM to reside in an ExPortal, where they colocalize with SecA. The ExPortal was found to reside in a region of the membrane enriched in anionic lipids, which can be visualized through staining with nonyl acridine orange (NAO) (Rosch et al., 2007). We used this fact to determine whether secretion of either M-protein or SfbI, which serve as models for the two secretion patterns observed in this organism, localize to the ExPortal.

To remove previously anchored surface proteins, *S. pyogenes* cells were again grown in media containing trypsin and pronase as described above, and were either fixed immediately or washed and allowed to regenerate surface proteins for five minutes in medium without proteases prior to fixation. M-protein and SfbI were labeled in separate experiments using specific antibodies and AF647 conjugate (red), the anionic lipid microdomain was labeled using NAO (green), and cell wall was labeled using WGA marina blue conjugate.

The observed distribution of M-protein and SfbI was consistent with the previously observed results, namely M-protein was anchored at the septum and SfbI was anchored at the poles. Co-localization of newly anchored M-protein and the ExPortal was sometimes observed, in cases where the anionic microdomain was found at the septum, however this was not a general phenomenon and in the majority of the cells no such co-localization was observed (Figure 4.4A). No regular co-

localization was observed between SfbI and the anionic lipid microdomain either, although occasional co-localization was visible at times (figure 4.4B).

Since the localization pattern of protein secretion *per se,* may be more accurately studied in a *srtA* mutant, in which no anchoring takes place, we repeated the experiment described above in the sortase mutant strain AR01. As in the WT strain, M-protein was secreted at the septum while SfbI was secreted at the poles, while the anionic lipid microdomain could be observed in any location in the cell. In these cells too, no obvious co-localization was observed between the anionic lipid microdomain and either of the surface proteins studied (Figure 4.5). Since the SfbI fluorescent signal was relatively weak in the sortase mutant AR01, it was enhanced as compared to the parent strain D471, but maintained at a range where the pre-immune serum control displayed only a low background signal.

One caveat of these experiments is that while the distribution of surface proteins represents the sum of the proteins secreted/anchored during a 5 minute interval, the localization of the ExPortal represents a snapshot of its location at the moment of fixation. We cannot therefore completely rule out a scenario, in which the lack of co-localization between the site of protein secretion, and NAO labeling results from rapid movement of the ExPortal between the septum and the poles. We consider such rapid movement unlikely however, since SpeB, which is the best-characterized ExPortal marker, is secreted at a single location (Rosch & Caparon, 2004), a pattern incompatible with such rapid movement.

Figure 4.4 M-protein and SfbI anchoring sites do not regularly localize to the anionic lipid microdomain location in D471

Cells were grown in TH+Y containing 0.35 mg/ml trypsin and 0.04 mg/ml pronase, and either fixed immediately upon reaching OD_{600} 0.5 (no regeneration) or washed and suspended in TH+Y for 5 minutes at 37^{o}C prior to fixation (5 min regeneration). The cells were stained using the M-protein specific monoclonal antibody 10B6, or SfbI-specific serum (AF647, red), NAO (green), and DAPI (blue). Pre-immune serum was used as control for the SfbI-specific polyclonal serum.

Figure 4.4

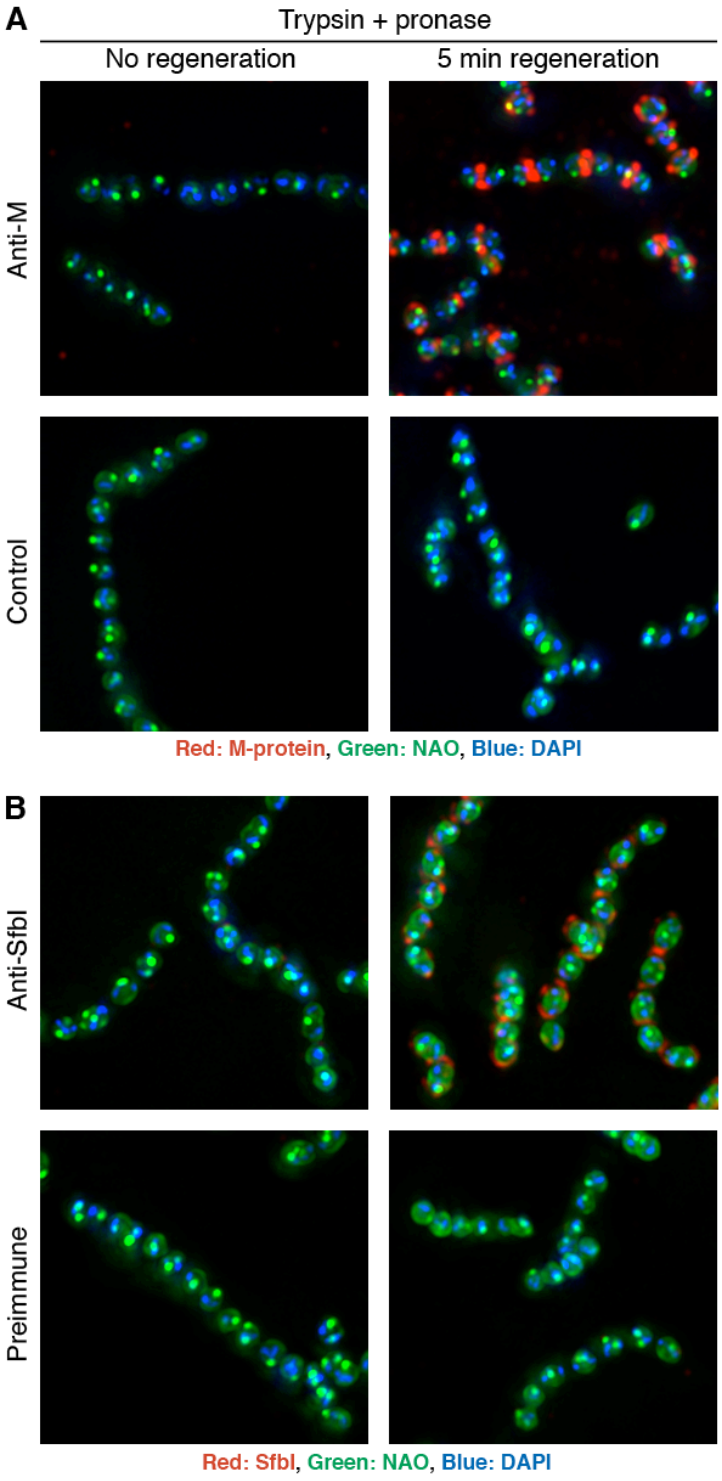
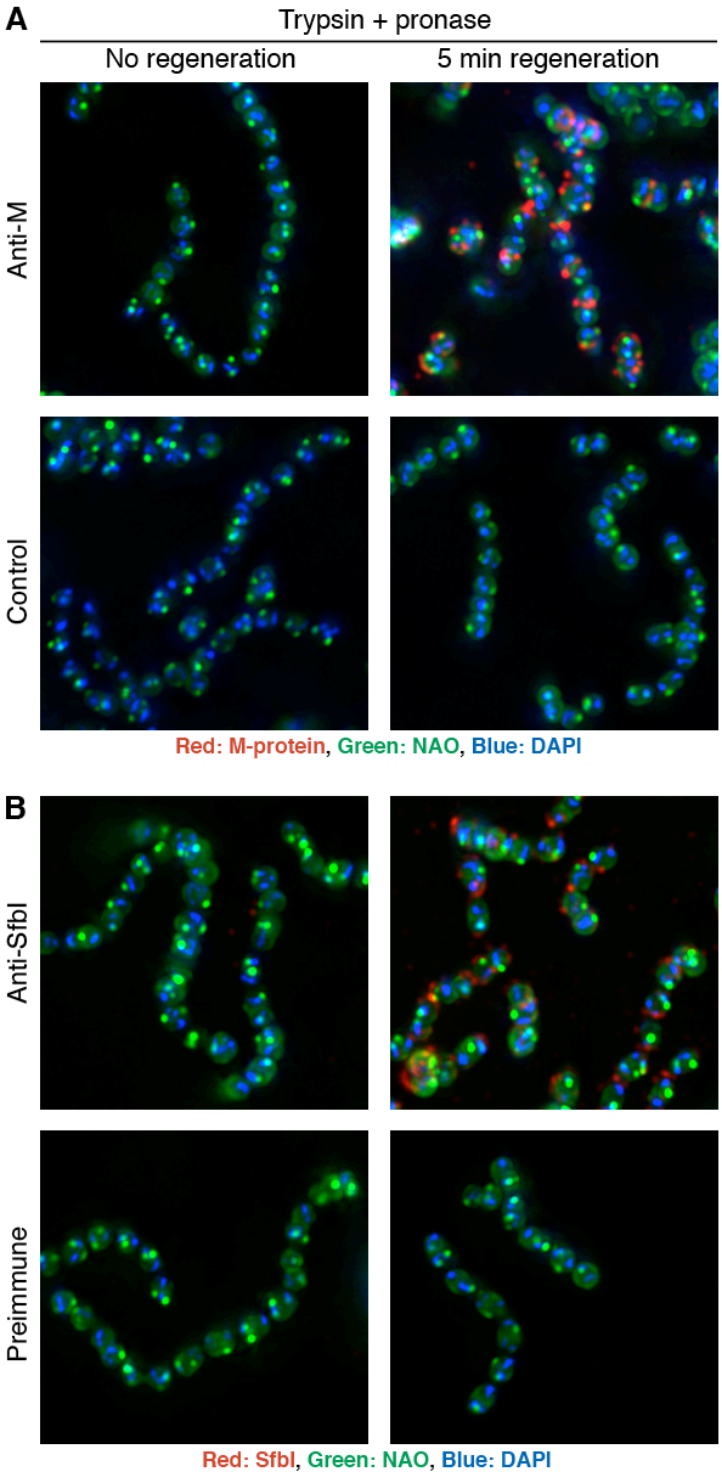

A
Trypsin + pronase
No regeneration
5 min regeneration
Anti-M
Control
Red: M-protein, Green: NAO, Blue: DAPI
B
Anti-SfbI
Preimmune
Red: SfbI, Green: NAO, Blue: DAPI

Figure 4.5 M-protein and SfbI secretion sites do not regularly localize to the anionic lipid microdomain location in sortase mutant AR01

Cells were grown in TH+Y containing 0.35 mg/ml trypsin and 0.04 mg/ml pronase, and either fixed immediately upon reaching OD_{600} 0.5 (no regeneration) or washed and suspended in TH+Y for 5 minutes at 37^{o}C prior to fixation (5 min regeneration). The cells were stained using the M-protein specific monoclonal antibody 10B6, or SfbI-specific serum (AF647, red), NAO (green), and DAPI (blue). Pre-immune serum was used as control for the SfbI-specific polyclonal serum.

Figure 4.5


A
Trypsin + pronase
No regeneration
5 min regeneration
Anti-M
Control
Red: M-protein, Green: NAO, Blue: DAPI
B
Anti-SfbI
Preimmune
Red: SfbI, Green: NAO, Blue: DAPI

4.6 Anchoring of M-protein is a very quick and efficient process while anchoring of SfbI is a slow and continuous one

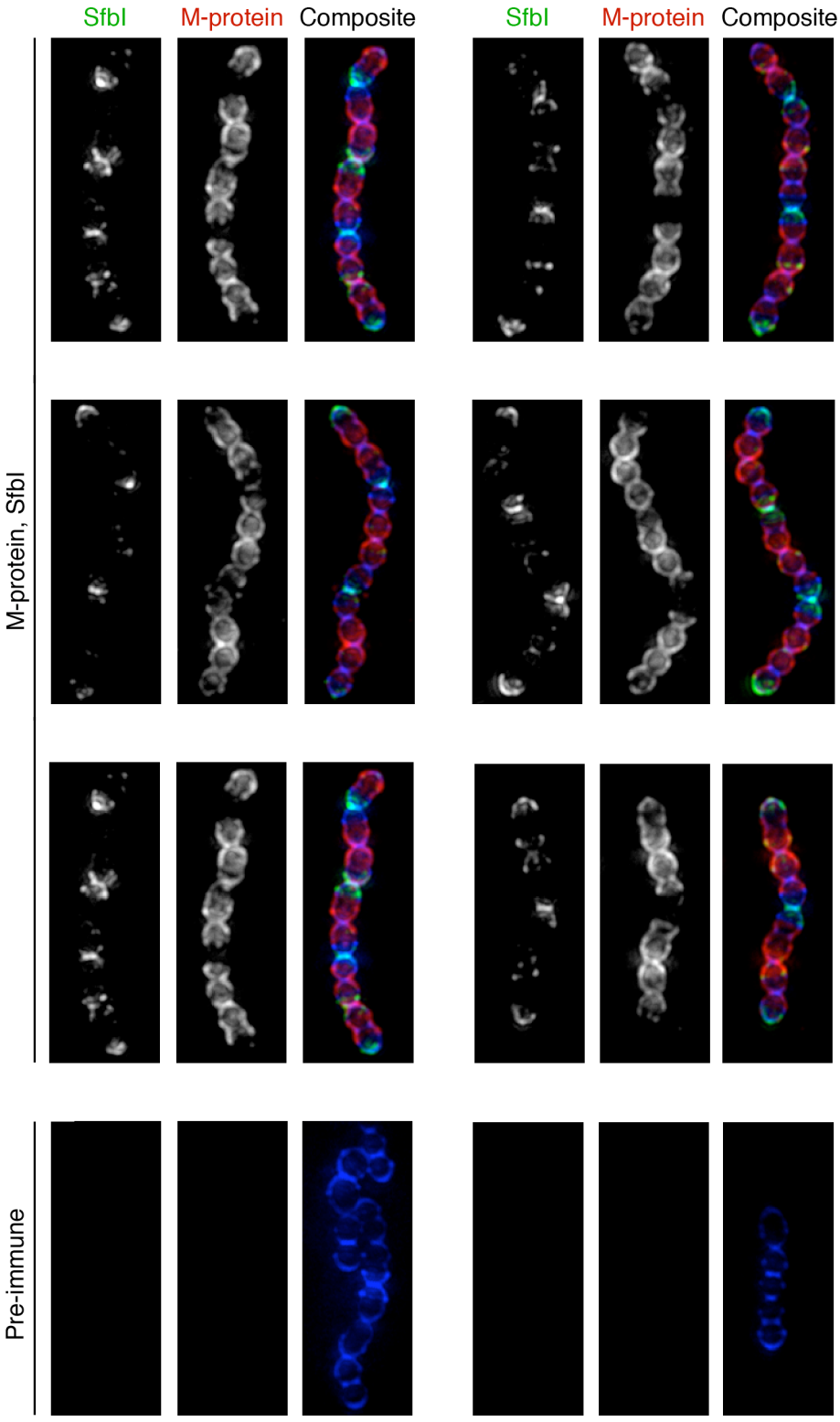
The sum of the results presented above suggest that secretion of M-protein and SfbI takes place at their respective positions simultaneously, and in a manner unrelated to the location of the ExPortal. While the anchoring of M-protein could be explained by the prevalence of sortase at the septum, we observed only a relatively small amount of sortase at the poles, where SfbI is anchored (Raz & Fischetti, 2008). One must keep in mind that M-protein is anchored solely at the septum, and that once the septum matures M-protein can no longer be anchored to that portion of the now peripheral cell wall. As a result there is a relatively short window of opportunity for the anchoring of M-protein. The anchoring kinetics of SfbI on the other hand, is not as well understood.

