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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Molecular Microbiology and Microbial Pathogenesis

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Organization of the Streptococcal ExPortal and its Interaction with
Cationic Antimicrobial Peptides

By

Luis Alberto Vega

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2012

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

Organization of the Streptococcal ExPortal and its Interaction with Cationic Antimicrobial Peptides

By

Luis Alberto Vega

Doctor of Philosophy in Molecular Microbiology and Microbial Pathogenesis
Washington University in St. Louis, 2012
Professor Michael G. Caparon, Chairperson

Streptococcus pyogenes and other Gram-positive pathogens are highly dependent on secreted virulence proteins for their ability to colonize a host and evade the immune effectors that act on pathogens to prevent this. Unlike eukaryotes and Gram-negative bacteria, all Gram-positive pathogens lack a cellular compartment dedicated to folding and processing secreted proteins once they have been translocated across the cytoplasmic membrane. *S. pyogenes* and other Gram-positive organisms overcome this challenge by clustering the secretory translocons at a defined anionic lipid microdomain of the cytoplasmic membrane that is enriched for accessory factors needed for protein biogenesis, referred to as the ExPortal. The manner in which *S. pyogenes* achieves and maintains spatial coordination of the factors that constitute the ExPortal is not understood. Given the importance of secreted virulence proteins to streptococcal pathogenicity, the ExPortal is an attractive target for the development of therapeutics that can efficiently counter streptococcal infections. I investigated the interaction of cationic antimicrobial peptides with the streptococcal ExPortal to provide insights into how this

secretory organelle is organized and how it may be targeted by this mechanism of innate immunity.

Cationic antimicrobial peptides (CAPs) targeted the anionic lipid microdomain of the ExPortal. Traditionally regarded as membrane permeabilizing compounds, CAPs interacted with the streptococcal membrane at concentrations that did not porate the membrane. Rather, exposure of *S. pyogenes* to sub-lethal CAP concentrations resulted in concomitant redistribution of anionic lipids, translocons and accessory factors to peripheral regions of the membrane. In addition, CAPs inhibited secretion of important virulence proteins, including the pathogenically relevant SpeB protease and Streptolysin O (SLO) cytolysin. One of the CAPs investigated also targets the lipid II precursor of extracellular peptidoglycan, suggesting the influence of CAPs on ExPortal organization extended beyond their affinity for negatively charged membrane lipids and that the streptococcal cell wall is involved in organizing the ExPortal.

Removal of extracellular peptidoglycan also resulted in redistribution of anionic lipids and ExPortal protein, indicating the cell wall is required for maintaining proper localization of both lipid and protein components of the ExPortal. Exposure of streptococci to lantibiotics that preferentially bind and sequester lipid II in the cytoplasmic membrane disrupted ExPortal organization and secretory function in a manner similar to CAP activity. Given that both CAPs and lantibiotics target lipid components of the cytoplasmic membrane, it appears that lipid segregation in the membrane is central to organization of the ExPortal. Establishment or maintenance of this lipid segregation could involve interaction with peptidoglycan synthesis in the streptococcal membrane, as two membrane-localized synthetic enzymes, one integral

(MraY), the other membrane-associated (MurN), that are required for lipid II production are localized in the same anionic lipid microdomain to where secretory translocons of the ExPortal are targeted to. This provides a potential model for the mechanism(s) organizing the ExPortal. Cytoskeletal proteins and cell wall biogenesis components which are observed to interact with cytoplasmic membrane structures to organize membrane localized processes required for cell growth and division influence the segregation of anionic lipids in the membrane, and by extension, where and how the ExPortal is organized.

Testing of this model by genetic and molecular approaches that target components of cell division and peptidoglycan will provide both further insights into how Gram-positive bacteria coordinate protein biogenesis with secretion as well as reveal novel potential targets for the development of effective therapeutics against pathogens like *S. pyogenes*.

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Chapter I

ExPortal Secretion, Cell Wall Biogenesis and Cationic Antimicrobial Peptide Involvement in *Streptococcus pyogenes* Pathogenesis

SCOPE OF THESIS

Streptococcus pyogenes and other Gram-positive bacteria circumvent the lack of a specialized cellular compartment for folding secreted proteins following their translocation by clustering the Sec translocons at a defined microdomain of the cytoplasmic membrane that is also enriched in anionic lipids and accessory factors for protein biogenesis, referred to as the ExPortal. The function of this secretory organelle appears to be that of spatially coupling secretion with protein maturation, but the mechanisms organizing the ExPortal are still poorly understood. In order to further knowledge of how spatial coordination of protein secretion and processing is achieved and maintained in streptococci, examination of cationic antimicrobial peptide (CAP) interaction with the anionic lipid-enriched membrane microdomain of the ExPortal was undertaken. Two CAPs, the therapeutically important polymyxin B and physiologically relevant human neutrophil peptide 1 (HNP-1), preferentially targeted the ExPortal at sub-lethal, non-porating concentrations. This non-permeabilizing interaction with the membrane resulted in disruption of ExPortal organization, as observed by the redistribution of ExPortal anionic lipids, the secretory translocon ATPase SecA and the chaperone protease HtrA into the peripheral membrane. Redistribution was associated with inhibition of secretion of certain toxins, including the SpeB cysteine protease and the Streptolysin O (SLO) cytolysin, but not SIC, a protein that protects *S. pyogenes* from CAPs. These data suggest a novel function for CAPs in targeting the ExPortal and interfering with secretion of factors required for infection and survival. This report also contributed to literature indicating CAPs can kill their bacterial targets in the absence of pore formation, exerting their lethal effect by interfering with the dynamic function of

lipid-associated complexes, such as the ExPortal, by a so-called “sand in a gearbox” mechanism.

Previous research showed that interaction of HNP-1 with the bacterial membrane is not limited to anionic lipids, since the peptide also binds the lipid II peptidoglycan precursor. As an emerging literature indicates that cell wall biogenesis components interact with cytoplasmic membrane structures to organize membrane-localized processes, involvement of the streptococcal cell wall in organizing and maintaining the ExPortal was investigated. Removal of extracellular peptidoglycan by the action of a streptococcal phage lysin resulted in redistribution of both lipid and aforementioned protein components of the ExPortal, suggesting the cell wall is required for maintaining proper localization of the secretory organelle. Exposure to a lipid II-targeting lantibiotic also concomitantly disrupted localization of anionic lipids and ExPortal proteins, as well as secretion of the streptococcal virulence factors observed to be affected by CAPs. These results support the hypothesis that lipid localization in the membrane is central to organization and maintenance of the ExPortal. Given that lantibiotic activity disrupts cell wall synthesis by binding lipid II and sequestering it away from the sites of peptidoglycan biogenesis, it was of interest to determine whether lipid II production and ExPortal secretion are spatially coordinated processes. Fluorescent tagging of MraY and MurN, two proteins involved in the membrane-associated steps of lipid II synthesis, revealed these to localize at foci in the streptococcal membrane that coincide with the anionic lipid microdomain and secretory translocons of the ExPortal. These results suggest a model for ExPortal organization in which peptidoglycan synthesis co-localizes with the ExPortal, influences its establishment and is required for its maintenance.

INTRODUCTION

Streptococcus pyogenes Pathogenesis

S. pyogenes is a gram-positive pathogen that can cause numerous diseases in humans that range from superficial infection of the skin and mucous membranes (impetigo, pharyngitis), to highly invasive and life-threatening diseases (necrotizing fasciitis), as well as serious post-infection sequelae (rheumatic fever, glomerulonephritis, reviewed in [1]). Annually, Group A streptococcus (GAS) infections account for an estimated 700 million cases of mild, non-invasive infections worldwide, of which approximately 650,000 progress to severe, invasive (at a sterile site) infections with an associated mortality of approximately 25% [2].

This ability to cause disease is dependent on the secretion of an extensive network of virulence proteins (Figure 1) [1, 3]. Among these are the proteases ScpC (SpyCEP), ScpA, EndoS and SpeB; surface-bound Protein F (SfbI) and M protein adhesins or polymeric structures such as pili; host-targeted cytotoxins such as Streptolysin O (SLO), streptococcal NAD glycohydrolase (SPN) and Streptolysin S (SLS); polypeptides like streptococcal inhibitor of complement (SIC) and extracellular DNAses that directly inhibit immune effectors [3, 4]. Extracellular DNAses degrade chromatin and break down the neutrophil extracellular traps (NETs) extruded by neutrophils to trap pathogens and subject them to microbicidal effectors, thus helping streptococci evade neutrophil killing [5, 6]. SIC is one of multiple defenses streptococci have developed against such microbicidal effectors. As its name indicates, SIC was first identified as a secreted polypeptide that can inhibit membrane attack complex of complement by preventing uptake of C5b67 complement complexes on bacterial membranes [7, 8]. SIC however,

also interacts with and inhibits multiple host microbicidal proteins, including lysozyme, cathelicidin LL-37, human neutrophil peptide-1 (HNP-1) and human beta-defensins (hBD1, -2, -3) [9-11]. Proteins bound to the streptococcal surface also play a major role in the evasion of phagocytic cells and their effectors [3]. Specifically, M protein inhibits opsonophagocytosis of streptococci by binding multiple host proteins [12-14] and promotes colonization alongside SfbI by interacting with extracellular matrix components and adhering to host epithelial cells [15-17]. Additionally, similar to other pathogenic bacteria, *S. pyogenes* assembles multimeric proteinaceous fibres on its surface known as pili, which are involved in the adhesion of the bacterium to host epithelia [18].

Like all Gram positive bacteria, Group A streptococci lack the specialized secretory systems and the accompanying host cell cytosol-targeted effectors that are necessary for virulence in some gram-negative pathogens (e.g. Type III secretion of *Salmonella enterica* [19]). However, the cytolysin SLO and NAD glycohydrolase SPN constitute a unique mechanism for specifically translocating the latter into the host cell via so-called cytolysin mediated translocation (CMT) [20]. The cholesterol-dependent cytolysin SLO oligomerizes to form large pores in host cell membranes and can thus acts on its own to induce cell death [21]. However, this pore-forming capacity is not required for the translocation of SPN by CMT [22] and mutations to SLO that do not interfere with pore-forming activity, or modifications to SPN that do not affect NAD-glycohydrolase activity render these proteins incompetent for CMT [23, 24]. The precise mechanism by which CMT of SPN occurs then is an area of active research, but it is clear that CMT is important for pathogenesis [20, 25], and that the process involves interaction of streptococci with the host cell surface as well as between SPN and SLO. Another

secreted toxin of streptococci, SLS, is cytolytic to erythrocytes, leukocytes and subcellular organelles [26-28], promotes evasion of phagocytic killing via its cytolytic activity on neutrophils [29] and inhibits neutrophil recruitment during the early stages of streptococcal infection [30].

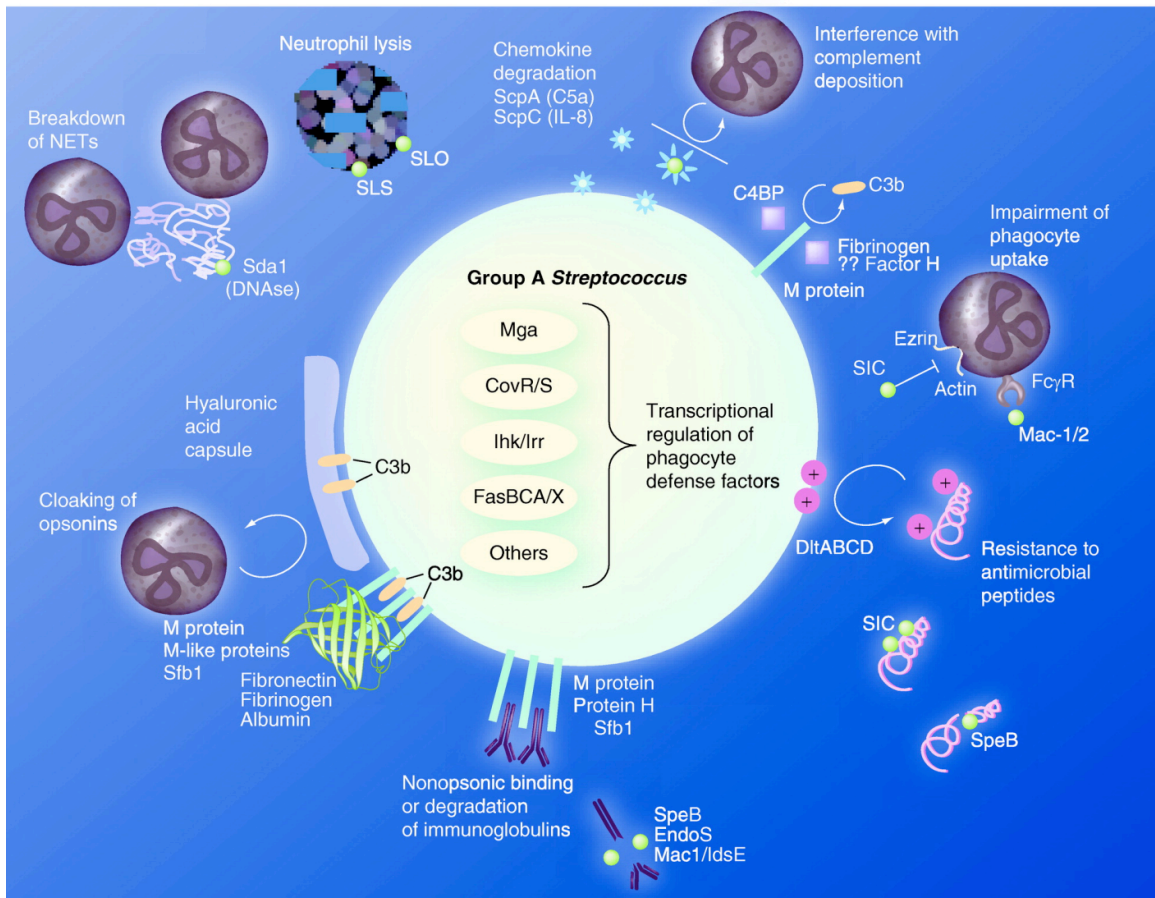


Figure 1. **Virulence factors secreted by *Streptococcus pyogenes*.** These factors include proteases (ScpA, ScpC, EndoS, SpeB, Mac1/IdsE), surface-bound adhesins (SfbI, M protein, Protein H) host-targeted cytotoxins (SLO, SLS), extracellular DNAses (Sda1) immunity effector-targeted polypeptides (SIC). A majority of these virulence factors are involved in the evasion of phagocytic killing [3].