To better understand the anchoring kinetics of SfbI in individual cells over time, we needed to distinguish between recently formed poles, and old poles. We took two approaches to address this issue. First, D471 cells were grown in medium containing trypsin and pronase, washed, and allowed to regenerate M-protein and SfbI for about two generations (50 minutes). Since M-protein can only be anchored to newly synthesized peptidoglycan at the septum, areas devoid of M-protein represent poles at least two generations old. When the distribution of SfbI was compared to that of M-protein, the SfbI signal tended to be strongest where M-protein was absent (Fig 4.6). This implies that the older the pole, the stronger the SfbI fluorescent signal. Also see figure 7.1 for a model representation.

Figure 4.6 SfbI anchoring kinetics – regeneration following protease treatment

D471 cells were grown in TH+Y containing 0.35 mg/ml trypsin and 0.04 mg/ml pronase, washed, and suspended in TH+Y for 50 minutes at 37^{o}C prior to fixation. The cells were stained using the M-protein specific monoclonal antibody 10B6 (red), SfbI-specific serum (green), and WGA (blue). Pre-immune serum was used as control for the SfbI-specific polyclonal serum. Also see figure 7.1 for a model representation.

Figure 4.6


SfbI
M-protein
Composite
SfbI
M-protein
Composite
M-protein, SfbI
Pre-immune

We next used a different assay to determine the relative age of the cell poles, without the use of proteases. D471 cells were labeled with wheat germ agglutinin (WGA) Alexa Fluor 594 conjugate in the growth medium for 30 minutes, and then washed, and placed in fresh medium for 25 or 50 minutes. While the "old" cell wall is labeled red, cell wall material deposited following the wash step is not labeled (Fig. 4.7). Interestingly, following 50 minutes growth, red WGA labeling could be detected almost exclusively at the equatorial rings, pointing to the possibility that while some peptidoglycan turnover occurs throughout the cell wall (leading to the loss of WGA labeling), peptidoglycan at the equatorial rings is inert (Fig. 4.7B). Labeling of the equatorial rings however was sufficient to determine which poles were those present at the time of WGA-labeling, and are as a result at least two generations old. Here too, older poles tended to display stronger SfbI labeling than recently formed poles.

The combined results of the two approaches suggest that the amount of SfbI anchored to the pole is in direct correlation to the pole's age, depicting SfbI anchoring as a slow and continuous process. It is likely therefore that a smaller amount of sortase could facilitate this type of anchoring kinetics.

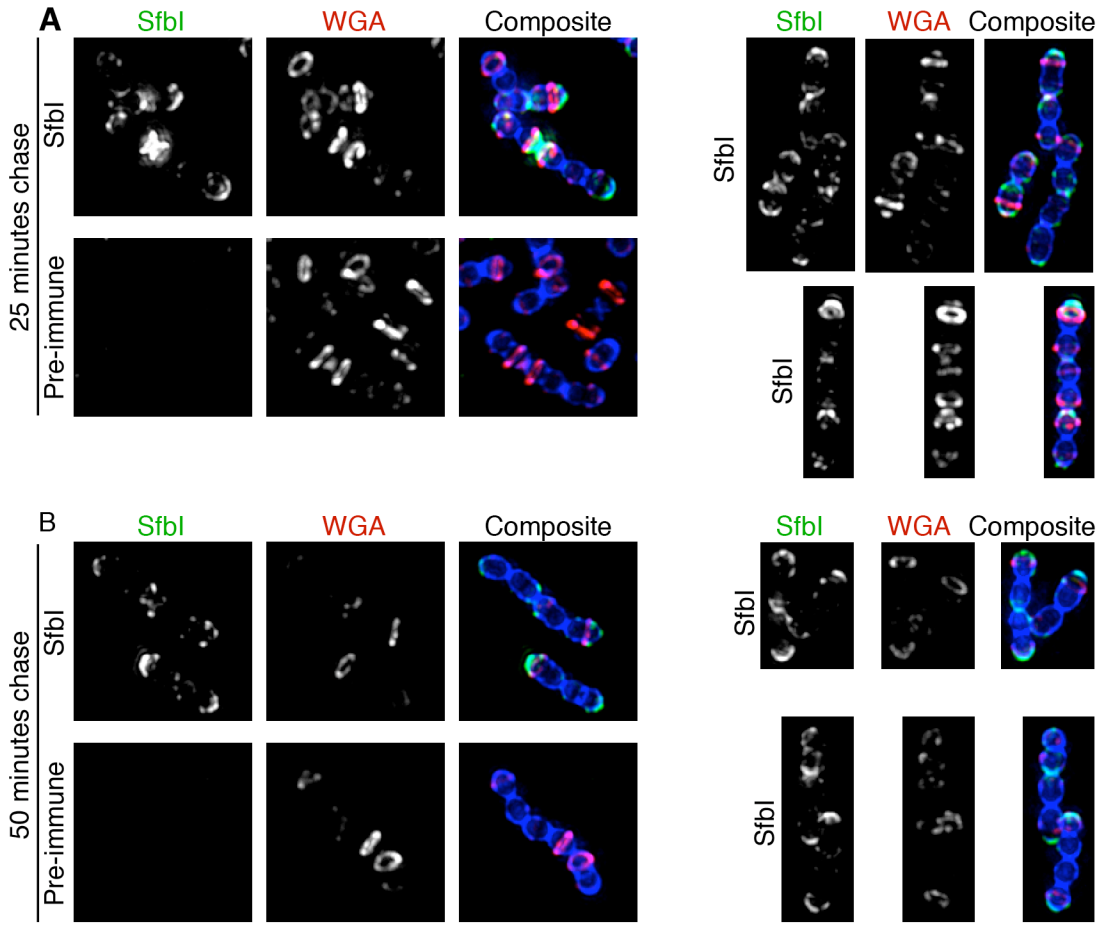


Figure 4.7 SfbI anchoring kinetics – WGA pulse-chase

Log phase D471 cells were pulsed with Alexa Fluor 594 – WGA (red) in the growth medium for 30 minutes. The cells were washed, grown in fresh medium for 25 or 50 minutes, and fixed. SfbI was labeled using specific serum (green). The cell wall was visualized using WGA marina blue (blue). Pre-immune serum was used as control.

5 Loss of M-protein expression in sortase mutant cells

5.1 M-protein negative variants appear spontaneously in the sortase A KO strain AR01.

While studying the distribution of M-protein on the surface of the sortase mutant AR01 we observed that on occasion, a chain of streptococci did not present any M-protein labeling on its surface. We first wanted to rule out the possibility that our AR01 stock is composed of a mixed population. For that purpose we sonicated a small aliquot of the initial stock and validated by light microscopy separation of the chains to single cells or diplococci. These cells were plated, and a culture raised from a single colony was tested by immunofluorescence and shown to display the same heterogeneity (Fig 5.1).

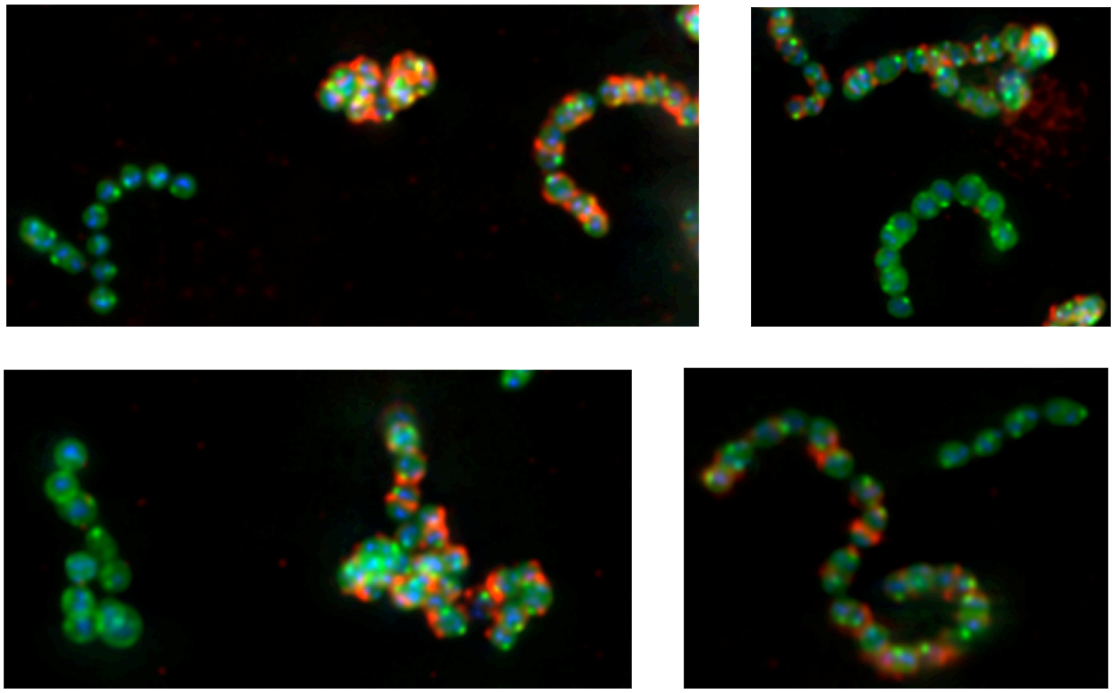


Figure 5.1 Occurrence of M-protein negative streptococcal chains in AR01

A small aliquot of the original AR01 stock was sonicated and plated on TH+Y. A single colony was grown overnight, diluted 1:100 into fresh medium, grown to OD_{600} 0.5, and fixed for immunofluorescence. Cells were labeled using the M-protein specific monoclonal antibody 10B6 (red), NAO (green), and DAPI (blue). Images are presented as 3D-projections of the relevant planes.

5.2 Loss of M-protein expression is a general phenomenon in AR01

Overexpression of a plasmid-encoded surface-anchored protein greatly reduces the viability of a staphylococcal *srtA* deletion mutant (Mazmanian *et al.*, 2001). We postulated that expression of M-protein may similarly be detrimental to sortase-negative cells, and that certain AR01 cells may spontaneously lose the expression of M-protein, thereby gaining a competitive advantage due to a faster growth rate.

To test this possibility we inoculated 10 AR01 sortase mutant colonies, derived from a sonicated culture, into 2ml liquid media. These cultures were allowed to grow for 24 hours at 37^{o}C, at which time a seed culture from each tube was inoculated into a new tube, and a 1 ml sample was harvested and frozen. This process was repeated for six days. At the first and last day, frozen glycerol stocks were prepared from each culture for future study. Additionally, at the final day the culture supernatant was saved for analysis.

To assess the amount of M-protein associated with the cells throughout the 6 days of the experiment, each of the frozen pellets was thawed and processed for semi-quantitative dot-blot as described in the methods section. While on the first day all 10 cultures displayed a substantial amount of M-protein associated with their surface (figure 5.2A), although not anchored to the cell wall (Fig 4.1), by the third day this amount was greatly reduced, and by the sixth day little or no M-protein could be detected attached to the cells of any of the cultures.

To test whether the reduction in the amount of cell-associated M-protein was the result of a reduction in M-protein expression, or an increase in the efficiency of

M-protein release from the cells, we tested the amount of M-protein in the supernatants collected at the last day (Fig 5.2B). We found that while in the majority of cultures M-protein could not be detected in either the supernatant or the lysate, one of the cultures showed a substantial amount of M-protein in the supernatant (Fig 5.1B, colony D). The deleterious effects of M-protein expression in the absence of sortase could therefore be mitigated either by the reduction of M-protein expression, or an increase in the efficiency of M-protein release from the cells.

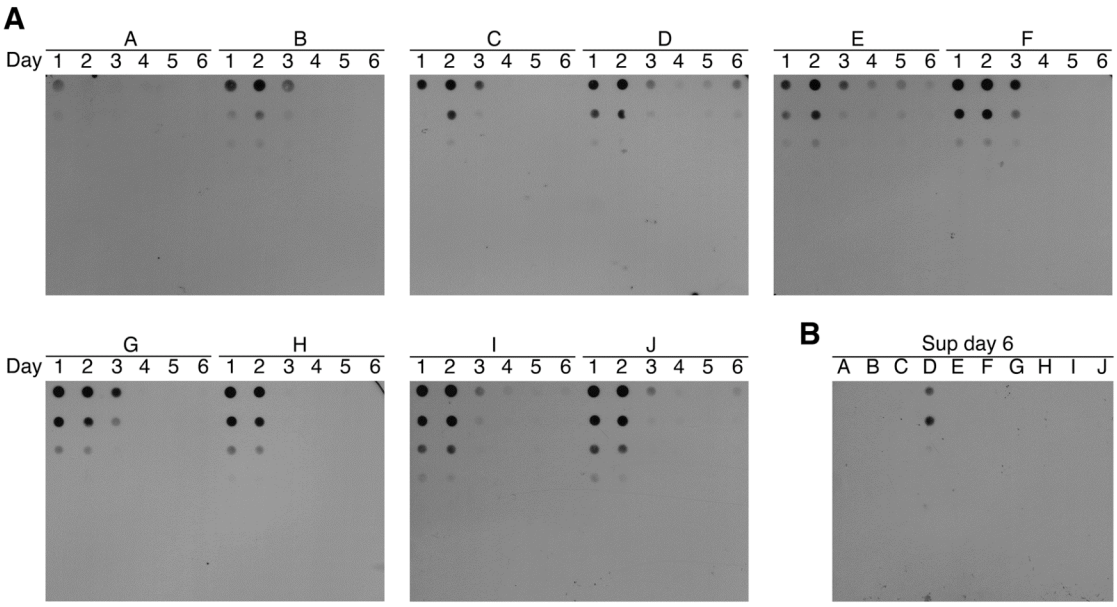


Figure 5.2 Loss of M-protein expression by the sortase mutant AR01

Original stock AR01 cells were sonicated to break the chains, and plated. Single colonies were grown at 37^{o}C for 24 hours, at which time a seed culture was transferred to a new tube, and the original culture was harvested. This process was repeated for 6 days. The amount of M-protein associated with the cells was assessed through semi-quantitative dot blot, as described in the method section (A). The amount of M-protein secreted into the supernatant at the final day of the experiment was similarly assessed through semi-quantitative dot blot (B).

5.3 Loss of M-protein is a general phenomenon to *srtA* KO cells

If loss of M-protein expression following extended growth in culture medium is a general phenomenon of *srtA* mutants, the *srtA* transposon insertion mutant JRS758 (a generous gift from June Scott) (Barnett & Scott, 2002) should behave in the same way. By the time we received this strain it had already been passaged several times in media, allowing ample time for the loss of M-protein expression. We initially used Western blot to compare the level of M-protein expression and its distribution in strains D471, passaged-JRS758, and stock-AR01 (Fig 5.3). While M-protein was found in the cell wall fraction of the WT strain D471, and about an equivalent amount of M-protein was found in stock-AR01, albeit missorted to the supernatant, wall, and spheroplasts fractions, the amount of M-protein found in JRS758 was markedly reduced, and only a faint band could be detected in the spheroplast fraction (Fig 5.3). GAPDH was used as loading control. Surprisingly, the amount of GAPDH found in the wall fraction of JRS758 was markedly greater that that found in D471 or AR01. This observation is not likely to be a loading artifact as the experiment was done in triplicates, yielding similar results. The reason for the expression of such a high amount of GAPDH on the surface of JRS758 is not clear.

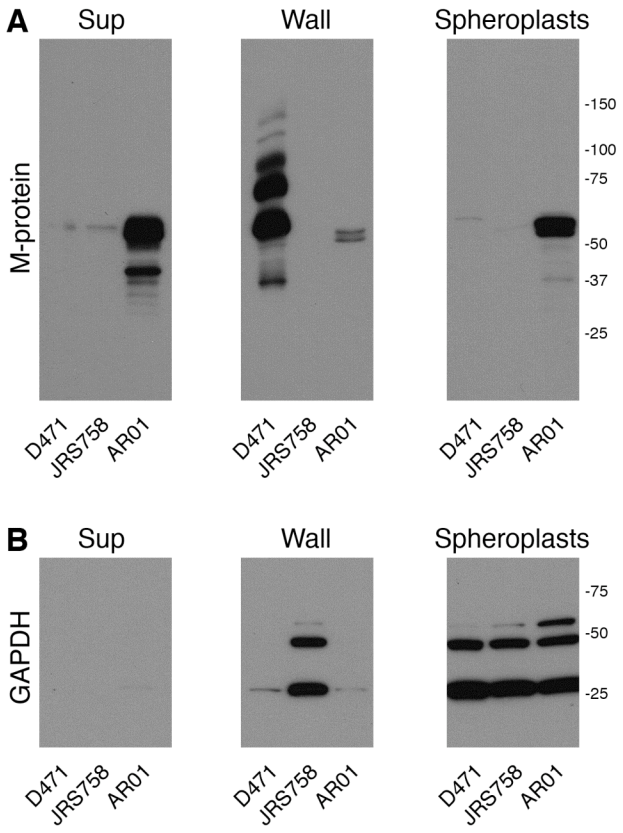


Figure 5.3 Comparison of M-protein distribution in D471, JRS758, and AR01

WT D471, the transposon integration sortase mutant JRS758 (passaged in media several times), and the sortase deletion mutant AR01 (original stock), were grown to OD_{600} 0.5 and fractionated to supernatant, wall, and spheroplasts, as described in the methods section. Fractions were analyzed by Western blot using the M-protein specific monoclonal antibody 10B6, and the 336 anti-GAPDH rabbit serum.

5.4 M-protein transcription is repressed in the sortase transposon mutant JRS758

One likely mechanism for the reduction of M-protein expression is through transcriptional repression. To test whether reduction of M-protein expression in JRS785 is due to transcriptional repression, we isolated RNA from both log phase and stationary phase cells of D471 and JRS758 and tested the level of M-protein mRNA by Northern blot. Equivalent amounts of RNA were loaded on a gel, which was subsequently stained with ethidium bromide to control for the amount and quality of RNA loaded (Fig 5.4A). The gel was transferred to a nylon membrane, and processed as described in the methods section, using a PCR product encompassing the M-protein gene as probe (Fig 5.4B). As expected, the D471 M-protein transcript could only be detected in log phase cells but not in the stationary phase. The M-protein transcript could not be detected in either growth phases of JRS758, confirming that the amount of M-protein in these cells was reduced through transcriptional repression.

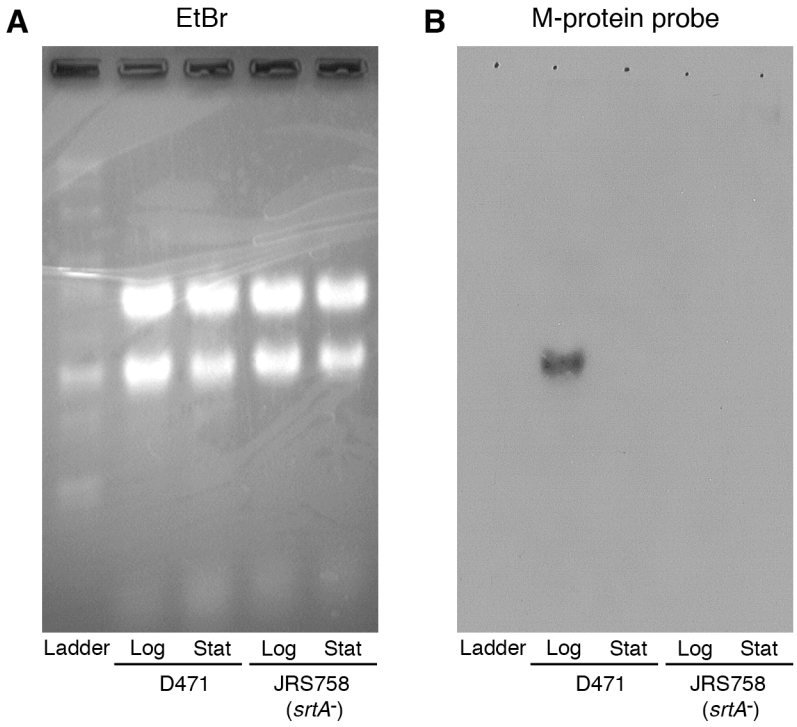


Figure 5.4 M-protein transcription is repressed in the sortase mutant JRS758

Log and stationary phase cultures of D471 and the media-passaged sortase transposon mutant JRS758, were harvested and processed for Northern blot analysis as described in the methods section. (A) Image of the ethidium bromide stained gel prior to its transfer to the membrane. (B) Development of the membrane following incubation with the M-protein probe.

6 Comparison of the specificity of sortase and LPXTGase

6.1 Introduction

Gram-positive bacteria typically possess a number of LPXTG-specific peptidases. *S. pyogenes* serotype M6 possesses two sortase genes, *srtA* the housekeeping sortase and *srtB* specific for T-antigen (Barnett & Scott, 2002), as well as LPXTGase, an LPXTG specific membranal peptidase, which is believed not to be genetically encoded (Lee et al., 2002). Sortases possess a transpeptidation activity, cleaving the LPXTG motif between the threonine and glycine residues and attaching the freed threonine to lipid II. While only peptidase activity has been demonstrated for LPXTGase, it is a much more potent enzyme than sortase (Lee et al., 2002), raising the possibility that these enzymes may cooperate in some way in the biogenesis of surface proteins. Despite the thorough *in vitro* characterization of LPXTGase (Lee et al., 2002, Lee & Fischetti, 2003, Lee & Fischetti, 2006), no *in vivo* activity has ever been assigned to this enzyme. This work is aimed at testing the activity of different LPXTG-specific peptidases on M-protein *in vivo*, as well as testing what parts of the LPXTG motif are critical for its recognition by each of the enzymes.

6.2 Effect mutations in the LPXTG motif have on the distribution of M-protein in JRS75 (M-, SrtA+)

To test the ability of each of the enzymes to recognize the LPXTG motif found in M-protein *in vivo*, we built a series of shuttle vectors each expressing M-protein under its native promoter, containing a defined set of mutations in the LPXTG motif, as described in the methods section. These include: wild type, reversed, or scrambled LPSTGE sequences, replacement of each amino acid in the LPSTGE sequence individually with an alanine, and replacement of the proline with an asparagine. These plasmids were termed pAR178-pAR187 (see table 2.2).

These plasmids were transformed into the M-protein negative D471 derivate, JRS75. Overnight cultures of the respective strains were diluted 1:100 into fresh medium and the cultures were grown to mid log phase, at which point a sample was fractionated into sup, cell wall, and spheroplasts as described in the methods section. The distribution of M-protein in the different fractions was assessed by western blot.

As expected, M-protein containing the native LPSTGE sequence was found attached to the cell wall, and no distinguishable amount was found in the supernatant or spheroplast fraction (Fig. 6.1A). Reversing or scrambling the LPSTGE sequence resulted in the secretion of most M-protein into the supernatant, with a substantial amount of protein found bound to the spheroplast. Surprisingly, replacement of any single amino acid with an alanine did not result in the abolishment of cell wall anchoring, although the expression levels observed in the leucine, proline, and serine mutated strains were sometimes reduced compared to the parental strain. Anchoring is demonstrated by the presence of a ~90kDa species, which results from the binding

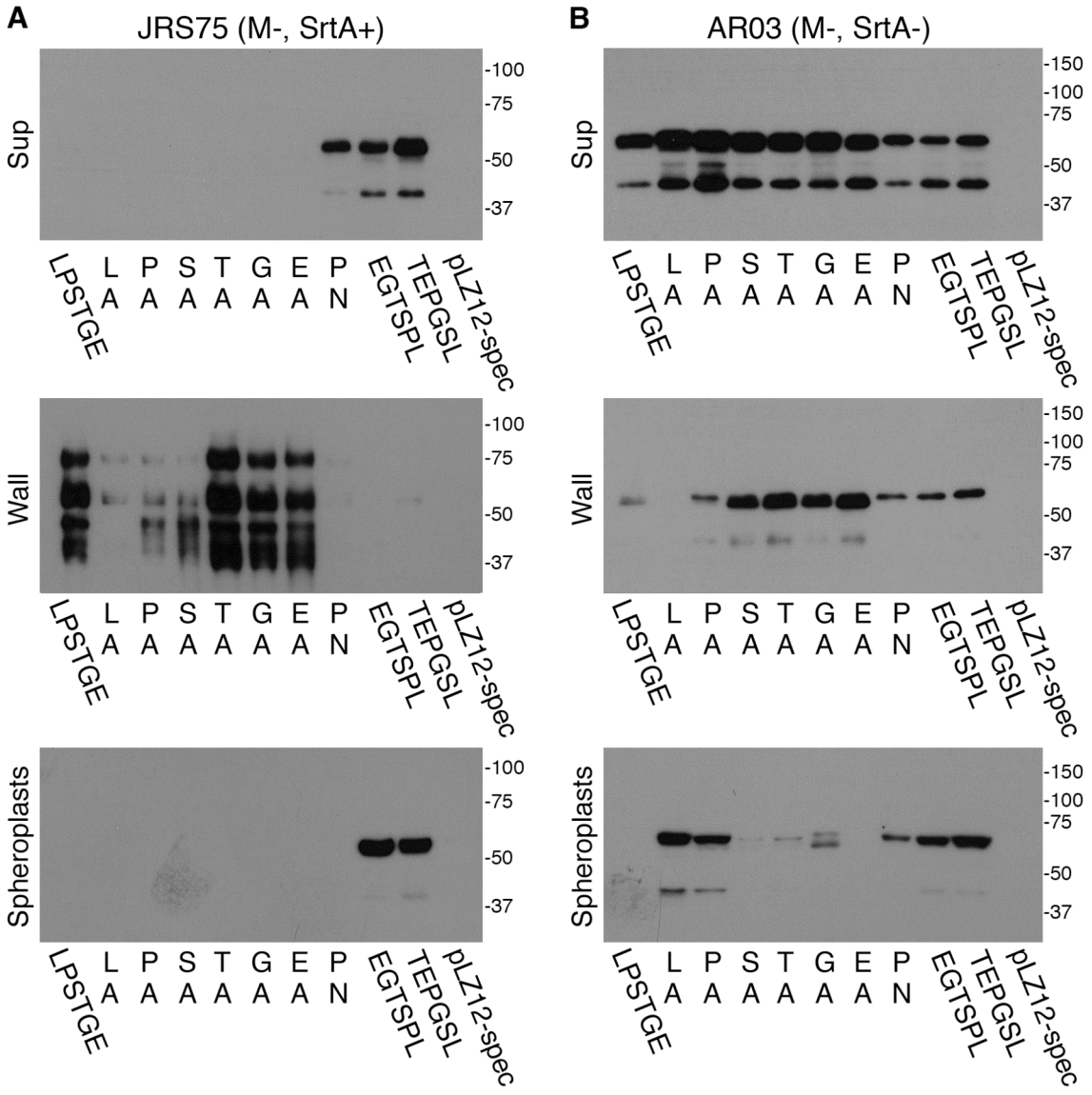
of M-protein to a fragment of cell wall. This 90kDa fragment is never observed in the sortase mutant AR03 (discussed below) (Fig 6.1B).

In a 1992 paper Schneewind et al. reported that replacing the proline residue in the LPXTG motif of *S. aureus* protein A with an asparagine, resulted in a product with a slightly higher molecular mass compared to that of WT protein A. This suggests that the altered sequence may be cleaved at a different location than that of wild-type LPXTG (Schneewind et al., 1992). Since we suspected that this alteration could be the result of cleavage by LPXTGase we produced a construct carrying a similar mutation. Replacing the proline residue in the LPSTGE motif of M-protein with an asparagine resulted in secretion of nearly 100% of the protein, an effect even more pronounced than is seen when the LPXTGE motif is reversed or scrambled (Fig. 6.1A).

Figure 6.1 Distribution of M-protein constructs carrying defined mutations in the LPSTGE motif in JRS75 and AR03

Plasmids pAR178-pAR187 and pLZ12-Spec (see table 2.2) were transformed into JRS75 (M-, SrtA+) (A), or AR03 (M-, SrtA-) (B). The strains were grown to OD_{600} 0.5 and fractionated into supernatant, wall, and spheroplasts as described in the methods section. Western blot analysis was carried out using the M-protein specific monoclonal antibody 10B6.

Figure 6.1


A
JRS75 (M-, SrtA+)
B
AR03 (M-, SrtA-)
Sup
Wall
Spheroplasts
-100
-75
-50
-37
-150
LPSTGE
LA
PA
SA
TA
GA
EA
PN
EGTSPL
TEPGSL
pLZ12-spec

6.3 Effect mutations in the LPXTG motif have on the distribution of M-protein in AR03 (M-, SrtA-)

M-protein is a SrtA substrate, and in its absence, the protein is not anchored to the cell wall, but instead missorted to the supernatant, wall, and spheroplasts fractions (Fig. 3.3A, and Fig. 4.1) (Barnett & Scott, 2002). It is not clear however how M-protein is released into the medium in the absence of SrtA. One possibility is that M-protein is released spontaneously, complete with its intact C-terminal sorting signal, while another is that it is released through the action of a different LPXTG-specific peptidase such as SrtB or LPXTGase, or even a non-specific protease. If the only enzyme effecting M-protein distribution is SrtA, mutation of the LPXTG sequence in the absence of SrtA should have no effect, and the distribution of all 10 constructs should be the same. If on the other hand a LPXTG-specific enzyme other than SrtA plays a role in the release of M-protein from the membrane, disruption of the LPXTG sequence may result in a different distribution pattern for each clone. Furthermore, if an LPXTG-specific enzyme is involved in the release of proteins from the membrane, it is reasonable to expect that mutations that completely disrupt this motif, such as a reversion or scrambling of this sequence, would have the same effect both in the presence and absence of SrtA. Such mutations are likely to prevent recognition by all LPXTG-specific enzymes, while more subtle mutations may or may not prevent recognition by one or more of the LPXTG-specific enzymes.

To test whether a LPXTG-specific enzymes other than SrtA could cleave the LPXTG motif found in M-protein *in vivo*, we deleted SrtA from the M-protein negative strain JRS75 through the use of pAR95, as described for the creation of

strain AR01 above. We termed the resulting strain AR03. We then transformed the 10 plasmids containing the different M-protein variants into AR03 and tested the distribution of M-protein as described above (Fig. 6.1B).

Constructs harboring reversed or scrambled LPSTGE motifs were missorted to the sup, wall, and spheroplast fractions either in the presence or absence of SrtA, demonstrating that SrtA does not act on these clones. A construct with a WT LPSTGE motif was, as expected, not anchored to the cell wall in the absence of SrtA, however the distribution pattern of M-protein was different than that of the constructs with a reversed or scrambled LPSTGE motif (Fig. 6.1B). While the latter displayed a substantial amount of M-protein attached to the spheroplasts, or found at the wall fraction (most likely trapped at the membrane-wall interface), a construct harboring an intact LPSTGE motif was primarily secreted to the supernatant. M-protein in the wall fraction is not likely to represent anchored molecules given the absence of a typical ~90kDa band (found in the sortase positive strain), as well as given results obtained from SDS boiling experiments conducted in the sortase mutant expressing genomic M-protein (Fig. 4.1). This point however would have to be verified in future experiments.