The secreted proteases of Group A streptococci (GAS) serve to inactivate all kinds of proteins involved in host immunity. ScpC cleaves and inactivates interleukin-8 (IL-8) [31] and ScpA acts as an endopeptidase to cleave the C5a polypeptide fragment of the complement cascade [32]. EndoS hydrolyzes the chitobiose core of the asparagine-linked glycan on Immunoglobulin G (IgG) to prevent recognition of IgG by phagocyte Fc receptors, while SpeB cleaves IgA, IgM, IgD and IgE antibodies as well [33]. In fact, SpeB degrades a myriad of host proteins, including extracellular matrix components, cytokine precursors and antimicrobial peptides [34, 35]. The protease activity of SpeB extends to extracellular and surface-associated streptococcal proteins, including M protein [36], various superantigens [37], streptokinase [38], SLO [39] and SIC [40]. Given such a variety of virulence-associated targets, the precise role of SpeB in streptococcal pathogenesis is still unclear and an area of active investigation.

The SpeB cysteine protease is one of the most intensively researched virulence factors of GAS, and these efforts have revealed that production of enzymatically active SpeB is a complex process involving a variety of regulatory controls and multiple accessory factors post-translation (reviewed in [41]). Expression of SpeB is growth phase dependent and influenced by environmental factors including pH [42], NaCl concentration [43] and nutrient availability [44]. Peptide transport also affects SpeB expression as was determined by the mutational inactivation of the oligopeptide permease (Opp) and dipeptide permease (Dpp) systems responsible for peptide acquisition [45, 46]. Multiple regulatory proteins influence *speB* expression by either directly activating (RopB, CcpA [47-49]) or indirectly inducing (Mga, CodY, SagP, LuxS [50-52]) or repressing (CovR/CovS, Srv, LacD.1, Nra, Vfr [53-58]) transcription; something that reflects the

variety of inputs that affect SpeB production and suggests that timely and appropriate synthesis of this protease is of great importance to streptococcal pathogenesis. The *speB* transcript is translated into a 40kDa zymogen, that through either inter- or intramolecular autocatalytic processing at nine reported cleavage sites is proteolytically processed into a 28kDa active enzyme [59-61]. Prior to this processing however, the SpeB zymogen is modified by the peptidyl-prolyl cis-trans isomerases (PPIase) RopA and PrsA [47, 62]. The PPIases isomerize one or more of the 13 proline residues in the SpeB zymogen and this activity is required for proteolytic processing of the polypeptide. Dysregulation of PrsA adversely affects both SpeB activity and pathogenicity of GAS [63]. Interestingly, the PPIase activity of RopA is dispensable for secretion of the SpeB zymogen, but loss of RopA expression inhibits secretion of SpeB [47], suggesting that aside from its processing role RopA is an accessory factor for secretion of the protease. Another accessory factor of SpeB secretion and processing is the serine protease HtrA. This extracellular membrane-anchored protease is involved in the folding and maturation of secreted proteins as well as the degradation of proteins misfolded during secretion in *Lactococcus lactis* [64]. In GAS functional HtrA is required for the efficient conversion of secreted SpeB zymogen into its active form [65], although HtrA does not directly process SpeB *in vitro* [66]. Additionally, mis-localization of HtrA out of the cytoplasmic membrane results in altered maturation kinetics of SpeB [67]. Given the multitude of streptococcal and host proteins SpeB acts on, it is not surprising that its biogenesis is a highly regulated process involving many factors. It is possible though that other secreted virulence proteins of streptococci that have not been as well characterized as SpeB also require the interaction of multiple accessory factors for their production. Most

importantly, existing research has demonstrated that loss or inhibition of these secreted proteins' activity results in reduced virulence [5, 68-71], highlighting the importance of understanding the mechanism(s) by which these proteins are secreted and processed into their active forms.

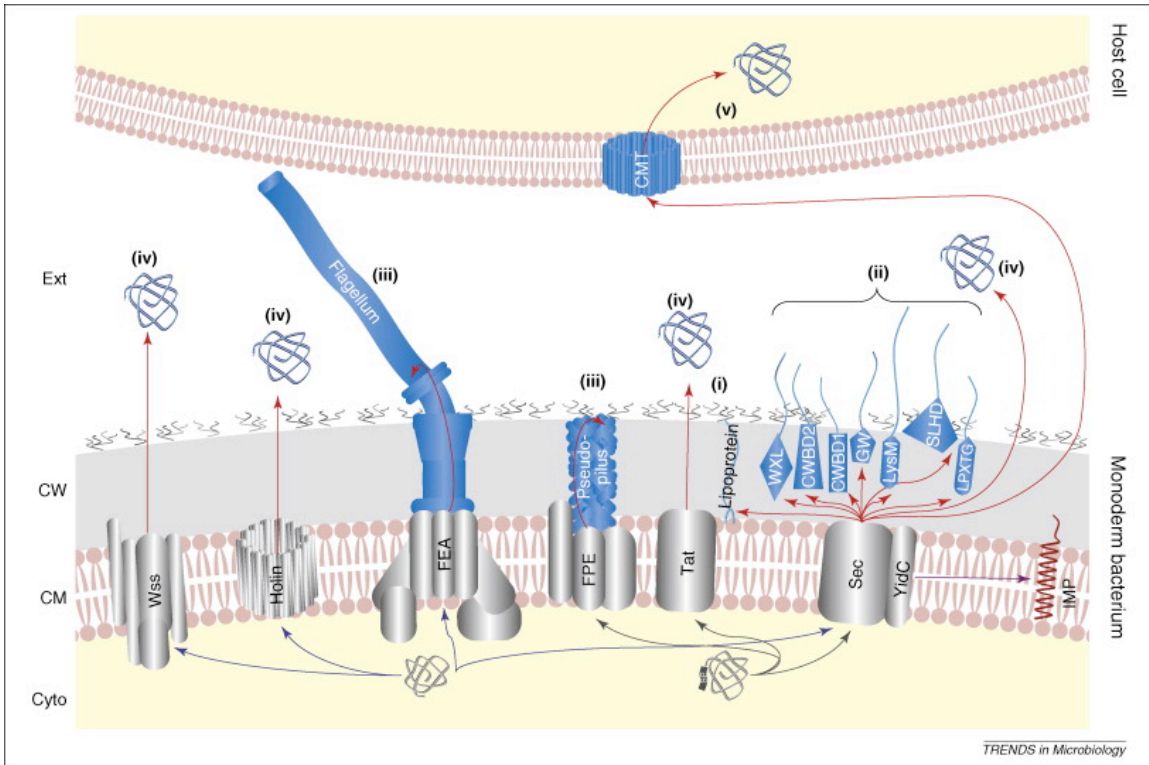


Figure 2. Secretion systems of Gram-positive bacteria. In gram-positive bacteria (Monoderm, as the figure states), secreted proteins can be (i) anchored to the CM (i.e. lipoproteins); (ii) attached to the CW either covalently (i.e. LPXTG proteins) or non-covalently (i.e. by LysM, GW, CWBD1, CWBD2, SLHD or WXL motifs); (iii) part of cell-surface appendages, such as pili (whose subunits are secreted via Sec), competence pseudo-pili (assembled via FPE) or flagella (assembled via FEA); (iv) released into the extracellular milieu via Sec, Tat, holin or Wss; or (v) translocated into a host cell after secretion via Sec of a cholesterol-dependent cytolysin, which integrates into the membrane of a host cell to permit transport of secreted effectors via cytolysin-mediated translocation (CMT). Black arrows show routes of proteins targeted to CM exhibiting an N-terminal signal peptide, blue arrows routes used by proteins lacking a signal peptide. Red arrows relate to secretion, and violet arrows refer to integration of membrane proteins (IMP). Secreted proteins are blue. Abbreviations: Cyto, cytoplasm; CM, cytoplasmic membrane; CW, cell wall; Ext, extracellular milieu; FPE, fimbrial-protein exporter; FEA, flagella export apparatus [72]. *S. pyogenes* possesses only the Sec secretory pathway.

Secreted protein biogenesis and the ExPortal of GAS

In bacteria, the most conserved route of protein translocation across the cytoplasmic membrane is the general secretory (Sec) pathway (for a detailed review see [73]). The membrane-embedded enzyme complex that constitutes the translocase is composed of a molecular motor, the peripheral adenosine triphosphatase (ATPase) SecA [74] and a trimeric protein complex that acts as the conducting channel. This channel is formed by the SecY [75], SecE [76] proteins that together associate with the integral membrane subunit SecG [77], or with another heterotrimeric complex composed of the SecD, SecF and YajC proteins encoded in the *secD* operon [78, 79]. *S. pyogenes* however expresses the SecYEG complex, SecA and YajC proteins, but its genome does not encode genes for SecDF [80]. Studies in *Escherichia coli* and *Bacillus subtilis* reveal that YajC is a membrane protein with a single transmembrane segment and a large cytosolic domain that, except for the observation that it associates with SecDF, is not needed for protein translocation or viability [81, 82]. Therefore, given that streptococci lack SecDF, the precise function of YajC in GAS remains unknown.

Proteins destined for secretion via the Sec pathway are synthesized as preproteins that are targeted to the Sec translocons by an 18 to 30 amino acid N-terminal signal sequence containing a positively charged N-terminal domain, a hydrophobic core and a more polar C-terminal domain [83]. This signal is cleaved off by a signal peptidase, a membrane bound protease that acts on the cleavage site in the C-domain of the signal peptide during the translocation reaction. Proteins thus produced can either be post-translationally or co-translationally translocated, and the mechanism in GAS for directing the latter process is the signal recognition particle (SRP) pathway. In bacteria the SRP is

a complex composed of a 4.5S RNA and the GTPase Ffh [84] that interacts with the signal sequence of nascent preproteins as they emerge from the ribosome [85]. This so-called ribosome nascent chain (RNC) complex bound to SRP in a GTP-dependent manner interacts with a membrane bound receptor, FtsY [86]. It is this complex that, upon GTP hydrolysis by both FtsY and SRP, transfers the RNC to the translocase to initiate export of the polypeptide [87]. For many bacteria the SRP pathway is essential to survival, but, as described in the Appendix, in GAS it was found to be dispensable for growth under *in vitro* conditions, yet necessary for virulence and the production of certain secreted virulence factors, including SLO, SPN and SpeB, the latter in a nutrient-dependent manner. [88].

The secreted proteins of streptococci that remain surface-bound are anchored to either the outer leaflet of the cytoplasmic membrane or to the streptococcal cell wall via sortases. These enzymes function to covalently join secreted surface factors to cell wall peptidoglycan or to polymerize the proteins that constitute multimeric structures like pili into their functional fibrous form (for a detailed review see [89]). Though not virulence effectors themselves, the role sortases play in targeting surface factors required for virulence like those previously described means sortases are crucial for pathogenesis. The known sortases that act on cell wall-anchored substrates all function as cysteine transpeptidases, joining proteins containing a cell wall sorting signal to an acceptor amino group on the outer cell surface [90]. The sorting signal consists of an LPXTG motif (*X* standing for any amino acid), followed by a segment of hydrophobic amino acids and a tail of predominantly positively charged residues. The enzymatic reaction carried out by these sortases consists of breaking the threonine and glycine peptide bond

in the LPXTG motif to form a substrate-sortase complex in order to transfer the surface protein to the peptidoglycan precursor lipid II, thus generating an isopeptide linked protein-lipid II product [91]. The transglycosylation and transpeptidation reactions that synthesize the cell wall then incorporate the lipid-II bound surface protein into the peptidoglycan polymer. The gram-positive sortases characterized to date are categorized into six different classes based on sequence homology and functionality [89]. Two of these classes are represented in *S. pyogenes*. One is the class commonly referred to as the ‘housekeeping’ Class A sortases, whose enzymatic activity anchors a diverse array of surface proteins to the cell wall, including M protein, SfbI and ScpA [92]. The other is the Class B sortases exemplified by Sortase C, which is involved in the assembly of the adhesive pili required for streptococcal biofilm formation and adhesion to host epithelia [93].

S. pyogenes lack all of the other known secretion systems of Gram-positive bacteria, diagrammed in Figure 2 [72, 80]; thus all secreted virulence proteins of GAS are exported via the Sec pathway. Streptococci however, like all Gram-positive bacteria, lack a specialized cellular compartment for folding secreted proteins following their translocation across the membrane by the Sec system. This problem is circumvented in *S. pyogenes* and several other species of Gram-positive cocci by clustering the Sec translocons and secretory accessory factors at a defined microdomain of the cytoplasmic membrane that has been termed the ExPortal [67, 94-97]. In *S. pyogenes*, secretion of the SpeB protease occurs at a unique site on the streptococcal surface, as does secretion of a non-native Sec pathway substrate (PhoZ) expressed from a plasmid [94]. This site of SpeB secretion coincides with the membrane localization of the SecA ATPase and of the

HtrA serine protease [67, 94]. Likewise SrtC, the sortase required for the polymerization of pili in *Enterococcus faecalis*, co-localizes with SecA in the enterococcal membrane, and loss of SrtC expression resulted in accumulation of pilus subunits (EbpA, EbpC) at unique foci on the bacterial surface [95]. Other examples of this coordination of Sec translocons and accessory proteins include the co-localization of SecA and the cell wall protein-anchoring Sortase A at unique foci in the membrane of *Streptococcus mutans* [97], whereas in replicating *Streptococcus pneumoniae* cells HtrA and SecA were observed to oscillate between septal and polar membrane microdomains [98]. The membrane microdomain to which SpeB secretion localizes to in *S. pyogenes* is also enriched in anionic lipids [99], while in *S. pneumoniae*, cells devoid of the cardiolipin synthase encoding gene *cls* failed to localize SecA to septal membrane microdomains [98]. A similar mutation in GAS did not affect secretion of SpeB, suggesting that in *S. pyogenes* it is the more abundant phosphatidylglycerol that localizes to the ExPortal [99]. Moreover, additional evidence from both enterococci and GAS indicates that sorting of cell wall-anchored virulence factors occurs in the immediate vicinity of their site of secretion and coincides with the site of *de-novo* peptidoglycan synthesis [95, 97, 100, 101]. Altogether, this suggests that a function of the ExPortal is to spatially couple secretion with protein maturation, and that this coordination could involve membrane lipid organization and cell wall synthesis as well.

Bacterial lipid membrane microdomains

As mentioned, the ExPortal of *S. pyogenes* has been shown to have an asymmetric lipid content enriched in anionic phospholipids [99] that may contribute to the preferential retention of certain proteins at the ExPortal vs. the peripheral membrane [95]. Other bacterial membranes have been known to contain localized microdomains enriched for specific phospholipids, as was shown in the case of cardiolipin and phosphatidylethanolamine-rich septal membrane domains of *B. subtilis* [102, 103]. These domains were absent in mutants disrupted in the cardiolipin synthase-encoding gene *clsA* [102] and in the genes encoding phosphatidylserine carboxylase (*psd*) and phosphatidylserine synthase (*pssA*) [103]. Interestingly, the majority of the lipid synthases localize to the septal membrane region at which cardiolipin and phosphatidylethanolamine are retained [103]. Membrane lipid spirals detected along the long axis of cells in *B. subtilis* co-localized with GFP protein fusions of MinD, thus hinting at possible involvement of lipid membrane microdomains in cell division [104].

Anionic lipids also play a multitude of roles in organizing the protein components of the bacterial membrane, including targeting secretory proteins by their signal sequence, directing membrane proteins via the SRP pathway, facilitating the interfacial insertion of peripherally attached protein domains, influencing or even determining the correct topology of transmembrane segments, and promoting efficient formation of protein complexes in the membrane (reviewed in [105]). Particularly with respect to protein secretion, phosphatidylglycerol is involved in optimal protein translocation via the Sec pathway by influencing interaction of the SecA ATPase with the SecYEG translocon [106-108], as well as interacting with pre-protein signal sequences during

protein translocation [109], and with the FtsY component of the SRP pathway, enhancing its GTPase activity [110]. Thus, bacterial membrane lipids play a vital role in the organization and functionality of the bacterial cell's secretory processes. However, in gram-positive bacteria secretion is one of several important cellular processes occurring at the surface for which lipid organization appears to be of particular importance. Another such process, as described below, is cell wall synthesis.

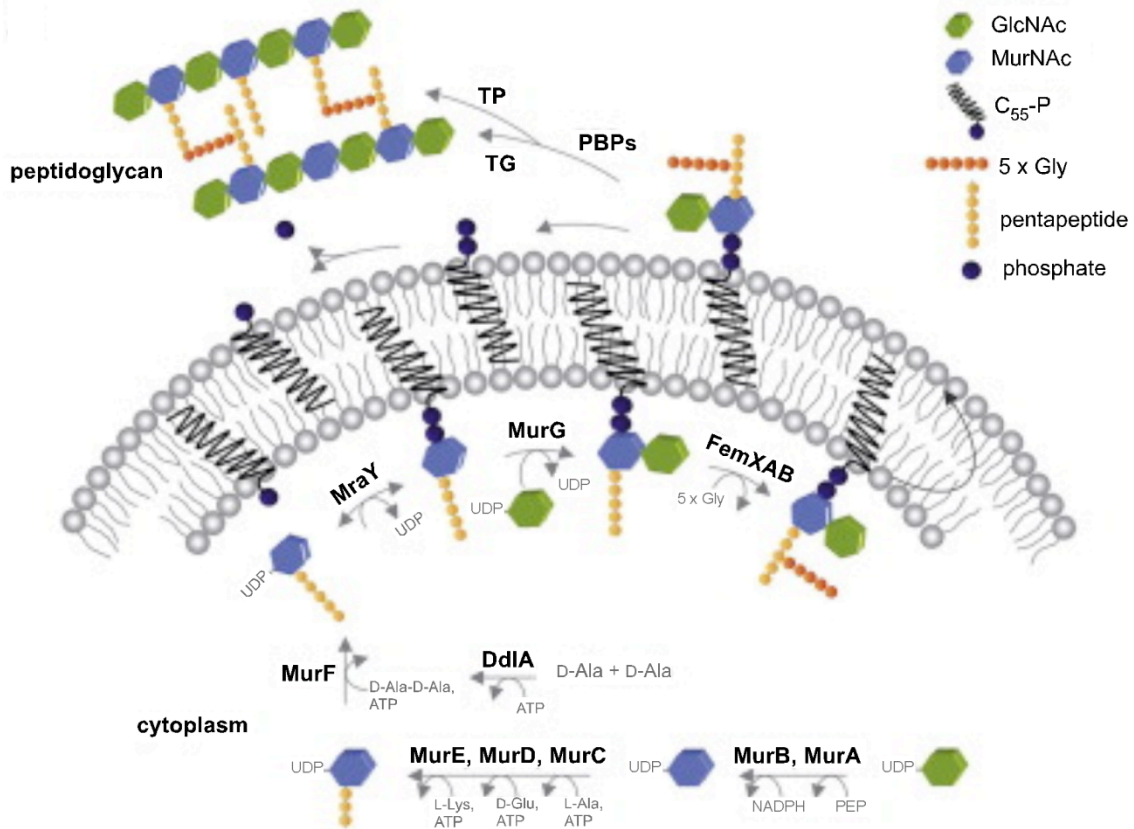


Figure 3. Gram-positive cell wall biogenesis. The enzymatic steps of cell wall synthesis in *Staphylococcus aureus*, a representative gram-positive pathogen, are shown. The cytoplasmic conversion of UDP-GlcNAc to the soluble precursor UDP-MurNAc-pentapeptide is carried out by sequential action of the MurA to MurF enzymes. The membrane-associated synthesis of lipid II by MraY and MurG involves conjugation of MurNAc-pentapeptide to undecaprenyl-phosphate (C_{55} -P) and addition of GlcNAc. Addition of the interpeptide crosslinker (5 x Gly) is catalyzed by the FemXAB transferases just prior to translocation of the lipid II precursor across the cytoplasmic membrane. Extracellular transglycosylation (TG) and transpeptidation (TP) reactions carried out by penicillin binding proteins (PBPs) assemble lipid II precursors into the cell wall peptidoglycan polymer [111].

Gram-positive cell wall synthesis

The cell wall of Gram-positive pathogens is an extracellular organelle that plays a multitude of roles essential to both survival and virulence (for a review see [112]). A rigid peptidoglycan exoskeleton made up of polymers of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) glycan units cross-linked via peptide bridges, its main purpose is to preserve cellular integrity against osmotic forces from without and within the bacterial cell [113]. The structural rigidity required for this is balanced by the flexibility necessary to allow for changes in cell shape during growth and division. This plasticity results from the combined activities of penicillin binding proteins (PBPs) and cell wall hydrolases. PBPs are the enzymes that carry out the transglycosylation reactions involved in the polymerization of disaccharide units to produce the individual strands of peptidoglycan, as well as the transpeptidation reactions that crosslink these strands via peptide bridges [114]. Their name stems from the fact that they are the targets of the β -lactam class of antibiotics to which penicillin belongs to. PBPs are classified as high molecular weight (HMW) Class A PBPs, which are bifunctional proteins with both glycosyltransferase and transpeptidase activity, HMW Class B PBPs with transpeptidase activity and low molecular weight PBPs with carboxypeptidase or endopeptidase activity [115]. Along with enterococci and other streptococci, GAS morphologically fit into the class of so-called ovococci, referring to gram-positive bacteria that are not true cocci due to their ellipsoid shape and divide in the same single plane perpendicular to the long axis of the cell, thus forming chains of cells [114]. To accomplish this most ovococci utilize a common set of PBPs: three class A PBPs (PBP1a, 1b and PBP2a), two class B PBPs (PBP2b and 2x) and a LMW PBP

(PBP3); GAS encodes all of the above with the exception of PBP2b. The hydrolases involved in modifying peptidoglycan during cell wall synthesis include N-acetylmuramidases and N-acetylglucosaminidases that digest the glycan backbone, amidases that cleave the peptide cross-links, and lytic transglycosylases [116]. In *S. pyogenes* these enzymes have remained largely uncharacterized; however a secreted protein containing a cysteine and histidine-dependent aminohydrolase/peptidase (CHAP) domain [117] common to N-acetylmuramidases was recently identified as a functionally active peptidoglycan hydrolase required for virulence [118]. Disrupting expression of this protein, termed CdhA (CHAP-domain containing and chain forming cell wall hydrolase), resulted in severe growth defects, including the inability to form chains, suggesting a role in coordinating cell division plane recognition and cell wall synthesis in streptococci. It is known that ovococci synthesize the cell wall mostly at the division site mid-cell, the new hemispheres of the daughter cells being synthesized between the parting parent hemispheres [119, 120]. The precise coordination of PBPs and other cell wall synthesis factors (i.e. hydrolases and cell division proteins) in order to achieve this is an area of active investigation and the organization of cell wall biogenesis appears to result largely from underlying cytoplasmic membrane organization and the positioning of cell division machinery [104, 121-123].

Synthesis of the cell wall by PBPs requires the production and translocation across the cytoplasmic membrane of peptidoglycan subunits in the form of undecaprenyl-pyrophosphoryl-MurNAc-(petapeptide)-GlcNAc (lipid II). The final steps of lipid II biogenesis occur at the inner leaflet of the cytoplasmic membrane (Figure 3) and involve membrane-associated (MraY, MurG) as well as cytosolic proteins (FemABX; for a

review see [124]). MraY is an integral membrane protein that carries out the first membrane-associated step in the synthesis of lipid II by catalyzing the transfer of the phospho-MurNAc-pentapeptide from the soluble cytoplasmic UDP-MurNAc-pentapeptide substrate to the membrane embedded undecaprenyl-phosphate (C_{55} -P) acceptor [125, 126], thus producing undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide (lipid I), the immediate precursor of lipid II. The subsequent step involves the extrinsic membrane transferase MurG, which catalyzes the transfer of the GlcNAc moiety from UDP-GlcNAc to lipid I in order to produce lipid II [127]. The peptidoglycan structure of many gram-positive bacteria contains an additional peptide cross-link between the pentapeptide chain and the cross-linked strand, something that requires the addition of interchain residues to the pentapeptide of the lipid II intermediate [128]. Cytosolic non-ribosomal peptidyltransferases carry out this reaction in *Staphylococcus aureus* (FemABX [129, 130]), *E. faecalis*, *S. pneumoniae* and *S. pyogenes* (MurMN [131, 132]) on the inner leaflet of the cytoplasmic membrane prior to translocation of lipid II across the membrane [124]. In *S. aureus* synthesis of these peptide bridges is required for the sortase-mediated attachment of surface virulence factors to the cell wall [133] and their loss results in reduced virulence [134].

The dynamic quality of the cell wall is also important for its function as an attachment site for extracellular factors that interact with the host environment, which aside from surface virulence proteins also includes lipoteichoic and teichoic acids. Given the role of sortases in processing the former, most research into the interaction of gram-positive secretion with cell wall biogenesis to date, particularly in the case of streptococci, has focused on the characterization of sortase function [89], rather than the

the coordination between secretion and cell wall synthesis, an area that holds great potential for important discoveries. The cellular processes described thus far (i.e. proteins secretion, membrane lipid segregation and cell wall synthesis) are clearly linked to one another in a variety of ways; whether it is that molecular substrates of one process (e.g. secreted surface factors) are inputs for another (cell wall assembly), or the organization of one (membrane lipid segregation) appears to inform the functionality of another (protein secretion). It follows then that certain disruptive influences on any one of these processes could have deleterious consequences on the others and, by extension, the most effective antimicrobials will be those that can influence any one of these processes in such a way as to affect the others as well. Such antimicrobial compounds exist in nature, are the subject of active investigation and a summary of their known properties and activities is given below.

Cationic antimicrobial peptides

Cationic antimicrobial peptides (CAPs) are gene-encoded peptides produced by nearly all known life-forms as part of an ancient nonspecific innate immune system that is the main defense mechanism during the initial stages of infection against many organisms (for a review see [135]). They display a broad range of activity across a range of pathogens, including bacteria, fungi, metazoans, parasites and viruses [136], and some are even active against cancerous cells [137]. Some are systemically expressed while others are localized to specific cells or tissue types most susceptible to infection by pathogens (e.g. histatins in human saliva [138]). In mammalian hosts, their contribution to overall immunity varies between different tissue sites (skin, oral, gastrointestinal and respiratory epithelia) within a host and from one organism to another [139].

Though they display a broad range of activity, have limited sequence homology and a wide range of secondary structures, the great majority of CAPs share similar physical properties. In general, they derive from larger precursors by proteolytic processing, are 12 to 50 amino acids in size, carry a net cationic charge (+2 to +11), possess a nearly 50% hydrophobic residue composition and are able to adopt an amphipathic structure in contact with lipid membranes [140]. Structurally, the majority of CAPs fall into either of two categories: amphipathic α - helices, and amphipathic β -sheets. The former group is made up of the majority of CAPs known so far, which are short linear cationic α -helical peptides (e.g. magainins, cathelicidins, cecropins [141-143]). The amphipathic β -sheets on the other hand present a defined number of β -strands organized in a common amphipathic manner, with relatively few or no helical domains, and constrained either by disulfide bonds (e.g. defensins, protegrins) or by cyclization of the peptide backbone

(gramicidin S or polymyxin B). The defensins make up most of the cysteine-containing peptides and are frequently formed by several antiparallel β -strands stabilized by a series of up to six disulfide bonds [144]. They are expressed by neutrophils and epithelial cells of humans and other mammals, functioning both as broad-spectrum microbicides and as modulators of the immune response by influencing epithelial cell proliferation, enhancing wound healing, regulating production of pro-inflammatory cytokines, and directing chemotaxis of several types of leukocytes [139, 145].

As described previously, the conjugation of lipids with proteins in supramolecular complexes is central to multiple biological processes. Thus, it is not surprising that the lipid membrane of pathogens is the principal target of CAP activity. It is generally accepted that the primary mechanism of antibacterial activity of CAPs involves interactions of their charged and hydrophobic residues with the hydrophilic charged head groups and the fatty acyl chains of phospholipids in the bacterial membrane. The consequences of this interaction include the dissipation of the electrochemical potential and lipid asymmetry, loss of important metabolites and cellular components due to the destruction of membrane permeability and eventual bacterial cell death [146-148]. The membrane-CAP interaction typically involves initial adsorption and binding of peptides to the membrane, followed by the accumulation of bound peptides that upon reaching a threshold level results in conformational transitions that permit peptide insertion into the membrane in order to exert their membranolytic activity [135]. Given their variety in size and structure, there is no unique mode by which all CAPs permeabilize bacterial membrane, rather a variety of mechanisms shared amongst different CAPs, as illustrated in Figure 4.