Mutation of the leucine or proline of the LPSTGE motif into alanines resulted in the retention of a substantial amount of M-protein in the spheroplasts fraction. This distribution resembles the one seen when the LPSTGE motif is reversed or scrambled. Mutating of any of the other four amino acids in the LPSTGE motif into alanines on the other hand, resulted in almost no retention of M-protein on the spheroplasts, but instead increased the amount of protein found in the cell wall

fraction. The nature of this material will have to be addressed by future studies. Replacement of the proline residue with an asparagine resulted in the secretion of most M-protein into the surrounding medium, while much less material was found attached to the spheroplasts, when compared to the proline to alanine mutation, and to the reversed or scrambled LPSTGE motifs. Secretion of most of the protein into the medium was also observed in the sortase positive strain (Fig. 6.1A), suggesting the possibility that the molecule is released into the medium through cleavage.

A difference is also visible in the size of the molecules released into the wall and supernatant fractions of AR03. Molecules with a reversed or scrambled LPSTGE motifs, as well as molecules with mutations in the leucine or proline residues of this motif appear to migrate slower, when compared to molecules with intact LPSTGE motif, or with mutations in the serine, threonine, glycine, or glutamic acid residues.

The sum of these results suggests that an LPXTG-specific enzyme other than SrtA is active in the release of M-protein from the membrane. This enzyme cannot act on molecules with reversed or scrambled LPSTGE motifs, as well as on molecules where the leucine or serine residues in this motif were changed to alanines. Mutations of other amino acids in the LPSTGE motif to alanine appear to be permissive. Release of the molecule containing a proline to asparagine mutation from the cell requires further study.

6.4 Analysis of the cleavage location in the LPXTG sequence of M-protein *in vivo*

The results presented above suggest that an enzyme other than SrtA might play a role in the release of M-protein into the medium. Determining whether M-protein is cleaved in the absence of SrtA, and at which location, can aid in finding the enzyme responsible for this cleavage activity. It is noteworthy that while sortase always cleaves the LPSTGE sequence between the threonine and glycine (Navarre & Schneewind, 1994), LPXTGase cleaves this motif both following the serine and following the glutamic acid residues (Lee et al., 2002).

Ton-that et al. used an elegant system to characterize the anchor structure of the *S. aureus* protein A (Ton-That *et al.*, 1997). In that system the secreted protein Seb was fused to the cell wall sorting signal of *S. aureus* protein A, and a methionine followed by a hexahistidine tag was inserted N-terminally to the LPXTG motif. Following cell wall anchoring, the reporter protein was purified and cleaved with cyanogen bromide (which cleaves at the methionine residue). This released a small peptide that was then analyzed by mass spectrometry to determine the anchor structure of the protein.

We initially tried using a similar method to characterize the cleavage site of M-protein, however we were not able to obtain a fragment of sufficient quality using cyanogen bromide due to excessive non-specific degradation of the molecule (not shown). We therefore inserted the cleavage site of the highly specific 3C viral peptidase (Cordingley *et al.*, 1990, Walker *et al.*, 1994) N-terminally to the hexahistidine tag, instead of methionine. Such constructs containing an intact

LPSTGE (pAR166), a reversed EGTSPL motif (pAR169), or a scrambled TEPGSL motif (pAR170), were produced as described in the methods section (Table 2.2).

The constructs were transformed into JRS75 (M-, SrtA+) and AR03 (M-, SrtA-), and the distribution of the proteins in log phase cells was examined by western blot following fractionation. Only the constructs containing an intact LPSTGE motif (pAR166, H6-3C-LPSTGE) showed anchoring to the cell wall of JRS75 (M-, SrtA+), while the constructs containing reversed or scrambled LPSTGE motifs were not anchored to the cell wall (Fig 6.2). None of the constructs was anchored to the cell wall of AR03 (M-, SrtA-). These results show that the LPSTGE sequence found in the context of the his-tag and 3C site is functional for cell wall anchoring, and that the various constructs behave in a manner similar to the respective constructs not containing the insertion of a his-tag and a 3C site.

When the molecular sizes of the molecules are compared, H6-3C-LPSTGE released into the supernatant appears to migrate faster than molecules containing reversed or scrambled LPSTGE motifs, indicating a possible cleavage at this motif. A lower band at the 50kDa range is likely to represent a cleavage product unrelated to the LPSTGE motif. It is of note that this non-specific cleavage product is more prevalent in constructs not containing a functional LPSTGE motif, and may therefore result from the activity of a quality control mechanism at the bacterial surface.

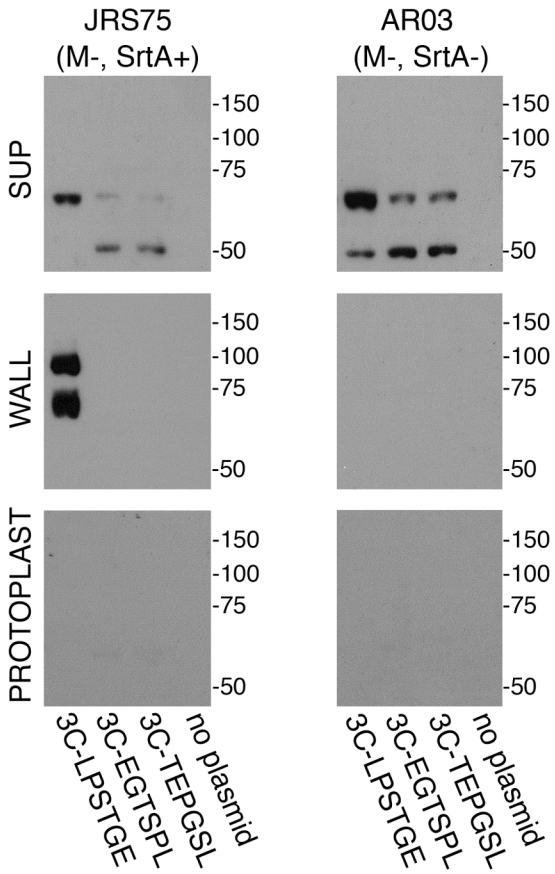


Figure 6.2 M-protein 3C-constructs are functional in cell wall sorting

JRS75 and AR03 strains expressing pAR166 (3C-H6-LPSTGE), pAR169 (3C-H6-EGTSPL), pAR170 (3C-H6-TEPGSL), or with no plasmid, were grown to log phase and fractionated to sup, wall, and spheroplasts fractions. Distribution of M-protein was determined by western blot using the 10B6 monoclonal antibody.

6.5 Purification of the 3C-H6-LPSTGE construct from the medium, 3C cleavage

Anchoring of the 3C-H6-LPSTGE construct to the cell wall of JRS75 demonstrates that the LPXTG motif of this strain is functional (Fig 6.2). To determine whether, and at which location, this motif is cleaved in the sortase negative strain AR03, we grew a 4-liter batch of this strain to late log phase, centrifuged and discarded the cells, and retained the supernatant. The supernatant was filtered and ammonium acetate was added to a final concentration of 65% to precipitated M-protein. Following several days at 4^{o}C, the precipitate was collected by centrifugation, resuspended in MCAC buffer (30mM tris pH 7.4, 0.5M NaCl, 10% glycerol, 1mM DTT), and subjected to metal affinity chromatography. Material eluted from the column with 100mM imidazole, which contained purified M-protein, was dialyzed overnight against 50mM tris, 200mM NaCl, pH 8.0. The sample was removed to a 50ml falcon tube and EDTA (10mM final concentration) and DTT (1mM final concentration) were added.

To 25ml of sample, 100µl of purified recombinant 3C-protease were added and the sample was left at 4^{o}C overnight. A control sample, to which no 3C-protease was added, was kept at similar conditions. 500µl of each sample were precipitated with 5% TCA, loaded on 10% SDS-PAGE and subjected to western blotting using either M-protein specific antibody (Fig. 6.3A), or his-tag specific antibody (Fig 6.3B).

Cleavage of the molecule is demonstrated by a downwards shift in the molecular size of the upper ~75kDa band following addition of 3C-protease, when 10B6 is used (Fig 6.3A). That no such change is observable in the lower ~50kDa non-

specific degradation product, demonstrates that this fragment does not contain the C-terminus of the molecule. Complete cleavage of the molecule by 3C-protease is also demonstrated by the disappearance of the His-reactive band following 3C-cleavage (Fig 6.3B). The fact that the ~50kDa band does not react with the anti-his antibody again suggests that this non-specific cleavage product does not possess its C-terminus. It is possible that the M-molecule is cleaved by a periplasmic protease at a position about 20kDa from its membrane anchor, to release this fragment, however cleavage of the molecule following its release into the surrounding milieu cannot be ruled out at present.

We next attempted to purify the peptide fragment released by 3C-cleavage in order to characterize the precise LPXTG cleavage site. To date however, we were not successful in isolating this fragment at a sufficient purity level to allow analysis by mass spectroscopy. This goal is currently being pursued.

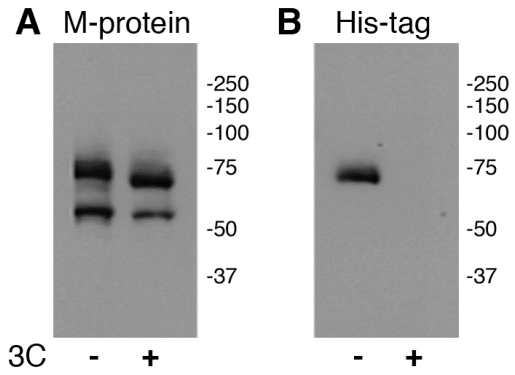


Figure 6.3 3C-cleavage of purified 3C-LPSTGE construct from AR03 (M-, SrtA-) supernatant

M-protein was purified form the supernatant of strain AR03 expressing pAR166 (3C-H6-LPSTGE), and incubated in the presence or absence of 3C protease. Samples were TCA- precipitated and examined by Western blot using either the M-specific monoclonal antibody 10B6 (A), or a his-tag specific antibody (B).

7 Discussion

When grown in ideal conditions *S. pyogenes* cells divide roughly every 30 minutes. In this time span the bacteria must not only duplicate and segregate their genome and cytoplasmic proteins, but also create two new hemispheres, complete with membrane, cell wall, and anchored proteins. Regulation of these processes in time and space is key for the high efficiency of the division process. Studies dealing with cell division, have mainly dealt with the regulation of septum placement and the localization of the peptidoglycan synthesis machinery, while much less is known about the way attachment of other components to the cell wall is regulated in space. In this work we developed a novel method for the detection of sub-surface antigens in *S. pyogenes* by immunofluorescence. This assay allowed us to address the regulation of protein anchoring to the cell wall of *S. pyogenes*.

7.1 Comparison of the new immunofluorescence protocol to previously available methods

The majority of the information available regarding the spatial regulation the cellular processes of the bacterial cell was gained through the use of fluorescent and electron microscopy, each offering a characteristic set of advantages and disadvantages. In the current work we presented a novel method for the localization of sub-surface antigens in *S. pyogenes* through the use of immunofluorescence. The following section will discuss the different localization methods and the advantages and disadvantages of the new method compared to previously available techniques.

7.1.1 Localization of antigens through the use of electron microscopy (EM)

Immuno-EM is a technique that uses gold conjugated antibodies to determine the location of antigens in EM samples. Sample preparation involves fixation and embedding of the cells in a carrying matrix, which is then cut into ultra-thin sections, and reacted with the antibodies. The gold conjugates which are seen as electron dense spots in the EM image, allow localization of the antigen. This method has several advantages, the most significant of which is the high sample resolution obtained by the electron microscope. Additionally, no specific permeabilization method is required since samples are sliced mechanically allowing antibodies to reach any point in the cell.

On the other hand this method also carries significant disadvantages. The fixation and embedding procedures required for the preservation of sample morphology are generally significantly harsher than those needed for immunofluorescence (Rosch & Caparon, 2004). Severe fixation of the sample, and in particular the use of a high concentration of glutaraldehyde, may lead to the destruction of certain epitopes, leading to reduced sensitivity (in our hands). Additionally, since the cells studied are sliced at random, the distribution of an antigen cannot be studied throughout an entire cell but only a limited portion. The cellular distribution therefore can only be inferred by studying a large number of sections.

While the sample resolution of electron microscopy appears to be superior to that of immunofluorescence, the resolution of the immune signal is generally much lower. The term immune signal resolution refers to the number of immunoglobulin

molecules that are represented in the observed signal. Each gold dot observed in immuno-EM is the result of a single immunoglobulin molecule, and the detection of 5-10 gold dots is generally considered sufficient to establish a concentration of the antigen at that location (Rosch & Caparon, 2004, Rosch & Caparon, 2005, Ping et al., 2008, Kline et al., 2009). The number of fluorescently labeled antibodies required to produce a significant fluorescent signal on the other hand, is much larger, as can be learned from the much higher complexity of the images obtained, and the ability to discern a wide range of antigen concentrations in different locations in the cell. Immuno-EM has been used successfully to study the localization of a number of antigens in *S. pyogenes* (Rosch & Caparon, 2004, Rosch & Caparon, 2005, Ping et al., 2008), and has been the only viable method for the study of cytoplasmic and membranal antigens at the time this study was undertaken.

Scanning immuno-EM was used by Carlsson et al. to detect the location of M-protein and SfbI (Carlsson et al., 2006) on the surface of *S. pyogenes*. In contrast to transmission immuno-EM discussed above, the fixed cells are not embedded in resin and sliced, but are instead attached to a glass slide, exposed to gold-labeled antibodies, and viewed directly on the scanning microscope. This method combines the advantages of high sample resolution, with the ability to view an entire cell rather than a thin section. However, since the cells are not permeabilized in any way, this method is only suitable for the imaging of surface exposed antigens, and therefore cannot be used to localize subsurface molecules such as sortase.

7.1.2 Localization of antigens by fluorescent microscopy

Localization of antigens by fluorescence microscopy is carried out either through the cellular expression of a recombinant protein fused to a fluorescent tag such as Green Fluorescent Protein (GFP), or through the use of fluorescently labeled antibodies (Giepmans *et al.*, 2006). In recent years the resolving power of microscopes has been greatly enhanced by the advent of both better microscopes, and the utilization of computerized image processing (i.e. deconvolution of the captured images) (Frischknecht *et al.*, 2006, Garini *et al.*, 2005, Swedlow & Greenfield Sluder and David, 2007, Bolte & Cordelieres, 2006).

The use of fluorescent protein tags has become increasingly popular in recent years, as many different variants of GFP and other fluorescent tags became available, covering the visible and infrared spectra (Giepmans et al., 2006, Shaner *et al.*, 2007, Shaner *et al.*, 2004). The fluorescent tag is typically fused to either the N- or C-terminus of the protein of interest and expressed from a plasmid or following integration into the genome. The most prominent advantage of this method is that there is no need for fixation or permeabilization. This enables live imaging of the cells and generally preserves the cellular morphology. On the other hand, fusion of the protein to a ~30kDa fluorescent tag may potentially cause alterations in its correct localization or to its interactions with other proteins in the cell. Additionally, most GFP variants form dimers and tetramers, which may cause aggregation of the protein; this problem is partly mitigated in newer generation fluorescent protein tags, which do not tend to dimerize (Shaner et al., 2007). While fluorescent protein tags may be suitable for the study of *S. pyogenes* cytoplasmic proteins, GFP fails to fold correctly

and become fluorescent following passage through the SecYEG channel (Feilmeier *et al.*, 2000), and is therefore not suitable for the localization of sortase or its substrates.

Immunofluorescence uses specific antibodies to localize native antigens in the cell. Cells are fixed, typically using a paraformaldehyde / glutaraldehyde mix, and reacted with antibodies specific for the antigen under study. These antibodies can be themselves conjugated to fluorescent dyes or be detected using a secondary antibody conjugate. If the antigens studied are found within the cell (i.e. membranal or cytoplasmic proteins), an additional permeabilization step must be employed. Treatment with detergents is usually sufficient to allow antibodies access into mammalian cells, however permeabilization of the bacterial cell wall requires treatment with a cell wall hydrolase (Levin, 2002).

The main advantage of immunofluorescence is that the protein being studied is the native protein, and is not altered in any way. Additionally, a whole plethora of dyes and reagents are available for the labeling of different components of the cell such as DNA and various membrane components. The main disadvantage of immunofluorescence is that since the cells are fixed only "snapshots" can be taken, making the study of dynamic protein localization in real time impossible. Additionally, the fixation and permeabilization processes are inherently harmful to the morphology of the cells to some extent, and care must be taken to keep this damage to a minimum.

Permeabilization of the cell wall of model organisms such as *E. coli* and *B. subtilis* is routinely performed through the use of the muralytic enzyme lysozyme (Maddock & Shapiro, 1993, Harry *et al.*, 1995, Levin, 2002). Lysozyme has also been

used successfully with *S. pneumonia* (Morlot *et al.*, 2003) although in this case deregulation of autolysins may also play a role in the permeabilization of the cell wall.

Organisms resistant to lysozyme require special treatment to allow antibodies to reach sub-surface antigens. Permeabilization of *Metabacterium polyspora* to antibodies has been achieved through an extended treatment with a combination of mutanolysin and lysozyme (Angert & Losick, 1998), and *S. aureus* has been permeabilized using lysostaphin, an enzyme specific for the peptidoglycan of this organism (Pinho & Errington, 2003). Due to the high resistance of *S. pyogenes* to lysozyme (Krause & McCarty, 1961, Gallis et al., 1976) (and our own observation), and the extremely poor cell morphology obtained following treatment with mutanolysin, which requires prolonged treatment with highly concentrated enzyme before permeabilization of some of the cells is observed (not shown), we sought a different muralytic enzyme with a high efficiency in the permeabilization of the streptococcal cell wall.

7.1.3 A method for the study of *S. pyogenes* cytoplasmic and membranal antigens by immunofluorescence

In this study we presented a method for use of the phage lytic enzyme PlyC in the permeabilization of *S. pyogenes* for immunofluorescence microscopy. PlyC is encoded on the genome of phage C1 (Fischetti *et al.*, 1971). It is composed of one large catalytic subunit and eight smaller subunits, which facilitate the binding of the enzyme to the *S. pyogenes* cell wall carbohydrate (Nelson et al., 2006). This enzyme

demonstrates a uniquely potent amidase activity in the degradation of the *S. pyogenes* cell wall (Nelson et al., 2006) (Koller *et al.*, 2008).

Cells are initially fixed with paraformaldehyde and glutaraldehyde, both of which are cross-linkers covalently linking proteins and other components in the cell. Compared to the fixation required for electron microscopy studies, this fixation is generally a gentle one, aimed at preserving the maximum amount of reactive epitopes in the cell. Since fixation of the cells does not permeabilize the membrane (Bone & Denton, 1971), methanol is used for this purpose. Methanol treatment was first suggested for use in immunofluorescence as a fixative by Hiraga et al. (Hiraga *et al.*, 1998), and has been used in immunofluorescence protocols in other bacteria (Morlot et al., 2003). When fixed cells not permeabilized with methanol are treated with PlyC, the high osmotic pressure within the cell results in membrane bulging through PlyC-generated holes in the cell wall and cell lysis. Membrane bulging as a result of lysin treatment has previously been described in *B. anthracis* (Schuch *et al.*, 2002) and *S. pneumoniae* (Loeffler *et al.*, 2001).

Several steps have been taken to ensure maximum preservation of the cellular morphology. The PlyC exposure time has been limited to 10 minutes, far less than common immunofluorescence protocols (Levin, 2002). Additionally, the amount of PlyC used was empirically set as the minimal amount that would allow permeabilization of the entire cell population. The morphological effects of this treatment were evaluated through scanning-EM imaging to ensure adequate results. Under the conditions used, treatment of fixed *S. pyogenes* cells does not result in complete degradation of the cell wall, but rather in weakening and fragmentation of

the peptidoglycan. However, when fixed cells lacking M-protein are treated with PlyC however, the cell wall is completely removed, leaving behind spheroplasts. This phenomenon is reproducible whether lack of M-protein on the bacterial surface is the result of a deletion of the *emm* gene, a deletion of the sortase gene (which prevents the anchoring of M-protein to the cell wall), or if M-protein is removed by trypsin treatment. M-protein therefore plays a critical role in the preservation of the morphology of the cells.

M-protein is a α-helical coiled-coil protein, which form fibrils that extend 60 nm from the cell surface (Fischetti, 1989). These fibrils can interact with each other, stabilizing both molecules (Frick *et al.*, 2000). We propose that the non-covalent interactions between M-protein molecules places them in an advantageous position to be cross-linked during fixation, forming a mesh that binds the fragmented cell wall together, and facilitate the preservation of the cellular integrity following PlyC treatment.

Spheroplasts produced when fixed cells lacking M-protein are treated with PlyC have several advantages compared to spheroplasts produced using traditional methods in suspension. Since the cells are fixed and attached to the slide prior to the application of PlyC, the spheroplasts that form preserve their correct cellular dimensions and chain orientation. This is in stark contrast to spheroplasts generated when unfixed cells are suspended in hypertonic solution containing a muralytic enzyme, a process that results in the breakdown of the chains and the loss of correct cellular dimensions, making the resulting spheroplasts poor candidates for study by fluorescence microscopy. Spheroplasts produced through our method often retain

some cell wall material trapped at the septum, which can serve as an excellent septal marker following WGA labeling. The reason for the preservation of wall material at the septum is unclear. One possibility is that the septum is less accessible to PlyC, a hypothesis supported by the fact that cell wall material is completely removed when a higher concentration of PlyC is applied. Other possibilities are that the septal invagination helps keep the cell wall in place following fixation, or that the septum is enriched in membranal proteins to which wall material in linked following fixation.

The clean removal of the peripheral cell wall from M-negative cells may be explained in part by the presence of a periplasmic space between the membrane and the cell wall of Gram-positive organisms (Matias & Beveridge, 2005, Matias & Beveridge, 2006, Zuber et al., 2006). Such clean removal would be less likely if the cell wall and membrane were directly adjacent, due to possible cross-linking of membranal proteins and cell wall moieties.

We propose that phage lytic enzymes could be useful in permeabilization protocols in many Gram-positive bacteria, and in particular those of medical importance. In recent years, a large number of lysins active against a wide range of Gram-positive pathogens have been studied, mostly for their potential as anti-bacterial agents and their use in the decontamination of mucosal surfaces. These include lysins specific for *B. anthracis* (Schuch et al., 2002, Yoong *et al.*, 2006), *S. pneumoniae* (Diaz *et al.*, 1990, Loeffler et al., 2001), *S. pyogenes* (Fischetti et al., 1971, Nelson et al., 2006), *Enterococcal* species (Yoong *et al.*, 2004), *S. aureus* (Daniel *et al.*), and many more (Fischetti, 2005, Fischetti, 2008). Owed to their medical potential, many additional enzyme are likely to be isolated. These enzymes

may prove as invaluable tools in the study of the cellular processes of major human pathogens, and in the discovery of potential cures.

Lysins possess one (or more) of four types of catalytic activities, each directed against a different major bond in the peptidoglycan. These include endo-β-N-acetylglucosaminidase, N-acetylmuramidase, endopeptidase, and N-acetylmuramoyl-L-alanine amidase activities (Fischetti, 2005). An ideal cell wall hydrolase would permeabilize the cell wall to antibodies while causing a minimal amount of damage to the general cellular morphology. The availability of numerous phage lysins for many of the medically important pathogens would allow an empirical determination of the best enzyme for the permeabilization of each. It would also be of interest to determine whether a certain peptidoglycan bond provides as a rule a better target for hydrolysis in terms of the preservation of cellular morphology.

7.2 Spatial distribution of the protein sorting reaction and its relation to other cellular processes

7.2.1 Introduction

The sorting reaction for surface proteins is initiated with the synthesis and export of a nascent wall-protein through the Sec apparatus. During protein export, the C-terminal anchor domain of a nascent surface protein is stalled in the secretion channel, leaving the LPXTG motif exposed on the outer surface of the membrane. The transpeptidase sortase then cleaves this motif between the threonine and glycine residues (Mazmanian et al., 1999), and attaches the freed threonine to the peptidoglycan precursor, Lipid II (Perry et al., 2002). The lipid II – surface protein complex then serves as substrate for peptidoglycan synthesis by penicillin binding proteins, leading to the covalent attachment of the protein to the cell wall.

Through the used of the immunofluorescence protocol described above, we found that sortase localizes to a number of foci in *S. pyogenes* cells, and that these foci are preferentially but not exclusively associated with the septum of this organism. Localization to the septum begins at a very early stage when sortase can be found associated with the equatorial rings of the future daughter cells. Sortase foci were always associated the outer surface of the *S. pyogenes* membrane.

Sorting of proteins to the cell wall however cannot be fully understood unless viewed in the context of other cellular processes, all of which function together in the course of cell division. Other factors that may interact with sortase include components of the secretion apparatus, lipid II translocase, and PBPs, which are all required for the sorting reaction. While some information is available regarding the

mechanism directing the peptidoglycan synthesis at the septum, it is not clear at present what governs the spatial distribution of sortase or how different proteins are targeted to specific regions of the cell. Better understanding of how all these distinct processes interact in the creation of the external bacterial envelope is the goal of future research.

7.2.2 Localization of sortase and the secretion apparatus

As mentioned above, sortase cleaves the LPXTG motif found in the C-terminus of nascent surface proteins while the protein is stalled at the secretion channel (Navarre & Schneewind, 1994). Colocalization of sortase and the secretion apparatus may therefore contribute to efficient protein sorting. Two groups have studied the localization of SecA, a soluble ATPase associated with the Sec channel, and have obtained conflicting results. While Caparon's group reported that SecA localizes to a single membranal micro-domain (Rosch & Caparon, 2004), Lindahl's group reported that SecA is randomly distributed in the membrane (Carlsson et al., 2006). The reason for this difference is not clear as both groups used immuno-EM and the same serum. One possibility however is that since SecA is a soluble protein that is dynamically distributed between membranal secretion channels and the cytoplasm (Cabelli et al., 1991), a small differences in growth conditions could have affected its distribution.

Caparon's group showed that both SpeB and the chaperone HtrA colocalize with SecA in a single microdomain, which they termed ExPortal (Rosch & Caparon, 2004, Rosch & Caparon, 2005). The ExPortal resides at a membranal region enriched

in anionic phospholipids, which can be visualized with nonyl acridine orange NAO (Rosch et al., 2007). Sortase A and SecA were found to colocalize in the ExPortal in *S. mutans* (Ping et al., 2008). In *E. faecalis*, both sortase A and sortase C (responsible for the polymerization of pili) were found to be colocalized with SecA at the ExPortal (Kline et al., 2009).

When compared to other organisms, targeting proteins to specific locations in the streptococcal and enterococcal cells through the localization of the Sec apparatus is unique. In most other documented cases, the ultimate distribution of the translocated proteins is not similar to that of the secretion apparatus. In these cases, secretion is either targeted to a particular region of the cell through interaction with factors other than the secretion apparatus, or through drift in the membrane and capture at the desired location (Janakiraman & Goldberg, 2004, Shapiro *et al.*, 2002, Brandon et al., 2003). In *Shigella* for example, the autotrasporter IcsA is localized to the pole despite being secreted through the Sec apparatus, which is circumferentially distributed in this organism (Brandon et al., 2003). Secretion in *Listeria monocytogenes* is not localized to a specific region of the cell but occurs at locations along the cylindrical wall of the bacterium. Nevertheless, ActA is localized asymmetrically to one of the poles, in a manner required for directed actin polymerization, responsible for bacterial motility within the infected host cells (Rafelski & Theriot, 2006).

While the localization data for *S. mutans* and *E. faecalis* sortases were obtained using Immuno-EM, we used immunofluorescence to study the localization of *S. pyogenes* SrtA (Raz & Fischetti, 2008). We found that SrtA in this organism

generally localizes to more than one focus per cell, and that the foci are predominantly associated with the division septum. It is not clear at the moment whether the observed difference in the localization patterns is the result of a different mechanism for surface-proteins anchoring in these organisms, or whether it could result from the difference in the microscopy method used. As discussed above, there are two major differences between immunofluorescence and immuno-EM that may lead to such a difference in the observed localization pattern. First, when using immunofluorescence the antigens distribution is studied in an intact cell, whereas only a thin section can be studied using immuno-EM. When a sortase focus is found in such a section, one can postulate from the occurrence frequency of such foci whether or not additional foci are likely to be found in the cell, but direct study of this question is possible only if all adjacent sections are examined, a procedure that was never employed in any of these studies (Ping et al., 2008, Kline et al., 2009). Secondly, the immunofluorescent signal is typically the result of many more antibodies, each contributing a smaller relative portion of the signal, when compared to the signal observed in immuno-EM. In the later case, each gold dot observed is the result of a single antibody, and only few such dots are required to establish a concentration of antigen at a given location. It is possible therefore that only very strong foci can be detected through immuno-EM, while smaller foci, which could be resolved through the use of deconvolution immunofluorescence microscopy, are discarded as noise when only one or two gold particles are observed.

When we tested the anchoring pattern of two different surface proteins, M-protein and SfbI, we found no correlation between the site of active anchoring of

either protein and the location of a NAO-stained membranal anionic lipid microdomain, in which the ExPortal resided (Rosch et al., 2007). M-protein anchoring sites coincided with the ExPortal only when the latter was found at the septum, which was not a general phenomenon. SfbI anchoring was more diffuse, and generally covered a much larger area than the compact ExPortal.

When sortase is deleted, surface proteins remain associated with the cell wall to some extent but are not anchored covalently to the cell wall. Following a trypsinization-regeneration type experiment, the signal observed better reflects the localization of active protein secretion since in this case protein anchoring does not affect the observed distribution. Kline et al. reported that sortase substrates accumulate at the ExPortal of *E. faecalis* when sortase is deleted (Kline et al., 2009). Deletion of sortase genes also resulted in an alteration of *S. pneumoniae* pilus assembly sites (Falker *et al.*, 2008). When the *S. pyogenes* sortase A mutant strain AR01 was tested however, the secretion patterns of M-protein and SfbI were similar to those observed in the WT strain, although the amount of SfbI expressed was reduced. As in the WT strain, we observed no correlation between regions where M-protein or SfbI accumulated, and the NAO-labeled anionic lipid microdomain. Since secretion of these two proteins occurred simultaneously at different regions of the cell, it is highly unlikely that they are both secreted through the same single ExPortal unless the ExPortal is highly motile.