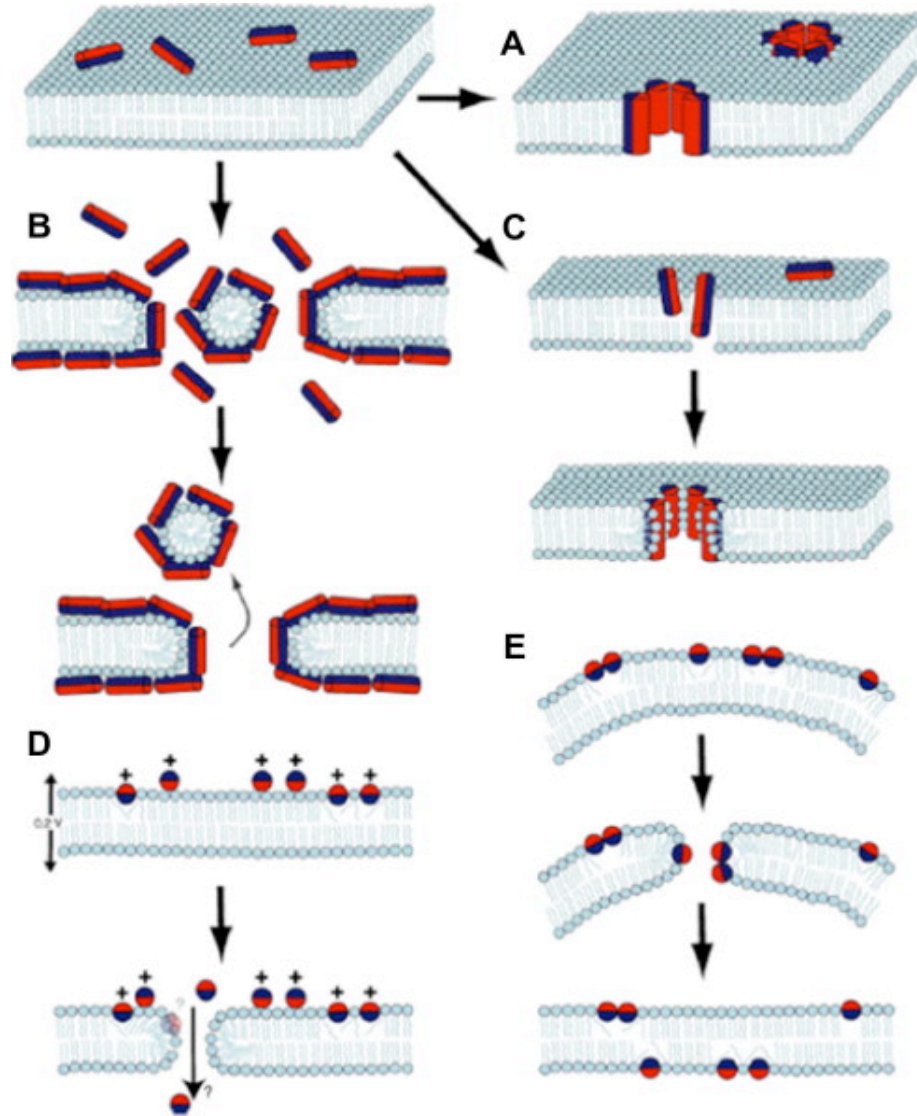


Figure 4. **CAP membranolytic mechanisms.** The principal models proposed for the pore-forming activity of CAPs are shown. Following binding to the outer surface of the cytoplasmic membrane (top-left), peptides can oligomerize and form pores according to the barrel-stave model (A), aggregate on the membrane surface in a detergent-like manner as in the carpet model (B) or distort the membrane to form lipid/peptide-lined pores as in the toroidal pore model (C). In the molecular electroporation model (D), lipid-bound CAPs promote an electrical potential difference across the membrane that induces transient poration. The sinking raft mechanism (E) proposes that peptide aggregation on the outer leaflet causes a mass imbalance between the two leaflets. The ensuing curvature gradient causes peptides to ‘sink’ into the membrane and form transient pores that permeabilize the cell and promote translocation of CAPs to the inner leaflet [135].

The membranolytic mechanisms of CAP activity include the formation of stable pores (barrel-stave and toroidal pore models), membrane thinning (via molecular electroporation or the sinking raft model) and micellization of membranes in a detergent-like manner (carpet model). In the barrel stave model (Figure 4A), peptide helices form a bundle in the membrane with a central lumen that constitutes the pore, a mechanism observed for alamethicin and zervamicin peptides [149, 150]. It is interesting to note that the formation of cytolytic pores by SLO and other cholesterol-dependent cytolysins like it involves a similar mechanism of oligomerization and protein conformational changes of the cytolytic polypeptide [151]. The toroidal pore model (Figure 4C) involves similar partitioning of peptides into the membrane, with the additional effect of inducing bending of the membrane leaflets such that the resulting pore is lined by both the inserted peptides and lipid head groups [148]. The molecular electroporation model (Figure 4D) describes the pore-forming activity of annexin V and consists of the aggregation of CAPs at the membrane surface with sufficient charge density to generate an electrical field that induces the formation of pores similar in size to those of polymyxin B and melitin peptides in the absence of overall membrane disruption [152, 153]. Another membrane thinning mechanism, the sinking raft model (Figure 4E), was described only recently for an α -helical peptide and involves an imbalance in mass ratio of the membrane due to binding of a particular lipid microdomain [154, 155]. This results in the formation of transient pores following translocation of the peptide and dissipation of the peptide induced membrane leaflet mass imbalance. The carpet model (Figure 4B), as its name implies, consists of adsorption of CAPs to the membrane such that the bilayer surface is ‘carpeted’ by the peptides. At the critical threshold concentration, peptides that act via

this mechanism (e.g. ovispirin, dermaseptin, magainins [156, 157]) will form toroidal pores lined by the peptide, allowing further access to the inner leaflet of the membrane by the CAP, and resulting in membrane disintegration through the formation of micelles in a detergent-like manner.

Recent studies however, suggest that many CAPs have multiple targets and/or much more complicated mechanisms of action than membrane poration alone (reviewed by [158]). In particular, some peptides can produce significant membrane perturbation by forming lipid-peptide domains, lateral phase segregation of zwitterionic lipids from anionic lipids and even inducing non-lamellar phases under physiologically relevant conditions [135]. A more in depth discussion of such CAP activity on bacterial membranes can be found in Chapter IV, especially as it relates to the effect of CAPs on ExPortal organization. Peptide-induced lipid segregation of anionic components has mainly been observed in gram-positive model membranes that are enriched in phosphatidylglycerol [159-162], the principal anionic lipid of *S. pyogenes* and other gram-positive pathogens [99, 163]. Another aspect of the peptide-pathogen interaction that was not often examined but is now of increasing interest is the effect CAPs have on their bacterial targets at levels below their minimum inhibitory concentrations. One studied effect is the induction of regulatory responses involved in resistance to CAPs. Bacteria have developed multiple factors to resist killing by CAPs, and expression of these factors is activated by regulatory mechanism responsive to sub-lethal amounts of CAPs. Researchers have exploited this to uncover previously unknown resistance mechanisms, by treating bacteria with sub-lethal peptide amounts and performing transcriptional analyses of the ensuing responses. Examples of this include studies on

Bacillus cereus resistance to enterocin [164], *Aspergillus niger* responses to caspofungin and fenpropimorph [165], and characterization of SigB-mediated antibiotic resistance in *Listeria monocytogenes* [166], to name a few. In the case of GAS responses to CAPs, sub-inhibitory concentrations of cathelicidin LL-37 stimulate expression of the *S. pyogenes* hyaluronic acid capsule synthesis operon (*hasABC*), which results in a more invasive phenotype [167]. This is a regulatory effect apparently exclusive to LL-37 among all the CAPs tested and involves direct sensing of the extra-cellular concentration of the LL-37 peptide by the CsrRS (CovRS) two-component regulatory system [167, 168]. Replacement of three acidic amino acids with uncharged residues in the extracellular domain of the CsrS (CovS) sensor-kinase abrogated induction of the *hasABC* operon by LL-37 [168], providing strong evidence of a close relationship between CAPs and streptococcal virulence. Mutagenic approaches combined with exposure to CAPs have conversely uncovered the regulatory networks controlling CAP resistance, as was the case in *S. aureus* with the identification of a two component regulatory system (GraRS) as the controller of key determinants of staphylococcal surface charge (*mprF* and *dlt*) required for resistance to multiple CAPs (HNP-1, polymyxin B) [169]. Another actively investigated effect of sub-lethal exposure to CAPs is the inhibition of antibiotic resistance factors produced by pathogens. For example, sub-lethal amounts of designer analogs of the CAP pyrrolicin inhibited the activity of the TEM-1 β -lactamase by targeting the heat-shock protein DnaK, thus restoring amoxicillin-sensitivity to β -lactam resistant strains of *E. coli* [170]. Also at sub-lethal levels, the enterocin peptide modifies the ion permeability of bacteria, dissipating the membrane proton motive force; this impairs the activity of multidrug efflux systems in *Listeria*

innocua, rendering it susceptible to traditional antibiotics [171], a potentially useful therapeutic tool given the troubling rise in multidrug resistance amongst pathogens.

Certain CAPs have additionally been demonstrated to act on intracellular targets or by non-permeabilizing mechanisms [172]. Examples include, activation of bacterial proteases by indolicidin [173], inhibition of DNA replication by PR-39 or microcin B17 [174, 175], or protein synthesis inhibition by pleurocidin [176]. However, the best characterized and most effective CAPs with additional non-permeabilizing activity are a class of lipid II-targeting bacterial defense peptides commonly referred to as lantibiotics (for a review, see [177]). The term lantibiotic designates gene-encoded peptides that contain the thioether amino acids lanthionine or methyllanthionine, which are formed by post-translational modifications that introduce intramolecular cyclic structures. The most representative lantibiotic, nisin, specifically binds lipid II in a defined stoichiometry in order to form pores that efficiently permeabilize bacterial membranes [178, 179]. More importantly however, non-porating variants of nisin have been shown to efficiently kill bacteria using an alternative mechanism by which lipid II is clustered into patches in the cytoplasmic membrane away from the regions where cell wall peptidoglycan synthesis occurs [180]. Other lantibiotics such as gallidermin, epidermin and mutacin have been observed to act via this alternative mechanism [180-182], thus inhibiting cell wall synthesis and causing bacterial cell death without permeabilizing the cytoplasmic membrane.

Characterizing ExPortal organization and its interaction with CAPs

Recent studies have shown that multiple human CAPs are lethal to *GAS in vitro* [183, 184], and that the CAP cathelicidin LL-37 is highly expressed in severe soft tissue infection in humans at sites coinciding with high tissue burdens of *S. pyogenes* [185]. Several studies have highlighted the important role of CAPs in innate immune defenses against *S. pyogenes*. These include the observation that soft tissue infection by *S. pyogenes* in mice deficient in a major CAP of the cathelicidin family (CRAMP) was significantly exacerbated [186] and that overexpression of a cathelicidin in murine skin provides enhanced protection [187]. It is not surprising then, that much recent research has been directed at identifying streptococcal factors that subvert the lethal effects of CAP activity [10, 167, 188].

The signature ability of CAPs to bind to the cytoplasmic membrane, offers numerous targets whose functions could be affected at sub-inhibitory concentrations. The cytoplasmic membrane is of particular importance to *S. pyogenes* since, as described previously, its pathogenicity relies on an extensive network of virulence proteins. Due to their cationic nature, CAPs predominantly bind to anionic phospholipids in bacterial membranes [147]. The ExPortal of *S. pyogenes* has been shown to have an asymmetric lipid content enriched in anionic phospholipids [99] that may contribute to the preferential retention of certain proteins at the ExPortal vs. the peripheral membrane [95]. These observations suggest that the ExPortal may thus be uniquely sensitive to the action of CAPs.

This presented the unique opportunity to expand understanding of the interaction of a strictly human pathogen like *S. pyogenes* with an important component of the innate

immune response, such as CAPs are, and of the mechanisms underlying the coordination of protein secretion and processing by the ExPortal in an organism as dependent as GAS are on secreted virulence factors. Thus, I investigated the interaction of CAPs at sub-lethal concentrations with *S. pyogenes* and the effects of said interaction on ExPortal organization and function. Given that lipid II-targeting CAPs can disrupt cell wall synthesis absent effects on membrane permeability and the potential for the ExPortal as a target for CAPs, this work was also designed to determine whether a link exists between cell wall biogenesis and organization of the secretory organelle that is the ExPortal.

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Chapter II

Cationic Antimicrobial Peptides Disrupt the *Streptococcus pyogenes*

ExPortal

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Cationic Antimicrobial Peptides Disrupt the *Streptococcus pyogenes* ExPortal

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ABSTRACT

Although they possess a well-characterized ability to porate the bacterial membrane, emerging research suggests that cationic antimicrobial peptides (CAPs) can influence pathogen behavior at levels that are sub-lethal. In this study, we investigated the interaction of polymyxin B and human neutrophil peptide (HNP-1) with the human pathogen *Streptococcus pyogenes*. At sub-lethal concentrations, these CAPs preferentially targeted the ExPortal, a unique microdomain of the *S. pyogenes* membrane, specialized for protein secretion and processing. A consequence of this interaction was the disruption of ExPortal organization and a redistribution of ExPortal components into the peripheral membrane. Redistribution was associated with inhibition of secretion of certain toxins, including the SpeB cysteine protease and the Streptolysin O (SLO) cytolysin, but not SIC, a protein that protects *S. pyogenes* from CAPs. These data suggest a novel function for CAPs in targeting the ExPortal and interfering with secretion of factors required for infection and survival. This mechanism may prove valuable for the design of new types of antimicrobial agents to combat the emergence of antibiotic-resistant pathogens.

INTRODUCTION

Cationic antimicrobial peptides (CAPs) are gene-encoded antibacterial peptides produced by nearly all known life-forms. A group of these, the defensins, are expressed by neutrophils and epithelial cells of humans and other mammals, functioning both as broad-spectrum microbicides and as modulators of the immune response [1]. It is generally accepted that the antibacterial activity of CAPs involves interactions of their charged and hydrophobic residues with the hydrophilic charged head groups and the fatty acyl chains of phospholipids in the bacterial membrane. The consequence of this interaction is the destruction of membrane permeability and bacterial cell death [2, 3]. However, more recent studies have suggested that many of these peptides may have multiple targets and/or much more complicated mechanisms of action (reviewed by [4]). In particular, one aspect of the peptide-pathogen interaction that is not often examined is the manner in which CAPs act on their bacterial targets at levels below their minimum inhibitory concentrations.

Interaction with CAPs plays an important role in host-pathogen interactions for infections caused by *Streptococcus pyogenes* (group A streptococcus). This Gram-positive pathogen can cause numerous diseases in humans that range from largely superficial infection of the skin and mucous membranes (impetigo, pharyngitis), to highly invasive and life-threatening diseases (necrotizing fasciitis), as well as, serious post-infection sequelae (rheumatic fever, glomerulonephritis, reviewed in [5]). Recent studies have shown that multiple human CAPs are lethal to *S. pyogenes in vitro* [6, 7], and that the CAP cathelicidin LL-37 is highly expressed in severe soft tissue infection in humans at sites coinciding with high tissue burdens of *S. pyogenes* [8]. Several studies have

highlighted the important role of CAPs in innate immune defenses against *S. pyogenes*. These include the observation that soft tissue infection by *S. pyogenes* in mice deficient in a major CAP of the cathelicidin family (CRAMP) was significantly exacerbated [9] and that overexpression of a cathelicidin in murine skin provides enhanced protection [10]. It is not surprising then, that much recent research has been directed at identifying streptococcal factors that subvert the lethal effects of CAP activity [11-13].

The signature ability of CAPs to bind to the cytoplasmic membrane, offers numerous targets whose functions could be affected at sub-inhibitory concentrations. The cytoplasmic membrane is of particular importance to *S. pyogenes*, as its ability to cause disease is dependent on the secretion of an extensive network of virulence proteins [14]. Lacking other known secretion systems, these virulence proteins are exported by the general secretory [15] pathway, which is highly conserved between Gram-positive and – negative bacteria and eukaryotes (for a review see [16]). However, unlike these latter two classes, Gram-positive bacteria lack a specialized cellular compartment for folding proteins following their translocation across the membrane by the Sec system. A solution to this problem used by *S. pyogenes* and several other species of Gram-positive cocci, is to cluster the Sec translocons at a defined microdomain of the cytoplasmic membrane that has been termed the ExPortal [17-21]. The ExPortal is also highly enriched with accessory factors for protein biogenesis, including sortases, and HtrA. The former are involved in the covalent attachment of proteins to the cell wall, while the latter is a multi-function protease and chaperone that is required for the biogenesis of the active form of the SpeB protease. These data suggest that one function of the ExPortal is to spatially couple secretion with protein maturation [17-19, 22, 23]. This is supported by the

observation that mutations causing the mis-localization of HtrA or sortase C outside of the ExPortal microdomain result in a highly reduced efficiency for maturation of secreted proteases and pili, in *S. pyogenes* and *Enterococcus faecalis*, respectively [18, 19].

Due to their cationic nature, CAPs predominantly bind to anionic phospholipids in bacterial membranes [3]. Of interest, the ExPortal of *S. pyogenes* has been shown to have an asymmetric lipid content enriched in anionic phospholipids [22] that may contribute to the preferential retention of certain proteins at the ExPortal vs. the peripheral membrane [19]. These observations suggest that the ExPortal may be uniquely sensitive to the action of CAPs. In the present study, we examined the interaction between several CAPs and *S. pyogenes* and report that CAPs preferentially interact with the ExPortal when examined at sub-lethal concentrations and that this results in a re-distribution of ExPortal components into the peripheral membrane. Furthermore, this disruption is associated with an inhibition of secretion of the SpeB cysteine protease and the Streptolysin O (SLO) cytolysin.

RESULTS

Polymyxin B binds to a single, unique site on the S. pyogenes membrane. To test the hypothesis that CAPs may interact with the ExPortal, we examined how the CAP polymyxin B interacts with the *S. pyogenes* membrane at sub-lethal concentrations. Readily available and well-characterized in its ability to bind anionic lipids [24], polymyxin B is a cyclic CAP antibiotic that is highly active against Gram-negative, but not most Gram-positive bacteria [25]. However, *S. pyogenes* is an exception and is highly susceptible to polymyxin B (see below), which likely reflects that unlike many other Gram-positive species its genome does not contain *mprF* [26], which encodes an enzyme that modifies the negative charge of phosphatidyl glycerol via lysinylation to reduce its affinity for binding CAPs [27, 28]. The interaction of polymyxin B with *S. pyogenes* was therefore examined using a biotin-labeled derivative of polymyxin B to treat cultures of the M1 serotype *S. pyogenes* strain SF370 in a “challenge assay.” In this assay (see “Experimental Procedures”), polymyxin B was added to cultures in the late logarithmic phase of growth (time = 0 h) and samples harvested for analysis after cultures had entered stationary phase (Fig. S3). The concentrations of polymyxin B used had no effect on viability in this assay and did not result in significant poration of membranes (see “Experimental Procedures” and Fig. S1). Bound polymyxin B was detected using a streptavidin-gold conjugate and examined by electron microscopy. This analysis revealed that rather than a uniform pattern of circumferential staining, cells consistently exhibited a single intense focus of gold particles at a discrete location adjacent to the membrane (Fig. 1A). An identical pattern was observed following treatment of the M14 serotype *S. pyogenes* strain HSC5 (data not shown) and minimal staining with the

streptavidin conjugate was observed in the absence of treatment with biotin-labeled polymyxin B (data not shown).

Polymyxin B targets the ExPortal. The pattern of polymyxin B binding was then examined following treatment with a fluorescent derivative of polymyxin B (dansyl-polymyxin B) at sub-inhibitory concentrations. Treatment with ≤ 60 μM polymyxin B under our challenge assay conditions (see “Experimental Procedures”) did not alter the viability of cultures at the end of the period of incubation as determined by enumeration of colony forming units (Fig. S1A). Examination of cultures treated with a fluorescent probe that is excluded by intact membranes (Live/Dead®) confirmed the viability of polymyxin B treated cultures and demonstrated that most cells had membranes that were not porated (Fig. S1B). Examination by fluorescent microscopy revealed that when treated at concentrations ≤ 15 μM the fluorescent CAP typically localized to a single discrete site of the membrane (Fig. 1B, C, D, E). Some cells with staining at multiple foci or with a more diffuse distribution around the circumference of the cell were also observed (data not shown). However, when examined quantitatively, the number of cells with single foci exceeded 60% and was significantly higher than that observed for any other staining pattern ($P < 0.0001$, Fig. 1F). To assess whether polymyxin B was targeting the ExPortal, co-staining was conducted to determine if these foci corresponded to the site of secretion of the SpeB cysteine protease, a signature feature of the ExPortal [17, 18]. This was conducted using an assay that employs a protease substrate that is intramolecularly quenched but becomes active when cleaved to visualize the site of secretion of active SpeB protease in cells that have been embedded in agarose [17, 29].

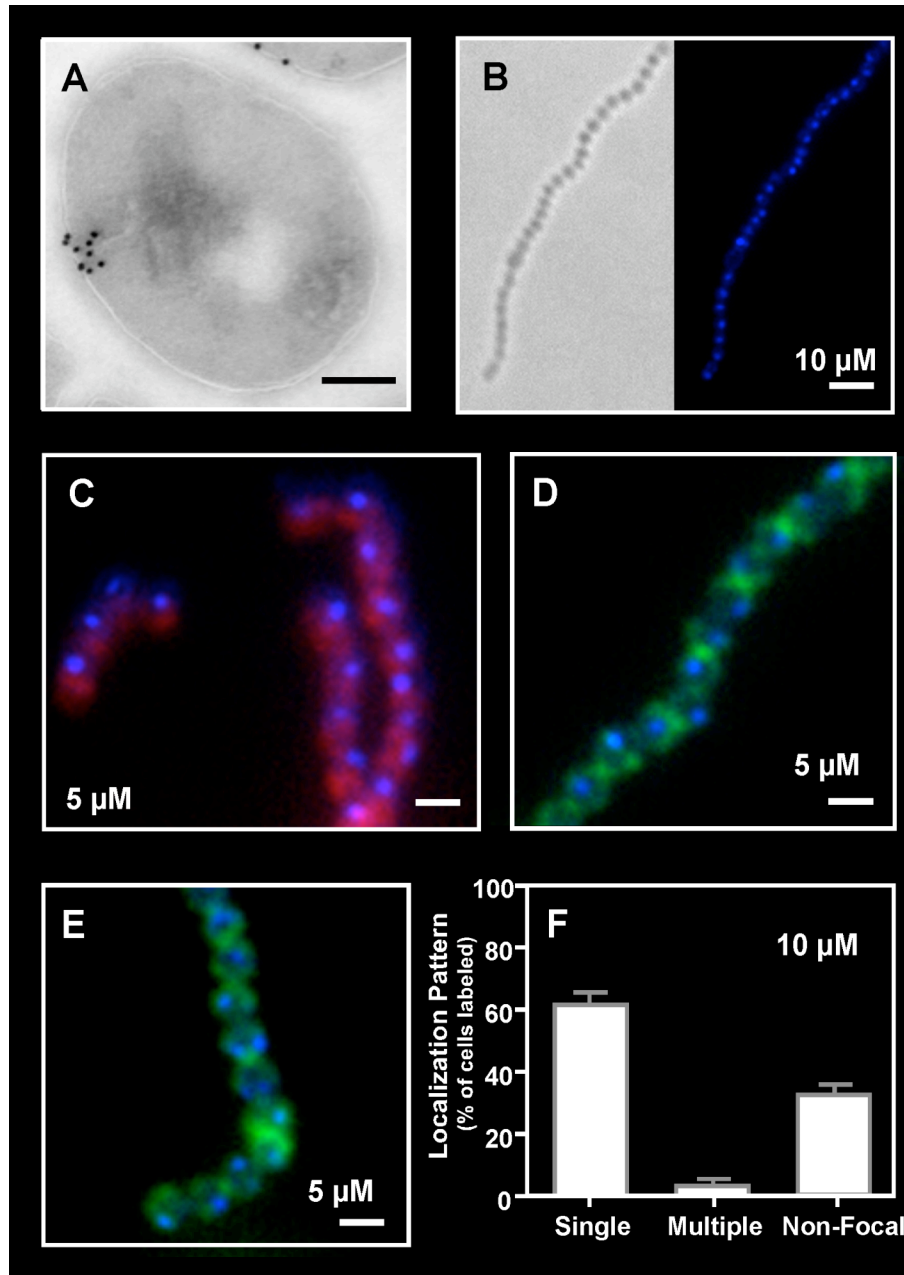


Figure 1. Focal binding of Polymyxin B to the *S. pyogenes* surface: The distribution of polymyxin B on the surface of *S. pyogenes* SF370 following sub-lethal challenge was revealed: (A) by treatment with biotinylated polymyxin B (1:10,000) and immunogold electron microscopy using staining with a streptavidin-gold conjugate (scale bar = 200nm) and (B, C, D, E) by fluorescent microscopy following challenge with dansyl-polymyxin B alone (B) at the concentration indicated in the Figure (scale bar = 1 μ m) or in cells counterstained with Nile Red (C), fluorescent vancomycin (D) or wheat germ agglutinin Alexa Fluor 488 conjugate (E). Staining patterns following challenge with dansyl-polymyxin B were quantitated as described in the Experimental Procedures (F). Data represents the mean and standard error of the mean (SEM) derived from at least 3 independent experiments and examination of a minimum of 1000 stained cells. The number of cells with a single focus was significantly higher than any other staining pattern ($P < 0.0001$).

When a sub-inhibitory concentration of dansyl-polymyxin B (5 μM or 10 μM) was included in this assay about 50% of the cells showed staining with both reagents. However, when co-stained (Fig. 2), the coincidence of the membrane site recognized by dansyl-polymyxin B and the site of SpeB secretion approached 100% (out of at least 200 co-stained cells observed).

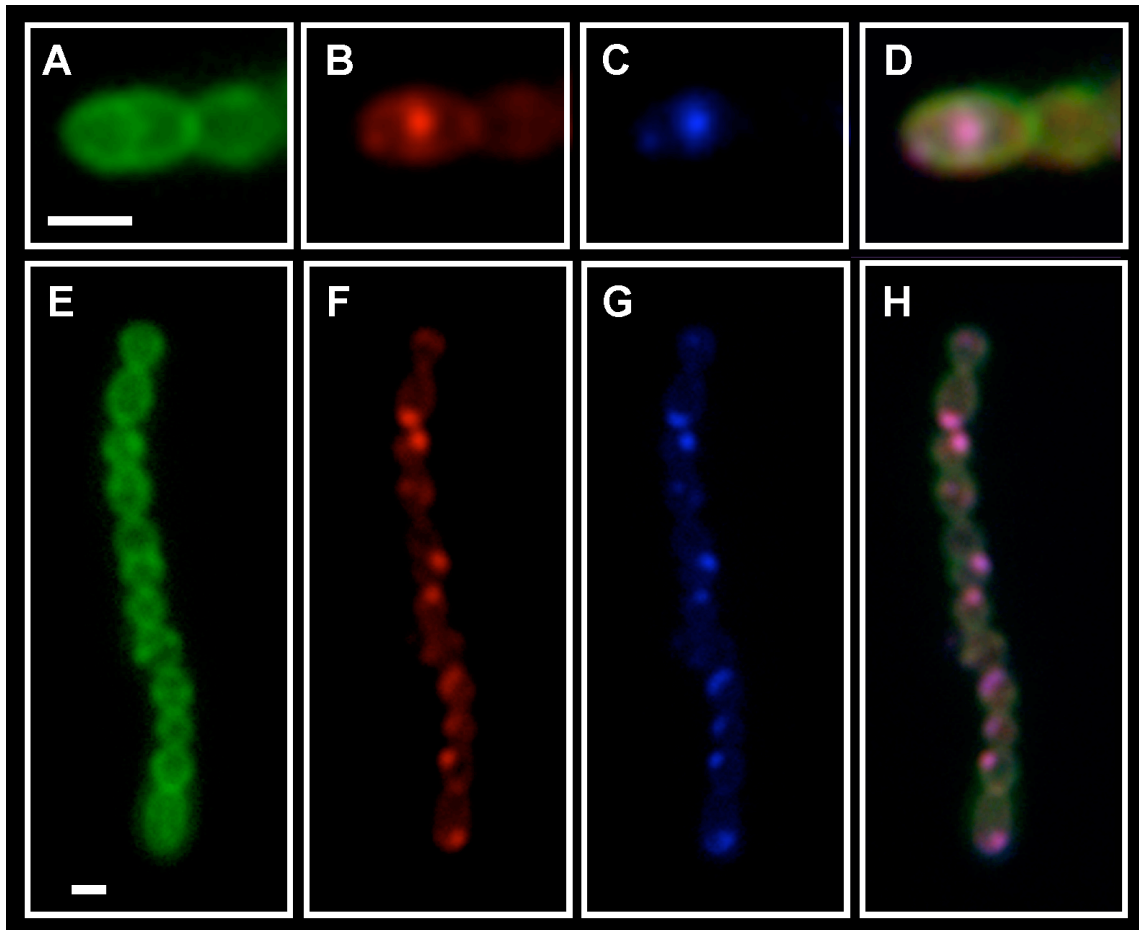


Figure 2. The site of polymyxin B binding is coincident with the site of SpeB secretion. Cells of *S. pyogenes* SF370 were challenged with dansyl-polymyxin B (10 μM), fluorescent vancomycin stained to visualize cell wall and subjected to the red protease assay, which monitors cleavage of BODIPY TR-X-casein by the SpeB protease. Stained cells were then examined by fluorescent microscopy. Panels are as follows: (A, E) fluorescent vancomycin, (B, F) BODIPY TR-X-casein, (C, G) Dansyl-polymyxin B and (D, H) Merge of Panels A, B and C. Scale bar = 1 μm .