We will approach this question through live imaging microscopy. NAO stained cells will be placed on agarose pads within cavity microscope slides, and allowed to grow and divide normally at 37^{o}C. The movement of the NAO-labeled

ExPortal will be monitored through time-lapse imaging. We consider such rapid movement of the ExPortal highly unlikely however, based on the results obtained when cells are embedded in agarose containing a quenched SpeB substrate (Rosch & Caparon, 2004). Presumably, rapid motion of the ExPortal would result in delocalized secretion of SpeB, a pattern different from the one-focus-per-cell distribution observed. The question of whether sortase and elements of the secretion apparatus are colocalized will ultimately be answered only when sortase distribution is compared to that of the secretion channel itself (SecY, SecE, SecG), rather than only to the soluble SecA.

7.2.3 Different secretion and anchoring patterns of M-protein and SfbI

The secretion and anchoring patterns observed for M-protein and SfbI were in agreement with previous observations, displaying M-protein anchoring concomitantly with peptidoglycan synthesis at the septum (Cole & Hahn, 1962, Swanson et al., 1969), and SfbI anchoring at the poles (Ozeri et al., 2001, Carlsson et al., 2006). These observations therefore fit better with the model suggested by Carlsson et al. according to which the localized secretion and anchoring of surface proteins is not governed by the localization of SecA to a single ExPortal, but rather, the proteins are directed to the correct region of the cell by information encoded in the signal sequence, within the protein itself (Carlsson et al., 2006). The mechanism targeting these proteins to their respective locations on the cell wall is not known at present. A YSIRK/GS motif is found in the signal sequence of M-protein but not that of SfbI. This motif was proposed to play a role in the targeting of M-protein to the septum,

however mutation of this sequence had no effect on M-protien targeting (Carlsson et al., 2006).

Another organism in which two distinct anchoring patterns were observed is *S. aureus*. In contrast to *S. pyogenes*, which always divide in parallel to the previous division plane, *S. aureus* cells divide in three alternating perpendicular planes, with sister cells remaining attached to each other after division (Tzagoloff & Novick, 1977). Despite this difference, there are striking similarities in the distribution of surface proteins between *S. pyogenes* and *S. aureus*. As in *S. pyogenes*, the signal sequence directs *S. aureus* surface proteins for secretion at specific regions of the cell (DeDent et al., 2008). Protein A, which possesses a YSIRK/GS motif in its signal sequence, is anchored to 2-4 discrete foci, which are distributed in a ring like structure associated with, but not necessarily parallel to, the division septum. (DeDent et al., 2007). This type of distribution is shared with other wall-anchored proteins possessing a YSIRK/GS motif, while proteins that do not possess this motif are anchored in a hemispherical distribution (DeDent et al., 2008). Given the similarities in surface protein distribution, it would be of interest to determine how *S. aureus* SrtA is distributed. We are currently studying this question. It is also of note that almost no *E. faecalis* proteins posses a YSIRK/GS motif in their signal sequence (Kline et al., 2009, Paulsen *et al.*, 2003), which may provide a possible explanation to the difference in sortase distribution between *S. pyogenes* and *E. faecalis*. Presumably if no proteins are targeted to the septum there is less need for the presence of sortase at this location.

7.2.4 How can sortase localization be explained in light of the different secretion and anchoring patterns of M-protein and SfbI?

Given that surface proteins are anchored at both the septum and the poles, it is not immediately clear why sortase is much more prevalent at the septum. The reason may lie in the anchoring kinetics of septal versus polar proteins. M-protein anchoring occurs solely at the septum (Cole & Hahn, 1962, Swanson et al., 1969). Newly anchored M-protein is always colocalized with areas of active cell wall synthesis, when labeled with vancomycin-BODIPY (Raz & Fischetti, 2008). This area of active anchoring represents only a very small portion of the overall cell wall. Since synthesis of peptidoglycan at the septum is a rapid process, there is a relatively short "window of opportunity" for the anchoring of M-protein at this location, before the peptidoglycan matures to become part of the peripheral cell wall. Such rapid anchoring at the confined space of the septum is likely to require a high concentration of sortase.

SfbI anchoring on the other hand occurs throughout a much larger portion of the cell wall, and the anchoring rate of this protein appears to be much slower. When cells from which surface protein has been removed by trypsin treatment, are allowed to regenerate surface proteins for defined periods of time, the intensity of M-protein labeling at the septum is as strong as that of the mature cell wall. SfbI labeling on the other hand is anchored in a much more diffuse fashion and only reaches mature level of fluorescence after some time has passed. Following regeneration of surface protein for two generations, the older poles, recognized through the fact that they lack M-protein labeling, generally showed stronger SfbI labeling compared to younger poles,

which were allowed to anchor SfbI for a shorter time period. In a different assay, cells labeled with fluorescent WGA, were washed, and allowed to divide for two generations. These cells too, displayed a prevalence of more intense SfbI labeling at older poles, labeled with WGA. The sum of these observations suggests that SfbI anchoring is a slow and continuous process, and is likely therefore to require a smaller amount of sortase. This slow kinetics is adequate however, since the time frame available for the anchoring of proteins to the pole is much longer. A model for the anchoring patterns of M-protein and SfbI is presented in figure 7.1.

While the slow rate of SfbI anchoring at the poles may be facilitated by the small sortase foci sometimes visible at the poles, we cannot rule out the possibility that a low basal level of membranal sortase, which may be below the detection limit of our current techniques, could also be functional in the anchoring of SfbI. When the sortase KO strain AR01 is complemented with the sortase gene containing plasmid pAR107, the resulting strain typically produces about 1/20 of the WT amount of sortase. Despite this fact, both M-protein and SfbI are efficiently anchored to the cell wall in laboratory conditions. It is possible that the apparent excess of sortase is needed for survival in the less ideal conditions found in the human host or that another cell wall protein, for which we do not have immunofluorescence data, requires a larger amount of sortase for its correct anchoring to the cell wall. The fact that such a small amount of sortase is functional in protein anchoring however, means that we cannot rule out the possibility that concentrations of sortase below our detection level could still be important for the cellular functions of *S. pyogenes*.

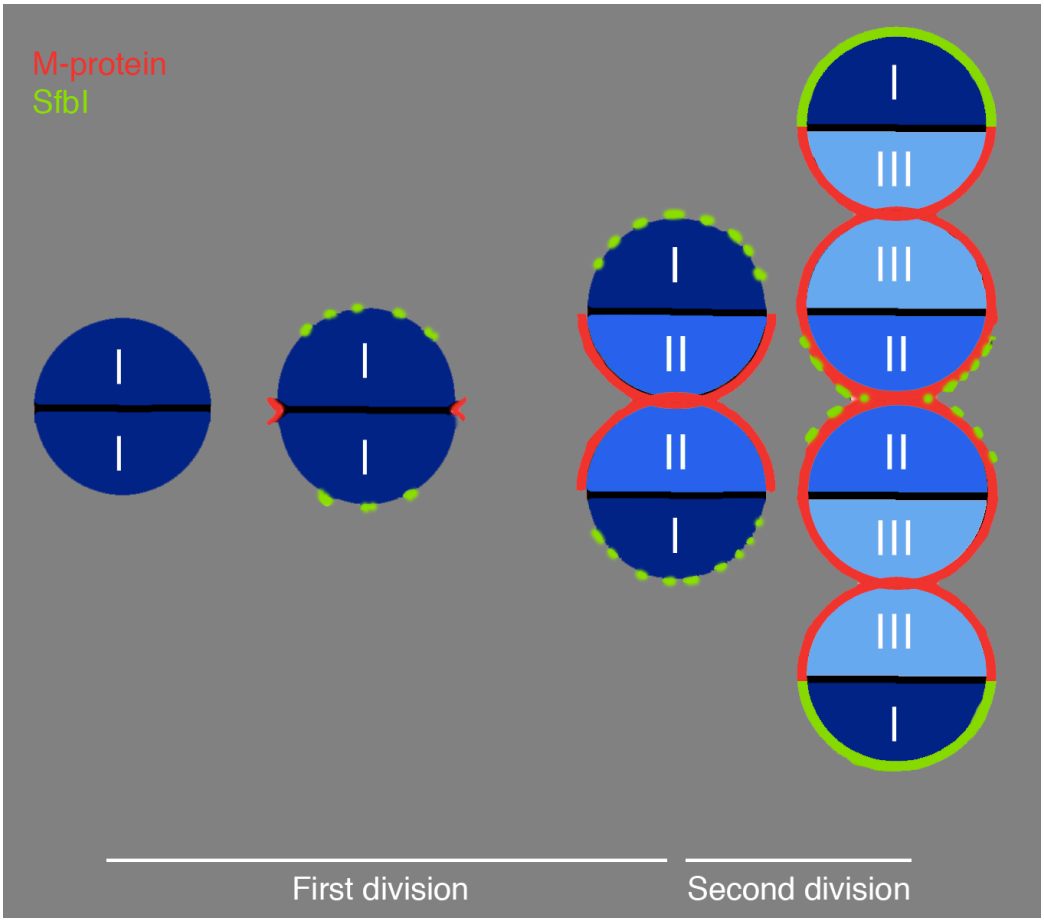


Figure 7.1 A model for the different anchoring kinetics of M-protein and SfbI

The fate of surface proteins is followed through two cell divisions. (I) old wall, (II) wall synthesized during the first division, (III) wall synthesized during the second division. On the left, a cell is displayed from which surface proteins were removed enzymatically. M-protein is anchored at a rapid pace solely at the septum, leading to coverage of newly synthesized cell wall. SfbI is anchored at a slow pace at the old poles. SfbI fluorescence gradually increases as the cell wall matures. Also see figure 4.6.

7.2.5 Correlations between the localization of sortase and the cell division machinery

Biochemical evidence suggests a close connection between the sorting reaction and cell wall synthesis machinery. Following cleavage of the LPXTG motif in a nascent surface protein by sortase, the freed threonine is attached to lipid II, the peptidoglycan precursor, which then serves as substrate for penicillin binding proteins (PBPs) resulting in the covalent attachment of the protein to the cell wall. Correct localization of the PBPs and the export mechanism for lipid II are therefore important for both cell wall synthesis and protein anchoring.

7.2.5.1 Regulation of septum assembly

The spatial regulation of cell division and peptidoglycan assembly has been studied in several model organisms including *E. coli*, *B. subtilis*, and *S. pneumoniae*. (Buddelmeijer & Beckwith, 2002, Errington *et al.*, 2003, Goehring & Beckwith, 2005, Sauvage *et al.*, 2008, den Blaauwen *et al.*, 2008, Zapun et al., 2008). Assembly of division proteins at the septum begins with the assembly of an FtsZ ring at this location. FtsZ is a tubulin homologue, ubiquitous among bacteria, that polymerizes into filaments that comprise the septal ring (Errington et al., 2003). The FtsZ ring serves as an internal scaffold that directs the placement of other division related proteins, such as FtsQ/DivB, FtsL, FtsB/DivIC, which are recruited in a stepwise process leading to the assembly of PBPs at the septum, and the synthesis of cell wall at this location (Goehring & Beckwith, 2005, Sauvage et al., 2008).

Placement of the FtsZ ring is regulated through various mechanisms. The nucleoid occlusion system (Mulder & Woldringh, 1989), mainly active in rod shape bacteria, does not allow the FtsZ ring to form in areas adjacent to the nucleoid. Following the segregation of the nucleoids in rod shaped bacteria, only the center of the cell and the poles are devoid of DNA and therefore permissive for FtsZ ring assembly (Errington et al., 2003). The nucleoid occlusion system does not appear to play a role in cocci since segregation of the daughter nucleoids typically only takes place after formation of the FtsZ ring (Morlot et al., 2003).

Another system that controls the placement of the FtsZ ring is the Min system, which is typically composed of MinC, MinD, and MinE. This system prevents the formation of an FtsZ ring near the poles. In *E. coli* the Min system functions by oscillation between the poles while in *B. subtilis* components of this system are permanently retained at the poles through interaction with the division protein DivIVA (Marston & Errington, 1999, Errington et al., 2003). Components of the Min system are absent from *S. pyogenes* however DivIVA is present. Deletion of *S. pneumoniae* DivIVA (Fadda *et al.*, 2003) and *E. faecalis* DivIVA (Ramirez-Arcos *et al.*, 2005) results in division and morphological defects. In *S. pneumoniae* DivIVA localizes to the septum and poles (Fadda *et al.*, 2007), suggesting that it may play a role in localizing downstream factors to these regions in this bacteria too.

Placement of the FtsZ ring in streptococci always occurs at the mature equatorial rings. Whether this is the result of factors bound to the equatorial rings that aid in FtsZ ring assembly, or merely from the fact that the equatorial rings are the regions with the largest diameter in the cell, thus making FtsZ ring assembly more

energetically favorable, is not clear at the moment. While the control of cell division in streptococci is not as well understood as that of *E. coli* or *B. subtilis*, both the FtsZ ring and DivIVA are interesting candidates that may have a role in the direct or indirect localization of sortase. Further study is required to test the relative distribution of sortase and these factors throughout the cell cycle.

7.2.5.2 Localization patterns of penicillin binding proteins (PBPs)

Following FtsZ ring assembly, other division factors are recruited to the FtsZ ring, and these in turn are believed to recruit the PBPs. Although no data is available regarding the localization pattern of *S. pyogenes* PBPs, *S. pneumoniae* high molecular weight PBPs were shown to localize to the division septum (Morlot et al., 2003, Zapun et al., 2008). Localization of PBPs to the septum occurs subsequently to the polymerization of the FtsZ ring, and they remain associated with the closing septum even after the formation of an FtsZ ring at the septa of the daughter cells. In many cases PBPs are associated with both the closing mother cell septum, and the forming daughter cells septa (Morlot et al., 2003). This localization pattern is interesting since sortase is also simultaneously localized to both mother and daughter cells septa.

In contrast to high molecular weight PBPs, the low molecular weight PBP3, which possess a DD-carboxypeptidase activity, localizes to the periphery of the cell but is not present at the septum. Deletion of this enzyme results in delocalization of other PBPs, raising the possibility that cleaving the D-ala – D-ala moieties in mature peptidoglycan by PBP3 may restrict the localization of high molecular weight PBPs to the septum by destroying their substrate at other locations in the cell (Morlot *et al.*,

2004). It would be interesting to compare the localization pattern of sortase to that of high molecular weight PBPs in a PBP3 mutant, to determine whether localization of sortase is also disrupted, and if so, whether it is distributed in the same manner as the PBPs. Additionally it would be of interest to test the effect deletion of PBP3 has on the anchoring distribution of M-protein and SfbI.

7.2.5.3 Lipid II translocation

Lipid II is not translocated across the plasma membrane at random, but rather at specific locations where it serves as substrate for peptidoglycan synthesis and protein anchoring. Vancomycin-BODIPY has been used to localize sites of lipid II translocation and peptidoglycan synthesis, owing to its ability to bind the D-ala – D-ala residues found in lipid II and young peptidoglycan (Pinho & Errington, 2003). As the peptidoglycan matures, the bond between the two D-ala moieties is cleaved through the action of high and low molecular weight PBPs, and vancomycin-BODIPY binding is diminished accordingly. Labeling of *S. aureus* cells with vancomycin-BODIPY reveals a strong septal band (Pinho & Errington, 2003). *S. pneumoniae* cells also display labeling of the equatorial rings at certain stages of the growth cycle, in addition to the labeling of the septum (Daniel & Errington, 2003, Ng et al., 2004). The vancomycin-BODIPY labeling pattern displayed by *S. pyogenes* resembles that of *S. pneumoniae* in that both the septum and mature equatorial rings are labeled (Raz & Fischetti, 2008).