Polymyxin B disrupts ExPortal Lipids. The data above suggest that at sub-lethal concentrations, polymyxin B preferentially targets the ExPortal. The consequence of this interaction was then examined using higher, but still sub-lethal concentrations of the CAP. ExPortal integrity was assessed by staining with 10-nonyl acridine orange (NAO), a fluorescent membrane probe that preferentially binds anionic phospholipids [22, 30]. In the absence of polymyxin B, NAO stained cells at single foci (Fig. 3A), which were previously shown to be co-incident with the ExPortal [22]. Following treatment with 30 μ M polymyxin B, most cells had either multiple foci or a more diffuse staining pattern (Fig. 3B), while this latter pattern predominated following treatment with 60 μ M polymyxin B (Fig. 3C). This result suggested that the CAP was altering the organization of ExPortal-associated anionic phospholipids. Consistent with this, treatment with a higher, but still sub-lethal concentration of biotin-labeled polymyxin B resulted in cells stained at multiple foci or more diffusely around the membrane when examined by electron microscopy, rather than staining at single foci as was observed at lower concentrations (compare Fig. 3E with Fig. 1A.). Similarly, treatment with a higher concentration of dansyl-polymyxin B resulted in a majority of cells stained at multiple foci (compare Fig. 3F, G to Fig. 1B, D, E). This shift was examined quantitatively (Fig. 3H), revealing that the number of cells stained at multiple foci was significantly higher than other staining patterns observed. Stressing membranes by subjecting cells to a condition known to induce the heat shock response (42°C, 30 mins.; [18]) did not alter the focal pattern of NAO staining (Fig. 3; compare panels 3D, 3A). Similarly, heat shock did not alter the focal binding pattern of a low sub-lethal concentration of bodipy-labeled polymyxin B (10 μ M, Fig. S2C,D), which contrasts with the disruption observed at a

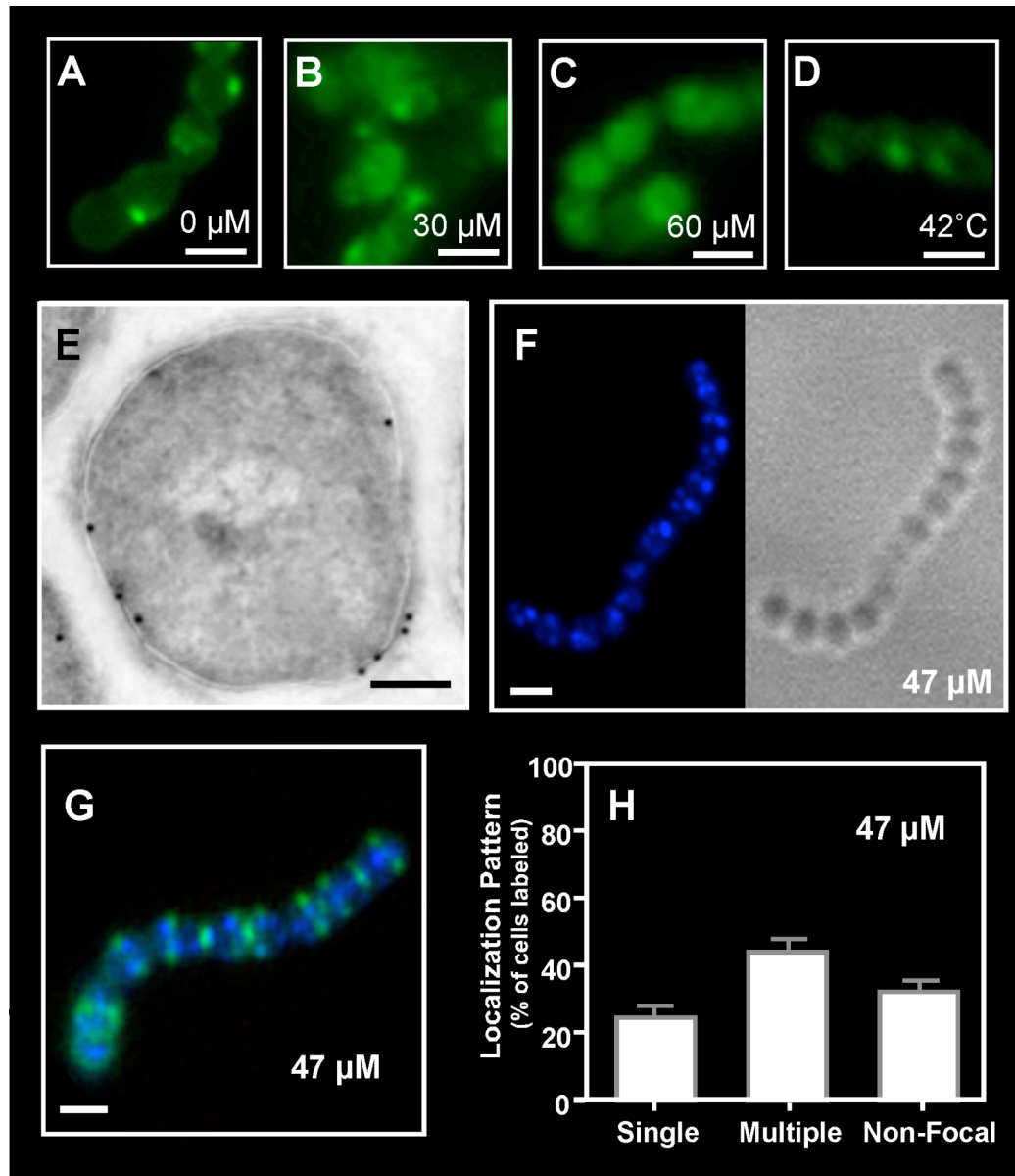


Figure 3. Sub-lethal challenge with polymyxin B alters the distribution of anionic membrane lipids. Cultures of *S. pyogenes* SF370 were stained with NAO following challenge with 0 μM (A), 30 μM (B) or 60 μM (C) polymyxin B or heat shock at 42°C (D) and examined by fluorescent microscopy (scale bar = 1 μm). The distribution of polymyxin B following higher, but still sub-lethal challenge, was revealed by treatment with biotinylated polymyxin B (1:500) and immunogold electron microscopy using staining with a streptavidin-gold conjugate (scale bar = 200nm) (E) and by fluorescent microscopy following challenge with dansyl-polymyxin B alone at the concentration indicated in the Figure (scale bar = 1 μm) (F) or costained with fluorescent vancomycin (G). Staining patterns following challenge were quantitated as described previously (H). Data represents the mean and SEM derived from at least 3 independent experiments and examination of a minimum of 1000 stained cells. The number of cells with multiple foci was significantly higher than any other staining pattern ($P < 0.0001$) at this polymyxin B concentration.

higher sub-lethal concentration (45 μM , Fig. S2E,F). Thus, these data suggest that disruption of the ExPortal-associated anionic lipid domain is specific to high sub-lethal concentrations of polymyxin B and does not result from a general membrane stress response.

Polymyxin B disrupts ExPortal Organization. There is evidence to suggest that the asymmetric lipid content of the ExPortal contributes to the retention of some ExPortal-associated membrane proteins [19]. This suggests that the polymyxin B-mediated disruption of ExPortal lipid structure observed above could result in an overall disruption of ExPortal organization. To test this, the distribution of several proteins known to be enriched at the ExPortal was examined in cells of strain SF370 exposed to a sub-lethal concentration of polymyxin B. The concentrations tested (30-60 μM) were those that produced a high frequency of cells with multiple NAO- or polymyxin B-stained foci (see above). Consistent with prior studies [17, 18], in the absence of polymyxin B, the translocon ATPase SecA localized to a single membrane site in a majority of cells when examined by immunogold electron microscopy (Fig. 4A). Similarly, the membrane-associated HtrA serine protease [18] localized to a single membrane site in a majority of cells when examined by immunogold electron microscopy (Fig. 4D) and by immunofluorescent microscopy (Fig. 4G, H). Adding polymyxin B at concentrations <30 μM did not alter this pattern for either SecA or HtrA. However, polymyxin B concentrations of 30 μM through 60 μM resulted in redistribution of SecA (Fig. 4B,C) and HtrA (Fig. 4E,F, I, J) as demonstrated by a more circumferential staining pattern and a significant increase in the number of cells demonstrating multiple stained foci (Fig. 4K;

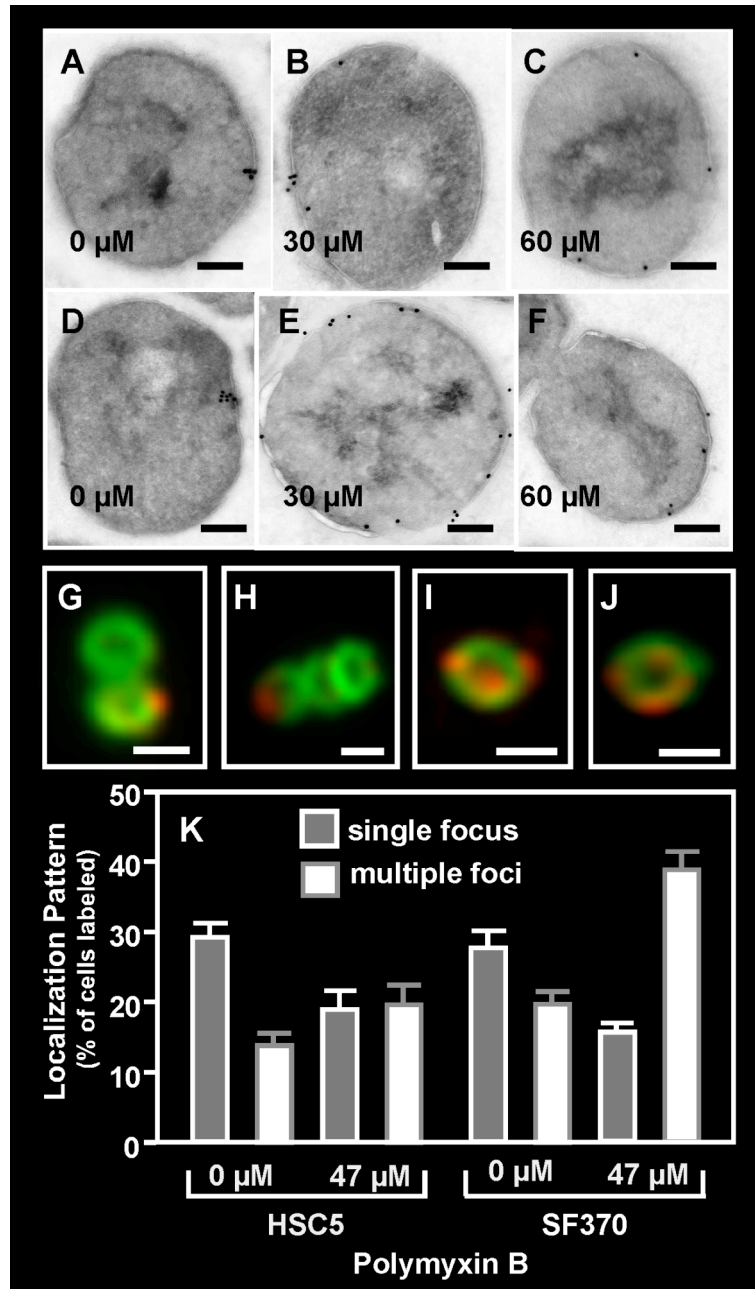


Figure 4. **Redistribution of ExPortal proteins following sub-lethal polymyxin B challenge.** The distribution of SecA (A, B, C) and HtrA (D, E, F) on *S. pyogenes* SF370 was assessed by immunogold electron microscopy following challenge with polymyxin B at the concentrations indicated in the Figure (scale bar = 200nm). The distribution of HtrA was also assessed by immunofluorescent microscopy in the absence of (G, H) and following challenge with polymyxin B at 47 μM (I, J) and was quantitated as described previously (scale bar = 500nm) (K). Data represents the mean and SEM derived from at least 3 independent experiments and examination of a minimum of 1000 stained cells. In untreated cultures, the number of SF370 or HSC5 cells with a single focus was significantly higher than any other staining pattern ($P < 0.05$), whereas in polymyxin B-treated cultures, the number of streptococcal cells with multiple foci was significantly higher ($P < 0.05$).

$P < 0.05$). Similar results were observed with the unrelated strain HSC5 (data not shown). Analysis of culture fractions indicated that the levels of SecA protein were unchanged in peptide-challenged streptococci and that the protein was retained in the same fractions in treated and untreated cells (Fig. S3A), suggesting that only localization of SecA at the membrane is affected. These data demonstrate that sub-lethal polymyxin B treatment can result in a disruption of ExPortal organization.

Polymyxin B inhibits ExPortal-mediated secretion of SpeB. Disruption of ExPortal organization could have a deleterious effect on protein secretion. Particularly vulnerable would be those proteins that require the ExPortal to coordinate the activities of multiple secretion and biogenesis factors. A prominent example of this class is the SpeB cysteine protease, which requires numerous accessory factors, including the ExPortal-localized HtrA, for its secretion and conversion of its 43 kDa zymogen into the 28 kDa mature form [18, 31-34]. In addition, because the gene encoding SpeB is expressed at the onset of the stationary phase of culture [35-37], its expression would provide a sensitive assessment of the effect of prior challenge with a CAP at the late logarithmic phase of growth. Treatment of strain SF370 with various sub-lethal concentrations of polymyxin B resulted in a dose-dependent reduction in the amount of cysteine protease activity in culture supernatant (Fig. 5A), to a level less than 20% of that observed in the absence of the CAP (Fig. 5A, 60 μ M). This reduction correlated with a dose-dependent decrease in the amount of SpeB polypeptide that was detected in treated culture supernatants (Fig. 5B). Secretion of SpeB in strain HSC5 was inhibited at lower concentrations of polymyxin B (Fig. S3B) and challenge with 60 μ M polymyxin B

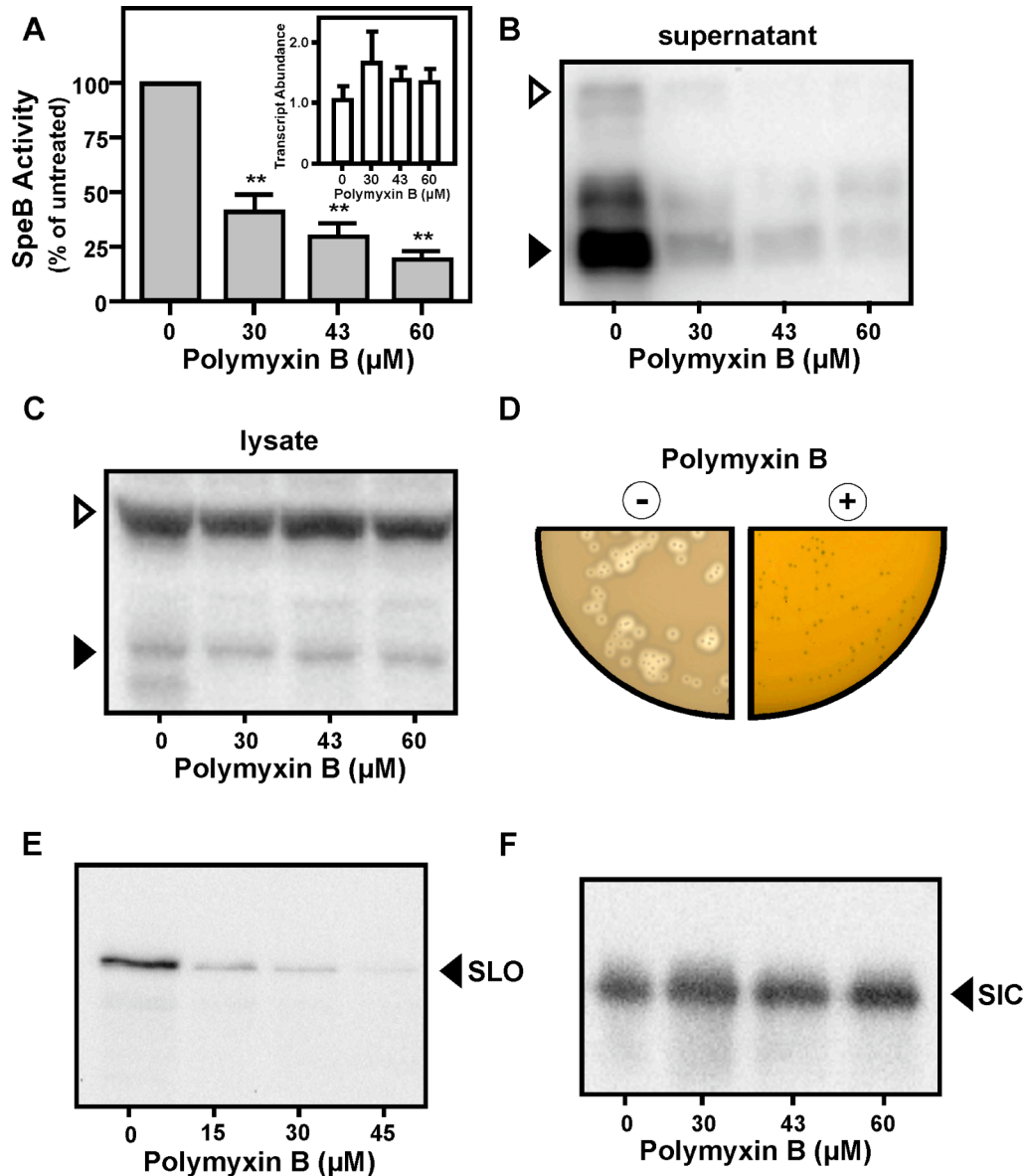


Figure 5. High sub-lethal challenge with polymyxin B inhibits secretion of SpeB and SLO, but not SIC. Expression of the SpeB protease in cultures of *S. pyogenes* SF370 was determined following challenge with the indicated concentrations of polymyxin B by quantitation of cysteine protease activity in culture supernatant (A), by real-time RT-PCR analysis of *speB* transcript abundance (A, inset) by Western blot analysis of culture supernatant (B) in whole cell lysates (C) and following overnight culture on protease indicator plates containing (+) or lacking (-) 150 µM polymyxin B (D). Expression of SpeB results in a zone of clearing around colonies. Western blotting was also used to analyze the amount of SLO (E) and SIC (F) present in culture supernatant following challenge with the indicated concentrations of polymyxin B. All samples for SpeB analysis were harvested at 3 hrs post-challenge; samples for SLO and SIC were harvested 2hrs post-challenge. Open and filled triangles indicate the migration of the zymogen and mature form of SpeB, respectively. The migration of the SLO and SIC polypeptides are also indicated.

resulted in a >95% inhibition of SpeB activity (data not shown). Several lines of evidence indicated that inhibition was not caused by an effect on growth, induction of stress or by suppression of *speB* transcription. Firstly, at the time of analysis of SpeB in culture supernatants (3 hrs post-challenge, Fig. S3C), the *speB* transcript was as abundant or present at higher levels as compared to untreated cultures (Fig. 5A, inset). Secondly, when transcription of *speB* was de-repressed and partially uncoupled from its growth phase-dependent control by mutation of the regulatory gene *vfr* [34, 38], polymyxin B challenge resulted in the same profile of inhibition of SpeB secretion that was observed for the wild type strain (Fig. S3D). Thirdly, sublethal polymyxin B did not induce a general stress response, as treatment had no effect on levels of the HtrA protease (Fig. S3E). Fourthly, while treatment reduced the amount of SpeB present in supernatants, the SpeB polypeptide was still produced and could be detected in cell lysates (Fig. 5C). Finally, incorporation of polymyxin B into protease indicator plates at concentrations that did not reduce numbers of CFU's, did inhibit expression of protease activity, as indicated by an absence of zones of clearance around colonies on treated media following overnight culture (Fig. 5D), although some activity was evident upon a prolonged period of incubation (not shown). Taken together, these data indicate that polymyxin B challenge inhibits SpeB expression at the level of secretion.

Polymyxin B inhibits secretion of SLO but not SIC. The Sec pathway delivers presecretory proteins to the translocons via either the post-translational pathway or the co-translational signal peptide recognition (SRP) pathway. While SpeB can be secreted via the post-translational pathway, the cytolysin SLO is targeted via the SRP pathway

[33]. Given that expression of this cytolysin begins during the early logarithmic phase of growth, the challenge assay was slightly modified (see Experimental Procedures) in order to detect SLO that was secreted post-challenge. Comparison of supernatant harvested from peptide-challenged and untreated cultures of SF370 revealed a dose-dependent decrease in the amount of SLO protein detected in treated cultures (Fig. 5E); an effect that was more pronounced in HSC5 (data not shown). Similar to *speB*, *slo* transcript levels in polymyxin B-challenged cultures were as abundant as in untreated cultures at the time of analysis (Fig. S3F). A prominent difference between SF370 and HSC5 is that the former possesses the Streptococcal Inhibitor of Complement (SIC), which has been shown to enhance the resistance of *S. pyogenes* to CAPs [13, 39]. In contrast to SLO, no apparent differences in secreted SIC polypeptide was observed between culture supernatants from untreated and challenged cultures of SF370 (Fig. 5F). Thus, inhibition of toxin secretion is selective for certain toxins and is not a universal feature of sub-lethal polymyxin B treatment.

Human Defensin HNP-1 also disrupts the S. pyogenes ExPortal. In order to assess if human CAPs could also disrupt ExPortal function, cultures were challenged with sub-lethal concentrations of the α -defensin HNP-1, the β -defensins hBD-1 and hBD-2, and the cathelicidin LL-37. A Western blot analysis of culture supernatant revealed that of these, only HNP-1 noticeably inhibited secretion of SpeB at the CAP concentrations tested (<50 μ M for all CAPs tested), and did so at concentrations lower than those required for similar inhibition by polymyxin B (Fig. 6A). Examination of the staining pattern of a fluorescent derivative of HNP-1 (5-FAM-HNP1) revealed that while staining

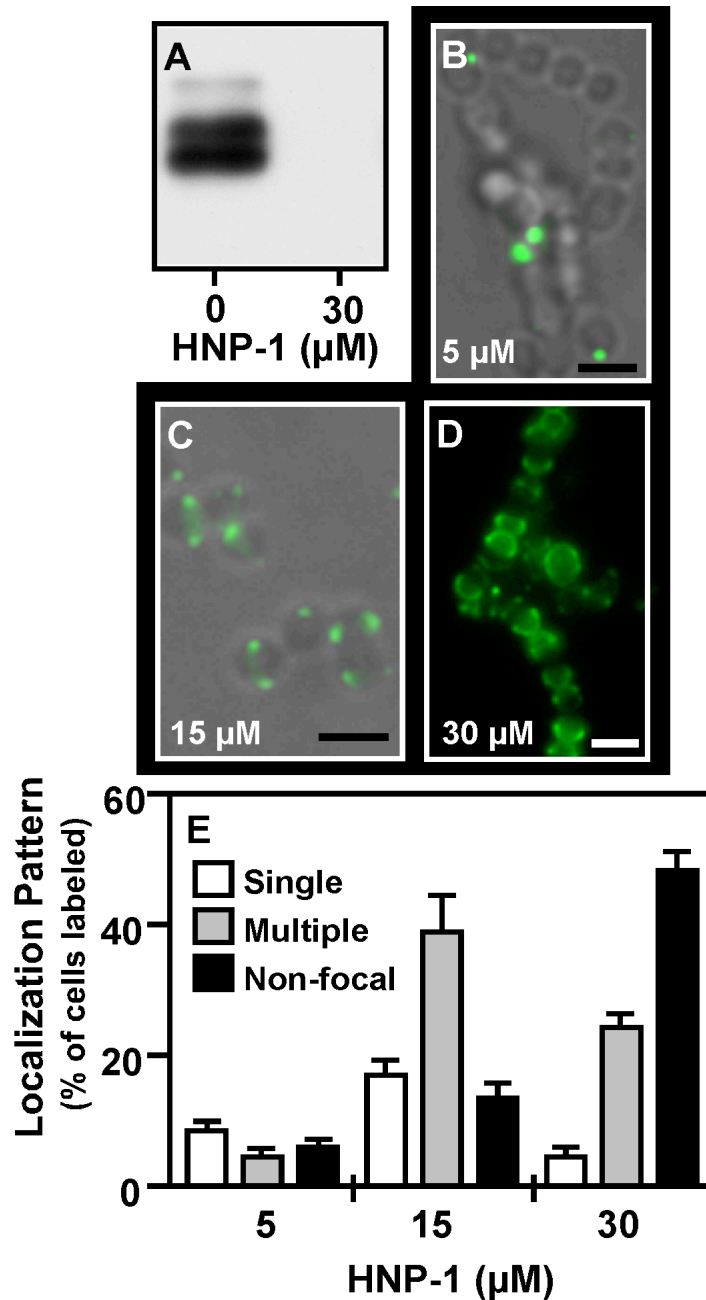


Figure 6. **Focal localization and inhibition of SpeB expression by HNP-1.** Cultures were challenged with HNP-1 or by 5-FAM-HNP-1 at the concentrations indicated. Expression of SpeB was evaluated by a Western blot analysis (A) and binding to the *S. pyogenes* surface was assessed using fluorescent microscopy (B, C, D). Presented are overlays of fluorescent and phase images (B, C) or the fluorescent image alone (D) (scale bar = 1 μm). Staining patterns were quantitated as described previously (E). Data represents the mean and SEM derived from at least 3 independent experiments and examination of a minimum of 1000 stained cells. At minimal concentration the number of cells with single foci is significantly higher than all other staining patterns, whereas multiple foci and hemisphere staining predominated significantly at higher peptide concentrations ($P < 0.05$).

was observed on a small population of cells at low concentration (5 μ M, Fig. 6B), that these predominantly stained at discrete single or multiple foci (Fig. 6E). Increasing concentrations (15 μ M) resulted in a higher percentage of cells with multiple foci (Fig. 6C, 6E), while at even higher concentrations (30 μ M) the CAP primarily localizes to one hemisphere of the cell (Fig. 6D). Finally, localization of HtrA by immunofluorescence demonstrated that similar to polymyxin B, treatment with HNP-1 at sub-lethal concentrations that inhibited SpeB secretion also significantly decreased the number of cells exhibiting HtrA at discrete foci ($P < 0.0001$, Fig. 7). These results suggest that a CAP to which *S. pyogenes* may be exposed to inside a human host can disrupt the organization and function of the ExPortal.

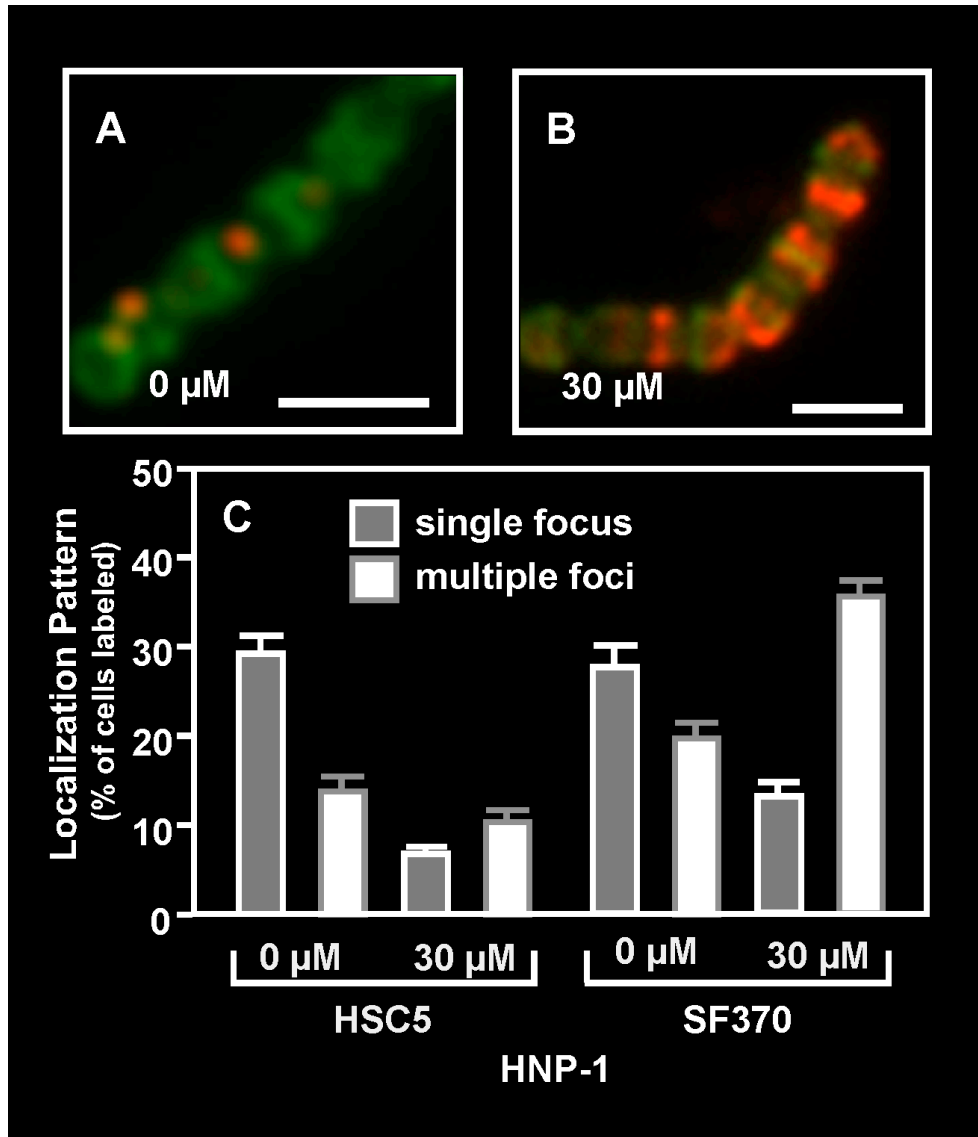


Figure 7. **Challenge with HNP-1 results in redistribution of HtrA.** Cultures of *S. pyogenes* HSC5 were challenged with the indicated concentrations of HNP-1 and subjected to immunofluorescent microscopy to assess the distribution of HtrA (A, B). Cell walls were visualized by staining with fluorescent vancomycin (scale bar = 1 μm). Staining patterns were quantitated as described previously (C). Data represents the mean and SEM derived from at least 3 independent experiments and examination of a minimum of 1000 stained cells. Challenge with the peptide significantly decreased the number of SF370 or HSC5 cells exhibiting HtrA at single foci ($P < 0.0001$) and resulted in a significant increase in the number of multiple foci ($P < 0.0001$) in the SF370 strain.

DISCUSSION

The ability of *S. pyogenes* to cause a wide range of diseases has been linked to its production of virulence factors that neutralize or subvert innate mechanisms of immunity, including the lethal effects of CAPs (for review, see [14, 40, 41]). However, by showing that sub-lethal concentrations of CAPs are able to disrupt the secretory ExPortal, the results of this study suggest that interaction with CAPs may play a more intimate role in *S. pyogenes* pathogenesis, serving to modulate virulence factor expression at both the transcriptional and post-transcriptional levels. Finally, the observation that disruption of ExPortal organization is associated with a defect in secretion of certain toxins provides additional support for the concept that the ExPortal serves a functional role in facilitating protein secretion.