Translocation of lipid II across the cytoplasmic membrane has been proposed to be carried out by members of the SEDS family (for shape, elongation, division and

sporulation), although this has not been experimentally proven (Ehlert & Holtje, 1996) (Ghuysen & Goffin, 1999). SEDS proteins are integral membrane proteins, with 10 membrane-spanning segments (Henriques *et al.*, 1998), which are usually found in the same operon as a class B PBP, and whose deletion generally has the same effect as the deletion the associated PBP (de Pedro *et al.*, 2001, Thibessard *et al.*, 2002). In *S. pneumoniae* FtsW is the SEDS protein associated with the septum (Gerard *et al.*, 2002), where it colocalizes with PBPs. Like PBPs, some FtsW is retained at the closed septum even after its assembly is initiated at the septa of daughter cells, and it can therefore be seen simultaneously at both locations (Morlot et al., 2004).

7.2.5.4 Protein anchoring and peptidoglycan synthesis – the septum

New cell wall is synthesized solely at the streptococcal septum (Zapun et al., 2008). Peptidoglycan synthesis at this location is fast and efficient, and is supported by the localization of high molecular weight PBPs (Morlot et al., 2003), as well as lipid II export (Daniel & Errington, 2003, Ng et al., 2004, Raz & Fischetti, 2008) at this location. FtsW, which is believed to be responsible for lipid II translocation, is also localized to the septum (Gerard et al., 2002, Morlot et al., 2004). Peptidoglycan synthesis at the streptococcal septum is divided into septal and peripheral, where septal synthesis forms the actual septum and peripheral synthesis is responsible for the elongated football shaped form of *S. pneumoniae* (Zapun et al., 2008). *S. pyogenes* on the other hand has no peripheral cell wall synthesis machinery, and is therefore much less elongated compared to *S. pneumoniae*. Components related to

peripheral wall synthesis that are lacking from the genome of *S. pyogenes* include the high molecular weight class B PBP2b, RodA (a SEDS family protein associated with PBP2b), and the MreC/MreD complex (believed to play a role in PBP2b localization) (Zapun et al., 2008).

As described above, M-protein is anchored solely at the septum (Swanson et al., 1969, Cole & Hahn, 1962, Carlsson et al., 2006) and is therefore coupled to septal peptidoglycan synthesis. Following removal of surface proteins by trypsin treatment, newly anchored M-protein appears in foci that localize exclusively to areas labeled by vancomycin-BODIPY (Raz & Fischetti, 2008), and are therefore the sites of active peptidoglycan synthesis. While M-protein initially appears in foci, vancomycin-BODIPY labeling tends to be even within the septal ring, suggesting that lipid II export and peptidoglycan synthesis are not likely to colocalize with sortase within the foci, but rather that sortase foci are targeted to the regions of peptidoglycan synthesis.

Interestingly, we found that in the later stages of cell division, M-protein is anchored simultaneously to the closing septum and the mature equatorial rings. This observation is in agreement with numerous other observations, all pointing to the possibility that division in streptococcal (and enterococcal) cells begins before the mother cell division is concluded. Vancomycin-BODIPY labeling of *S. pyogenes* cells showed that when simultaneous M-protein anchoring at the mother and daughter cells septa occurred, both septa were always labeled with Vancomycin-BODIPY, demonstrating simultaneous lipid II export at both locations, as is also the case in *S. pneumoniae* (Ng *et al.*, 2004). The distribution pattern of sortase is also in agreement with simultaneous M-protein anchoring at both locations, as foci begin to assemble at

the equatorial rings at an early stage, when the majority of sortase is still associated with the mother cell septum. In *S. pneumoniae* high molecular weight PBPs (Morlot et al., 2003, Zapun et al., 2008) as well as FtsW (Morlot et al., 2004) are also distributed simultaneously to both the closing mother cell septum and forming daughter cells septa at certain stages of the cell cycle. Finally, in *Enterococcus faecium* the initiation of cell division begins at a constant cell size. In the presence of DNA synthesis inhibitors, chromosome replication is delayed, and as a result closure of the mother cell septum is inhibited. Nevertheless splitting of the equatorial rings and cell division is initiated in the daughter cells as soon as the proper cell size has been reached (Gibson et al., 1983).

The combination of these observations suggest that the cell division of streptococci is a continuous process, where the division in the daughter cell, and with it the anchoring of proteins to the new septum, occurs simultaneously with the last lag of septum closure in the mother cell. This mechanism explains the retention of the cells in a chain, and may contribute to the division efficiency in this organism, by shortening the effective time required for cell division.

7.2.5.5 Protein anchoring and peptidoglycan synthesis – the poles

Since biochemical evidence suggests that lipid II and PBPs are required for the covalent attachment of surface proteins to the cell wall, and since cell wall synthesis in streptococci is confined to the septum, anchoring of SfbI at the old pole is less easily explained. Nevertheless, the possibility that some peptidoglycan turnover occurs at the poles should not be ruled out entirely.

First, bacteria possess an array of enzymes dedicated for peptidoglycan recycling (Park & Uehara, 2008). Although not much is known about how these enzymes are localized, it is possible that they may act at the cell periphery in addition to the septum. Secondly, evidence exists that PBPs may add material to the inner face of the cell wall resulting in its thickening (Higgins & Shockman, 1976, Lleo *et al.*, 1990). It is not clear however whether this mechanism also plays a role in *S. pyogenes* since as mentioned above, this organism lacks a secondary mechanism for peripheral peptidoglycan synthesis, including PBP2b and related factors. Additionally, as mentioned above, some *S. pneumoniae* high molecular weight PBPs (Morlot et al., 2003, Zapun et al., 2008) and FtsW (Morlot et al., 2004), linger at the closing mother cell septum even after division has started in the daughter cells. It may be possible that a small amount of these factors may still be functional in the anchoring of SfbI.

The fact that SfbI is anchored to the old pole is within itself a supporting evidence for the presence of some peptidoglycan turnover at the poles, since the action of PBPs is required for the incorporation of the surface protein – lipid II complex into the cell wall. Additionally, if no cell wall turnover takes place, it is not clear how SfbI becomes exposed on the cell surface following ifs anchoring at the membrane-wall interface. Presentation on the cell surface is not likely to be spontaneous since *S. aureus* SrtB substrates, related to the heme acquisition machinery of this organism, are not presented on the cell surface while SrtA substrates are, despite the fact that they are both covalently bound to the cell wall (Mazmanian *et al.*, 2003). It appears therefore that surface presentation requires

binding of a specific peptidoglycan structure that may direct the protein to the surface through peptidoglycan turnover.

Additional evidence for peptidoglycan turnover at the poles comes from the results of the WGA pulse-chase labeling experiment. In this experiment cells were pulsed with fluorescently labeled WGA, washed, and allowed to grow and divide in medium without WGA. Surprisingly, following two generations in media without WGA, WGA labeling could only be detected at the equatorial rings suggesting that these structures contain inert cell wall, while peptidoglycan at the peripheral wall may undergo slow turnover.

If cell wall turnover at the old poles indeed exist, it is likely to be very slow, due to the low presence of PBPs, and lipid II to this area. Additionally, if peptidoglycan turnover rate at the poles were significant, one would expect the gradual loss of covalently anchored M-protein from the cell wall, which is generally not encountered. It is plausible that while M-protein is anchored deep within the peptidoglycan, WGA is only bound to the outermost layer, resulting in its more rapid dissociation.

Future experiments will use live imaging to further study the kinetics of possible peptidoglycan turnover at the poles, and its relation to the anchoring of SfbI. These studies would apply in addition to WGA, fluorescently labeled PlyC binding domain, which bind the cell wall carbohydrate (Nelson et al., 2006), as well as N-reactive dyes that would covalently bind components of the cell wall (de Pedro *et al.*, 2004).

7.2.6 Future experiments

The availability of an immunofluorescence method for the study of sub-surface antigens in *S. pyogenes* will allow for a much more thorough analysis of the cellular functions of this organism. This method could therefore be used to determine the relative distribution of sortase as compared to a plethora of elements that either act together with sortase in the protein-sorting pathway, or are directly responsible for the localization pattern observed. Such factors may include components of the secretion apparatus, including SecA but more importantly the membrane embedded translocation channel itself (SecY, SecE, SecG), FtsW (due to it possible role in lipid II translocation across the plasma membrane), and the high molecular weight PBPs: PBP1a (spy1649), PBP1b (spy0097), PBP2a (spy2059), and FtsI (spy1664). It would also be interesting to compare the localization pattern of LPXTGase and sortase due to the similar substrate specificity of the two enzymes (Lee et al., 2002). Factors that may be involved in SrtA localization may include FtsZ due to its importance in septum organization (Vicente *et al.*, 2006) and DivIVA, due to its role in the localization of proteins to the septum and poles of *S. pneumoniae* (Fadda et al., 2007).

Depletion of FtsZ in *S. aureus* results in the delocalization of the cell wall synthesis apparatus, leading to the enlargement of the cell to up to eight times the ordinary cell size and eventually to cell lysis (Pinho & Errington, 2003). FtsZ depletion studies have not been carried out in streptococci, however the application of Zantrins which inhibit FtsZ ring formation resulted in cell enlargement in *S. pneumoniae* (Margalit *et al.*, 2004). It would be of interest to test the effect of Zantrins on SrtA localization, as well as on the sites of M-protein and SfbI anchoring.

Particularly, since Zantrins cause the delocalization of PBPs and the sites of lipid II translocation, it would be interesting to determine whether SrtA, and the sites of surface protein deposition, would be delocalized to the same sites as those of peptidoglycan synthesis, which may indicate regulation be the same machinery.

Additional experiments involve an attempt to target sortase to defined locations within the cell, and to determine the resulting effect on the anchoring pattern of different surface proteins. Sortase would be expressed as a fusion protein whose signal sequence / transmembrane domain is replaced with one from M-protein (septal localization), SfbI (polar localization), or HtrA (ExPortal localization). Signal sequences of some constructs will be modified as to prevent cleavage by signal peptidase (von Heijne & Abrahmsen, 1989) to retain sortase at the plasma membrane. This system is currently under construction.

7.3 Loss of M-protein in the sortase mutant

The initial expression level of M-protein in the *S. pyogenes* sortase mutant AR01 is about similar to that found in the parent strain when tested by Western blot, although M-protein is missorted to the supernatant, wall, and spheroplast fractions. When tested by fluorescent microscopy however, a small sub-population of cells not expressing M-protein is commonly visible. This is not the result of an initial mixed population since when a small aliquot of the initial batch is sonicated to break up the chains, and streaked on an agarose plate, cultures resulting from single colonies present the same mixed population.

7.3.1 Expression of M-protein is toxic, when not anchored to the wall

We propose that expression of M-protein in the SrtA-negative strain is toxic, and as a result spontaneous M-negative variants possess a competitive advantage, allowing them to gain prominence in the population over time. During the translocation of a nascent surface protein across the plasma membrane, the C-terminal cell sorting signal remains stalled in the secretion channel, placing the LPXTG motif exposed at the outer surface of the membrane (Navarre & Schneewind, 1994), where it is cleaved by sortase (Mazmanian et al., 1999). The fate of such a stalled surface protein in the absence of sortase is not absolutely clear, however fractionation experiments reveal that a much larger fraction of the protein is found attached to the spheroplast fraction compared to the wild type, suggesting a considerable delay in the protein release from the secretion channel. Such stalled proteins may clog the secretion channel, thereby preventing the secretion of other secretory proteins. If

proteins are released from the secretion channel intact, the presence of the C-terminal hydrophobic region is likely to lead to protein misfolding and aggregation. Release of the protein from the membrane by non-specific cleavage may lead to equally dire consequences. Expression of an M-protein variant that cannot form a coiled-coil structure, and is therefore misfolded, was shown to be toxic in *E. coli* (Ryan et al. unpublished). Aggregation of misfolded proteins in the eukaryotic ER (Schroder & Kaufman, 2005) and the periplasm of Gram-negative bacteria (Raivio & Silhavy, 2001, Duguay & Silhavy, 2004), which are equivalent to the Gram-positive membrane-wall interface (Matias & Beveridge, 2005, Matias & Beveridge, 2006, Zuber et al., 2006), were shown to have deleterious effects. It is likely therefore that sortase mutant cells not expressing M-protein would have a growth advantage that will manifest as a faster growth rate allowing them to eventually take over the population.

In agreement with this hypothesis, *S. pyogenes srtA* mutant cells showed slower growth rate compared to the wild type strain. In *S. aureus*, over-expression of a plasmid-encoded surface-anchored protein greatly reduces the viability of a *srtA* deletion mutant (Mazmanian *et al.*, 2001). Deletion *of S. aureus* sortase also results in the lowered expression of certain surface proteins (Marraffini et al., 2006). The mechanism, through which reduction of surface protein expression occurs however, has never been addressed.

To test this hypothesis we followed the expression level of M-protein in different AR01 (M+, SrtA-) cultures over a 6-day period. In all cases M-protein expression on the surface of the cells was lost within three growth cycles, 24 hours

each. In nine of the ten cases the expression of M-protein was altogether lost, supporting our hypothesis. The single colony that did express M-protein showed enhanced secretion into the medium, and almost no M-protein attached to the cells. This suggests that it is not the expression of M-protein *per se* that is causing the slower growth, but rather the accumulation of M-protein at the membrane-wall interface. The parental strain D471 never displayed this kind of rapid loss of M-protein expression despite numerous experiments conducted.

One possible mechanism for the loss of M-protein expression is through the transcriptional repression of the *emm* gene. This mechanism was shown to take place in JRS758, a transposon insertion mutant, that was produced in a different lab and using different methods. Loss of M-protein expression in this strain demonstrates that this is a general phenomenon and not the result of the construct we used. The fact that 9 out of 10 colonies showed no M-protein expression suggests that transcription repression may be a common mechanism.

7.3.2 Future experiments

While we provided initial characterization of the loss of surface protein expression as the result of sortase deletion, a much more thorough examination is needed. We believe that M-protein expression is repressed through suppressive mutations that arise randomly, and provide a growth advantage compared to the parental SrtA-negative strain. To further establish this mechanism, the growth rate of stocks of the same culture, before and after the loss of M-protein expression will be compared. If the derived strain will show an increase in growth rate following the loss

of M-protein expression, it will provide support for the hypothesis. Additionally, M-protein will be over-expressed from a plasmid transformed into AR03 (M-, SrtA-), and the growth rate will be monitored compared to a strain containing a mock plasmid. If expression of M-protein will result in a slower growth rate, it will provide additional support for the hypothesis. This experiment will be repeated in JRS75 (M-, SrtA+) to control for the possibility that expression of M-protein slows the rate of growth regardless of sortase. Additionally, AR01 (M+, SrtA-) will be complemented by a plasmid expressing sortase, or a mock plasmid. Isolated clones will be subcultured for 6 days as described in the results section. Loss of M-protein in the mock plasmid strain, but not the sortase-complemented strain will support our hypothesis.

It would also be of interest to determine what kinds of mutations typically result in the loss of M-protein expression. For that purpose we froze glycerol stocks of different sortase mutant clones, before and after the loss of M-protein expression. The gene expression pattern of each one of these clones will be compared to the progenitor strain by Northern blot or RT-PCR. The level of various surface proteins will be measured, as well as the expression level of global gene regulators such as *mga*, and *covR-covS* (Kreikemeyer *et al.*, 2003).

The phenomenon described above has a practical value in the field of anti-infective agents. As bacterial pathogens become increasingly antibiotic-resistant, new targets such as sortase are pursued (Maresso & Schneewind, 2008). Our results show that anti-sortase drugs would not only prevent the anchoring of virulence factors to the cell wall of the pathogens, but would also directly reduce their viability through

the overburdening of the secretion system or the accumulation of unfolded proteins at the membrane-wall interface. Prolonged exposure to such drugs would actively select for variants not expressing anchored proteins on their cell wall, which would be less virulent. Since such escape mutants however, are not likely to survive *in vivo*, anti-sortase drugs would confront the bacteria with conflicting selective pressures that would lead to their elimination (manuscript in preparation).

7.4 A mechanism of surface protein release in the absence of SrtA, and a possible *in vivo* role for LPXTGase

S. pyogenes of serotype M6 possess three enzymes capable of cleaving the LPXTG motif found in the C-terminus of surface proteins. SrtA, the housekeeping sortase, anchors M-protein, SfbI, GRAB and ScpA to the surface, while SrtB is specific for T-antigen (Barnett & Scott, 2002). The third enzyme, LPXTGase, possesses several unique characteristics. It is highly glycosylated, and contains amino acids in both the D and L conformations, as well as non-canonical amino acids, suggesting a possible non-ribosomal mechanism of synthesis (Lee et al., 2002, Lee & Fischetti, 2003). While the biochemical properties of LPXTGase from both *S. pyogenes* and *S. aureus* have been studied in detail (Lee et al., 2002, Lee & Fischetti, 2003, Lee & Fischetti, 2006), very little is known of the role it plays *in vivo*.

At present, there is no known gene that encodes LPXTGase, and no such gene is likely to exist given the presence of D-conformation and non-canonical amino acids in its peptide backbone. Conventional techniques such as gene knockout and recombinant protein expression are therefore difficult to perform. Despite several attempts, production of monoclonal antibodies was unsuccessful, possibly due to the prevalence of sugars on the enzyme's surface. In part due to these difficulties, we decided to test whether LPXTGase can cleave the LPSTGE motif found in the C-terminal sorting signal of M-protein in its normal surroundings in the cell.

7.4.1 LPXTG-dependent SrtA-independent release of M-protein from the cell

The C-terminal anchoring motif of M-protein, is a known substrate of SrtA (Barnett & Scott, 2002), and can also be cleaved by LPXTGase *in vitro* (Lee et al., 2002). M-protein cannot be anchored to the cell wall by SrtB (Barnett & Scott, 2002), however the possibility that the LPSTGE motif of M-protein could be cleaved by SrtB has not been ruled out conclusively. We were interested in determining whether LPXTGase or SrtB could cleave the LPSTGE motif in the C-terminus of M-protein *in vivo*, as well as to determine which residues within the LPSTGE motif are critical for the activity of each one of the enzymes. For that purpose we built a series of M-protein constructs, harboring defined mutations in the LPSTGE motif.

Complete destruction of the LPSTGE motif by reversing or scrambling the amino acids, prevented anchoring of the protein to the cell wall. Replacement of any single amino acid with alanine was tolerated for cell wall anchoring, although with possible reduced efficiency when the N-terminal part of this motif was mutated. Toleration of mutation in the LPSTGE motif is surprising considering that the leucine, proline, threonine, and glycine residues were all essential for the cleavage of a LPXTG substrate by *S. aureus* sortase A in an *in vitro* assay (Kruger *et al.*, 2004). The reason for this difference in not known. One possibility is that *S. pyogenes* SrtA is more promiscuous than the *S. aureus* enzyme, while another is that the *in vivo* environment is conducive to protein sorting and tolerates mutations in the LPXTG motif more easily.

Expression of the M-protein constructs in AR03 (M-, SrtA-) revealed that mutations in the LPSTGE motif could effect the protein's cellular distribution even in

the absence of SrtA, and in particular the amount of M-protein that remains attached to the spheroplasts. We found that when an intact LPSTGE sequence is used, deletion of SrtA results in a mild increase in the amount of M-protein that remains attached to the spheroplast, while constructs with reversed or scrambled LPSTGE motifs display the retention of a much more substantial amount at the spheroplast fraction. Retention of M-protein on the spheroplasts was also observed when the leucine or proline residues were mutated to alanines, but not when other amino acids in the LPSTGE motif were mutated. Additionally, molecules released to the supernatant when the LPSTGE motif was scrambled or reversed, or when the leucine or proline residues were mutated, migrated slower on an SDS-PAGE compared to the wild type and molecules mutated in other residues of the LPSTGE motif. These observations are indicative that there may be an LPXTG-specific peptidase that aids in the release of M-protein from the membrane in the absence of SrtA, although it is not clear at this stage whether this enzyme is SrtB or LPXTGase.

Interestingly mutations in the STGE part of the LPSTGE motif resulted in an increase in the amount of M-protein found in the cell wall fraction. The reason for this increase is not clear, and further experiments are required to test the possibility of covalent anchoring to the cell wall.

The leucine and proline residues, whose mutation resulted in retention of a considerable portion of the M-protein attached to the spheroplasts, are also the only amino acids in the LPSTGE motif that are completely conserved (Fig 7.2). Moreover, based on the sorting signals of the two SrtB substrates in the M6 serotype (LPSTGS and LPSSGG), recognition of amino acids other than the leucine and proline appears

to be quite promiscuous. Based on the current data therefore, we cannot rule out a role for either LPXTGase or SrtB.

Mutation of the proline residue of the *S. aureus* protein A LPXTG motif into an asparagine, resulted in a slower-migrating molecule on an SDS-PAGE (Schneewind et al., 1992). An equivalent mutation in *S. pyogenes* M-protein resulted in a similar shift in molecule size, as well as an increase in the release of the molecule from the cell both in the presence and absence of SrtA. How this molecule is released from the cell is unclear at the moment, however it is an interesting candidate for further testing.

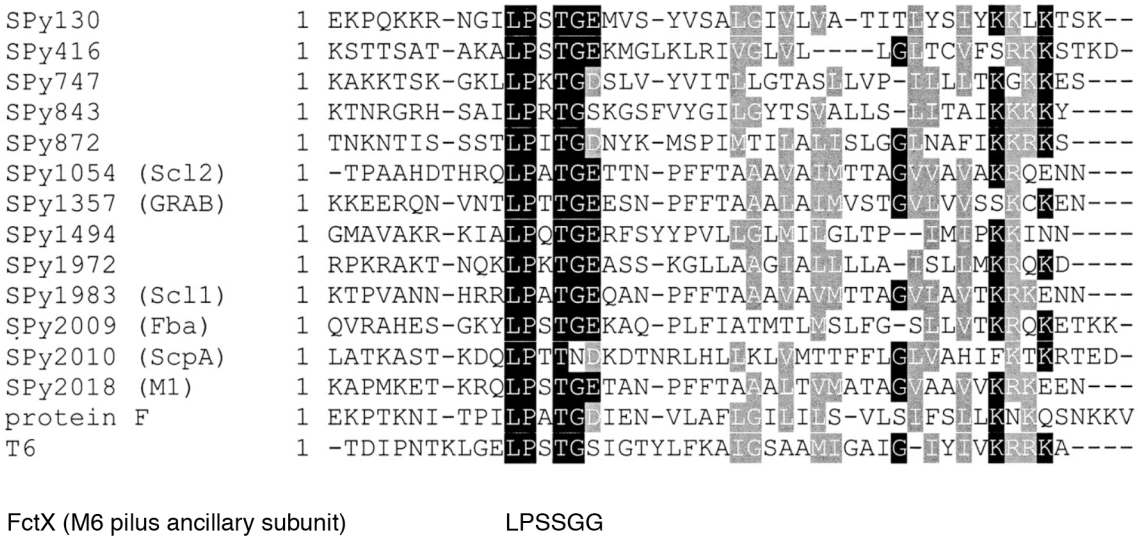


Figure 7.2 Conservation of amino acids within the *S. pyogenes* LPXTG motif

Alignment of amino acids in the C-terminus of *S. pyogenes* surface proteins using ClustalW. Adopted from Barnett and Scott (Barnett & Scott, 2002). FctX is an M6 pilus ancillary subunit not included in the initial analysis (Mora et al., 2005).

7.4.2 Addressing the identity of the LPXTG-specific peptidase

While it appears that the LPXTG motif found in the C-terminus of M-protein can also be recognized by another LPXTG-specific peptidase, it is not clear at the moment whether this enzyme is SrtB or LPXTGase. To address this question a strain containing a triple deletion (M-, SrtA-, SrtB-) is currently under construction. If the distribution pattern of the different M-protein clones in this strain is similar to that observed in AR03 (M-, SrtA-), it would provide strong evidence that LPXTGase might be involved in the cleavage of the M-protein LPSTGE motif *in vivo*. If on the other hand no LPXTG-specific cleavage activity could be detected in this strain, namely all clones would show the same distribution as a clone with a destroyed LPSTGE motif, it would provide strong evidence that SrtB possess in addition to its role as pilus polymerase, the ability to cleave the LPXTG motif found in SrtA substrates, but not the ability to anchor them to the cell wall.

7.4.3 Analysis of the cleavage site of the LPXTG-specific peptidase

Due to the inherent difficulty in deleting LPXTGase from the genome of *S. pyogenes*, direct evidence for the role of this enzyme is difficult to obtain through standard techniques. When tested *in vitro*, LPXTGase cleaves the LPSTGE motif of M-protein in a manner different than that of sortases (Lee et al., 2002). Being able to determine which bond is cleaved within the LPSTGE motif *in vivo* would therefore aid in determining which enzyme is responsible for this cleavage activity.

To determine the cleavage site within the M-protein LPSTGE motif *in vivo*, we used a procedure similar in concept to that used by Ton-That et al. to characterize the anchor structure of *S. aureus* surface proteins (Ton-That *et al.*, 1997). M-protein constructs were produced in such manner, as to include a 3C-protease site followed by a hexahistidine-tag, upstream of the LPSTGE motif. We have shown that the insertion of 3C-His-tag does not interfere with the anchoring of the resulting protein to the cell wall, and that destruction of the LPSTGE motif by reversion or scrambling of the amino acid sequence abolishes anchoring. When expressed in a SrtA-negative strain, the constructs are secreted into the supernatant and can be purified and cleaved with 3C-protease to release a small peptide, which could be used in mass spectrometry studies to find the exact site of LPSTGE-cleavage.

While the purity level of the cleaved peptide obtained thus far did not allow good enough results to determine the cleavage site of this protein, this goal is being actively pursued. The cleavage site will be compared between AR03 (M-, SrtA-) and the future (M-, SrtA-, SrtB-) strains, to better define the enzyme involved. In addition, since the proline to asparagine mutant seems to be a promising candidate for the study of LPXTGase activity *in vivo*, a 3C-type construct harboring this mutation will be produced and tested in a similar manner.

7.4.4 Analysis of the cleavage sites of SrtA, SrtB, and LPXTGase *in vitro*

To complement the *in vivo* results, we will develop an *in vitro* system to determine how mutations in the LPSTGE motif affect cleavage by SrtA, SrtB, and LPXTGase. The system will be based on recombinant fusion proteins expressed in *E. coli*. Each construct will possess an N-terminal GFP fused to the sorting signal of M-protein, in which the C-terminal hydrophobic region is replaced by a hexahistidine tag. The *in vitro* constructs would reflect the same mutations used in the *in vivo* assay to allow direct correlation between the two assays. Following purification, the constructs will be attached to NiNTA beads, and incubated with purified SrtA, SrtB, or LPXTGase. Cleavage of the LPSTGE motif found in these constructs will result in the release of GFP into the medium, and the relative increase in medium fluorescence could be detected using a spectrophotometer. Although the method proposed here, has a significantly lower throughput in terms of work per mutation tested, when compared to the use of synthetic peptides as done by Kruger et al. (Kruger *et al.*, 2004), the much longer portion of the sorting motif used would lower the risk of failure of one of the enzymes to recognize the sorting motif due to the absence of required neighboring sequences. In addition, since the cleaved portion of the LPSTGE motif will remain bound to the NiNTA beads through the C-terminal his-tag, the cleavage site could be determined using Edman degradation. This would allow direct correlation between the *in vivo* results, and the results obtained *in vitro* with each one of the enzymes (manuscript in preparation).

7.5 Concluding remarks

Surface proteins are critical for the *in vivo* survival of *S. pyogenes* and other Gram-positive pathogens. In this work we have introduced a new immunofluorescence protocol for the study of sub-surface antigens in *S. pyogenes*, which allowed us to examine the spatial regulation of protein anchoring. We found that sortase, the enzyme that covalently anchors surface proteins to the cell wall, is found in membranal foci, and that the majority of these foci are associated with the division septum.

Protein anchoring takes place at two distinct locations in the cell, the division septum and the poles. The two pathways differ in anchoring kinetics and the ultimate distribution pattern of the surface protein. Due to the rapidity of peptidoglycan synthesis at the septum, only a limited time is available for M-protein anchoring at this location before the wall material becomes part of the peripheral wall. To facilitate effective anchoring despite this short "window of opportunity", many factors required for the anchoring process are found at high concentration at the septum. These include the localized secretion of M-protein, sortase foci, lipid II (and FtsW which may be involved in its export), and high molecular weight PBPs. The division of streptococcal daughter cells begins before the previous division cycle is concluded. Sortase begins to assemble at the equatorial rings at the later stages of cell division, when active M-protein anchoring and lipid II are simultaneously visible at the mother cell septum and at the equatorial rings.

Anchoring of SfbI at the old pole follows a different rout, demonstrating a diffuse anchoring pattern at a slow and continuous rate. The slow anchoring pace at

the poles is in agreement with the much-reduced presence of sortase at these locations, and the scarcity of lipid II and PBPs, indicating a very slow rate of peptidoglycan turnover. Correct localization of M-protein and SfbI secretion at their distinct locations on the cell wall is not related to the ExPortal, a microdomain found to play a role in SpeB secretion, and is also independent of sortase.

Surface proteins are translocated through the secretion channel, where they are stalled until sortase cleaves the LPXTG motif found in their C-terminus, which is subsequently attached to the cell wall precursor lipid II. We found that in the absence of sortase, another LPXTG-specific peptidase in the cell can cleave the C-terminal LPXTG motif and release the protein from the secretion channel. Reversing or scrambling the LPXTG motif, or replacing any of the first two amino acids with alanines, abolished this cleavage activity. The identity of this enzyme is currently under study.

The LPXTG-cleavage activity described above however, is not sufficient to prevent surface proteins from overburdening the secretion apparatus and accumulating at the membrane-wall interface in the absence of SrtA. While the deletion of *srtA* is not lethal *in vitro*, the resulting dislocation of surface proteins has deleterious effects on the cells. This results in a selective pressure towards repression of surface proteins expression, and indeed variants lacking M-protein expression occur reproducibly in this strain. Drugs targeting sortase or other elements of the sorting pathway may therefore exert similar pressures on the secretion and folding apparatuses, in addition to preventing surface protein anchoring.

Today, multi-drug resistant bacterial pathogens are becoming commonplace both in the hospital, and the community-acquired settings. Due to the vast importance of surface proteins for the survival of Gram-positive bacteria in the mammalian host, studying the regulation of protein anchoring and related processes may lead to the discovery of promising new targets for the development of anti-infective agents.

8 References

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