A literature is emerging indicating that individual CAPs have a considerable diversity in how they interact with and kill bacteria using both lytic and non-lytic mechanisms (reviewed in [42-44]). However, an initial step common to all pathways analyzed to date, involves the binding of the CAP to anionic lipids in the membrane. While little is currently known about the mechanism(s) by which sub-lethal concentrations of CAPs may affect bacterial physiology, the data presented here indicate that disruption of ExPortal organization and inhibition of SpeB and SLO secretion were also associated with membrane binding by the CAPs. Most models of CAP action postulate that at low sub-lethal concentrations, the peptides bind in a parallel orientation relative to the lipid membrane and begin to aggregate as concentrations increase, until a lethal concentration is reached and the peptides reorient to attack the membrane (reviewed by [4, 43]). The observation that CAP binding to the ExPortal leads to

redistribution of anionic lipids in the absence of pore formation contrasts with CAP behavior in model membranes, where they typically cluster anionic lipids (reviewed in [45]). Additionally, this redistribution of anionic lipids does not appear to induce or result from generalized disruption of the cytoplasmic membrane, as our data show that the integrity of the membrane is not compromised. Furthermore, numerous functions that depend on membrane integrity are also not compromised, including energy-dependent processes such as gene transcription and cell-growth, the latter of which also requires the assembly of macromolecular complexes at the membrane. The binding of polymyxin B to the membrane appears to only affect the physical location of anionic lipids and SecA within the membrane relative to each other and to accessory factors like HtrA. This suggests that the initial binding and/or aggregation of the CAPs is sufficient to disrupt the factor(s) that maintains ExPortal organization. If the anionic character of these lipids plays a fundamental role in organization, then it is possible that CAP binding serves to neutralize their charge to promote disruption. Alternatively, the interaction of CAPs with ExPortal lipids may disrupt the insertion or translocation of factors that function to maintain ExPortal lipid organization. The redistribution of ExPortal-associated membrane proteins may then occur subsequent to disruption of lipid structure, as analysis of a bitopic membrane protein (sortase C) has suggested that interaction between anionic lipids and a high density of positive charge in the cytoplasmic tail of its transmembrane helix is responsible for its localization at the ExPortal [19].

Many bacterial pathogens segregate charged lipids into microdomains (reviewed in [46, 47]) and these support the function of several multi-enzyme complexes required for secretion, membrane protein biogenesis and cell division [48-50]. Since some CAPs

can kill in the absence of pore formation and can also target charged lipids, it has been proposed that they exert their lethal effect by interfering with the dynamic function of these lipid-associated complexes, referred to as the “sand in a gearbox” mechanism [51]. This concept is supported by the observation that these CAPs can elicit enhanced expression of some components of these complexes [51]. By documenting the physical disruption of a multi-component organelle in the absence of poration, the results of the present study provide strong support for this mechanism. Furthermore, they extend this emerging concept to show that disruption can also occur at sub-lethal exposure to CAPs, and as a consequence, significantly influence how the bacterium interacts with its environment.

To date, modulation of signaling via the CovRS (CsrRS) two-component transcription regulator has been the most widely studied consequence of sub-lethal *S. pyogenes*-CAP interaction. Analysis of this mechanism has shown that from among a large panel of CAPs that included HNP-1, only human LL-37 functioned to induce signaling [12]. Similarly, ExPortal targeting was not a property that was shared by all the CAPs tested in the present study, including LL-37. While the reason for this is not known, it was not unexpected, as the considerable diversity of CAP structure is reflected in differences in selectivity, binding efficiency, mechanism of killing and the physical conditions required for optimum activity (reviewed by [42]). Furthermore, these behaviors have not been extensively investigated at sub-lethal concentrations for any CAP.

Of the human-derived CAPs tested in the present study, HNP-1 proved more potent than polymyxin B in ExPortal disruption. This α -defensin is expressed

constitutively in the azurophilic granules of neutrophils [1] and can be detected at concentrations of up to 63 μM [52] in secretions from nasopharyngeal tissue, a niche that can be colonized by *S. pyogenes*. Thus, it is likely that *S. pyogenes* encounters HNP-1 during infection. Also, while HNP-1 targets anionic lipids, it may have additional targets, including the lipid II molecule involved in cell wall synthesis [53]. Binding to lipid II likely explains why higher concentrations of HNP-1 produced more heterogeneous patterns of staining, including a tendency for staining one hemisphere of the cell. In “ovococci” like *S. pyogenes*, which divide in successive parallel planes perpendicular to their long axis, this pattern is consistent with the pattern of new cell wall synthesis (reviewed by [54]).

The ability of *S. pyogenes* to secrete numerous toxins likely plays a central role in its ability to cause disease [55]. Thus, inhibition of SpeB and SLO may represent a physiologically relevant property of sub-lethal CAP concentrations. Since secretion of essential proteins will be required to support viability, it is unlikely that sub-lethal CAP treatment would produce a global blockade of protein secretion. It is known that biogenesis of SpeB requires secretion factors that are not required for other Sec-secreted proteins [31-34]. Similarly, as an SRP substrate [33] SLO's secretion requires coordination between FtsY, the membrane receptor for the SRP, and the Sec translocons [56]. In contrast SIC secretion in the SF370 strain was unaffected by CAP treatment, a result that suggests that its secretion may not require additional factors in addition to Sec. If the ExPortal functions to coordinate interactions between various secretion components, then disruption of ExPortal organization could lead to the secretion defect. Alternatively, it has been shown that some CAPs can act on internal targets (reviewed by

[4]), raising the possibility that sub-lethal CAPs may interfere with secretion factors in the cytoplasmic compartment of the streptococcal cell.

Interestingly, analysis of the recently introduced antibiotic daptomycin has suggested that it principally targets regions of membrane enriched in anionic phospholipids [57], suggesting that these domains can be exploited for the development of even more potent antibiotics. Thus, further analysis of CAP-ExPortal interaction will be valuable for uncovering the fundamental organizing principles of the ExPortal, how the ExPortal informs the coordination of protein secretion and maturation and the importance of modulation of secretion in response to host factors during infection.

EXPERIMENTAL PROCEDURES

Strains, plasmids, media and growth conditions: As indicated, experiments utilized *S. pyogenes* HSC5 [58] or SF370 [26]. Localization of HtrA was analyzed in HSC5 following transformation by pHtrA-HA, which expresses a HA-tagged derivative of HtrA [18]. Strain GCP682 contains an in-frame deletion ($vfr_{\Delta 15-289}$) in *vfr* (SPy_0887) (G. Port and M. Caparon in preparation). Routine culture was at 37°C in Todd-Hewitt broth (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium). Functional assays utilized cultures grown in unmodified C medium as described previously [59]. To produce solid media, Bacto Agar (Difco) was added to a final concentration of 1.4%. Expression of the secreted SpeB cysteine protease was evaluated on skimmed milk agar medium [31]. Liquid cultures were grown without agitation in closed containers and solid cultures were incubated under anaerobic conditions using a commercial gas generator (GasPak, cat. #260678, BBL) as described [59]. The SpeB protease was inhibited in cultures used to analyze the abundance of secreted streptococcal proteins by including the cysteine protease inhibitor E64 [31]. In selected experiments, media were supplemented with CAPs, including polymyxin B (cat. #P0972-50MU, Sigma), Human Neutrophil Peptide-1 (HNP-1, cat. #60743, AnaSpec), Human β -defensin 1 (h β D-1, cat. #072-53, Phoenix Pharmaceuticals), Human β -defensin 2 (h β D-2, cat.#072-48, Phoenix Pharmaceuticals), and Human cathelicidin LL-37 (LL-37, cat. #61302, AnaSpec) at the concentrations indicated in the text.

Challenge with CAPs: Unless otherwise indicated, bacteria were cultured overnight in liquid THY medium, diluted 1:100 in fresh C medium and then cultured to the late-

logarithmic phase of growth (Fig. S3C, 0h). The CAPs were then added to the various final concentrations indicated in the text and incubation continued until cultures reached stationary phase (3 hrs post-treatment, Fig. S3C) as determined by monitoring optical density (OD_{600}). Control cultures were grown in parallel, but were not treated with any CAPs; control and treated cultures entered stationary phase at approximately the same time (Fig. S3C). For this challenge assay, the minimum concentration of polymyxin B and HNP-1 that resulted in a greater than one-log reduction in the number of SF370 viable cells (determined by enumeration of colony forming units as described below) were 145 μ M and 67 μ M, respectively. This assay differs from a conventional assay for determining the minimal inhibitory concentration, which employs an inoculum of stationary phase cells from an overnight culture at a low cell density. As expected, SF370 was more sensitive to polymyxin B in the conventional assay (22 μ M). In contrast, SF370 was more resistant to polymyxin B on protease indicator medium (see Fig. 5), which is likely due to its increased lipid content. For analysis of proteins whose expression begins in early logarithmic phase, the challenge assay was modified in order to remove proteins secreted into the supernatant prior to challenge as follows: cultures were grown to mid-logarithmic phase (Fig. S3C), harvested by centrifugation, immediately resuspended in an equivalent volume of warm fresh medium and then challenged with CAPs as indicated in the text. Samples were collected and processed for assessment of viability, for microscopy and for analysis of protein secretion, as described below. Data presented are representative of those obtained over the range of sub-lethal concentrations of CAPs.

Analysis of Viability: At selected time points following challenge with CAPs, aliquots were removed from cultures and viability assessed by determination of CFUs following brief sonication to disrupt streptococcal chains, serial dilution in PBS and plating on C medium agar as described previously [60]. Viability was also assessed by staining with a fluorescent vital dye (LIVE/DEAD®, BacLight™) as recommended by the manufacturer (Invitrogen). Viability was quantitated by examination using a fluorescent microscope (Leica model DM IRE 2) with enumeration of the percentage of live bacteria in randomly chosen microscopic fields totaling >1000 cells for each condition examined. Data reported represent the mean and standard error of the mean derived from a minimum of 3 independent experiments. Differences between calculated means were evaluated for significance using a one-way Analysis of Variance (ANOVA).

Cellular staining and fluorescent microscopy: Streptococcal cultures were challenged with various CAPs as described above, stained with various fluorescent reagents and then analyzed by fluorescent microscopy as follows: Analysis of the location and integrity of a membrane microdomain enriched in anionic phospholipids was assessed by staining with 10-nonyl acridine orange (NAO, cat. #A7847 Sigma) as described [22]. Localization of the discrete membrane site of secretion of the SpeB cysteine protease was conducted using the red protease assay [17]. Analysis of CAP binding involved the substitution of native CAPs in the challenge assay with sub-lethal concentrations of fluorescent derivatives, including dansyl-polymyxin B (5, 10, 15 and 47µM, cat. #P13238, Invitrogen), polymyxin B BODIPY®FL conjugate (10 and 45µM, cat. #P13235, Invitrogen) or 5-FAM-HNP-1 (5, 15 and 30µM, custom synthesis by

AnaSpec). Samples were examined using a Leica model DM IRE 2 fluorescent microscope and images captured using a QImaging Retiga 1350 EX charged-coupled device camera and Openlab software (Improvision). Where indicated, simultaneous treatment with 2 reagents was conducted in order to assess co-localization of staining. In these experiments streptococcal cells walls were visualized by staining with fluorescent vancomycin ($1 \mu\text{g ml}^{-1}$, cat. #V34850, Invitrogen), or wheat germ agglutinin Alexa Fluor 488 conjugate ($5 \mu\text{g ml}^{-1}$, cat. #W11261, Invitrogen) and neutral membrane lipids were visualized by staining with Nile Red ($2.5 \mu\text{g ml}^{-1}$, cat. #N1142, Invitrogen). Focal localization in images of cells was quantitated as described in detail elsewhere [19] and scored as staining at a unique focus, multiple foci, or non-specifically (staining was of homogeneous intensity around the cellular circumference). Co-localization in the red protease assay was quantitated as the percentage of vancomycin-stained cells that exhibited focal staining with each individual reagent where the two foci were superimposable. Data presented for each condition represents the mean and standard error of the mean derived from at least 3 independent experiments and examination of a minimum of 1000 stained cells. Images were processed for publication using Adobe Photoshop CS3.

Immunofluorescent microscopy: The localization of native and HA-tagged HtrA protein (in SF370 and HSC5, respectively) was detected by immunofluorescent microscopy, as described [18]. Aliquots from untreated or CAP-treated ($47 \mu\text{M}$ polymyxin B or $29 \mu\text{M}$ HNP-1) challenge assay cultures (see above) were treated with PlyC lysin (prepared as described, [61]) and fixed according to the method of Raz and Fischetti [23]. Antisera

used included a polyclonal rabbit anti-HtrA antiserum (a gift from Mark Walker, University of Wollongong, Australia) used at a dilution of 1:100 that was detected using an AlexaFluor 594-labeled goat anti-rabbit IgG (Invitrogen) at 1:500 and an AlexaFluor 594-conjugated rabbit IgG anti-HA epitope antiserum (Invitrogen) used at 1:500. Wheat germ agglutinin (WGA)-AlexaFluor 488 (Invitrogen) at a final concentration of $5 \mu\text{g ml}^{-1}$ was used to visualize the streptococcal cell walls. Slides were mounted in an anti-fade reagent (Prolong Gold, Invitrogen) and images captured and staining patterns quantitated as described above. Differences between means were analyzed for significance using a two-tailed Student's t-test.

Immunogold electron microscopy: Localization of polymyxin B binding was examined by electron microscopy, as follows: Cultures in the assay described above were challenged with biotin-conjugated polymyxin B (HyCult Biotechnologies) at the various dilutions indicated in the text. At 1 hr post-challenge, aliquots were removed and prepared for immunoelectron microscopy as described [17, 18]. Sections were stained using a streptavidin-gold conjugate (20nm, BBI International) and examined by electron microscopy as detailed [17, 18]. Localization of SecA and HtrA following treatment in the challenge assay with the concentrations of polymyxin B indicated in the text was determined at 2 hr post-challenge by immunogold electron microscopy [17, 18]. Focal staining was defined and quantitated as described in detail elsewhere [19].

Analysis of protein expression and secretion: Expression of the transcript for the SpeB cysteine protease was determined at various time points following challenge with

polymyxin B by real time RT-PCR as described [59, 62]. Data represents the mean and standard error of the mean derived from 3 independent experiments conducted on different days, with each sample analyzed in quadruplicate. Supernatant fractions from cultures challenged with polymyxin B were analyzed for SpeB proteolytic activity as previously described [18] via the relative increase in fluorescence generated by the cleavage of fluorescein isothiocyanate-casein (cat. #C3777, Sigma). Supernatant, cell wall and protoplast fractions from cultures challenged with CAPs were prepared and analyzed for the presence of SpeB and HtrA by Western blotting as described [33]. Blots were developed using a Chemidoc XRS imager (BioRad) and relative protein concentrations determined using Quantity One software (BioRad, version 4.6.7). Data are expressed relative to untreated cultures and were derived from a minimum of 3 independent experiments. Differences between means were evaluated for significance using one-way ANOVA.

SUPPLEMENTARY FIGURES

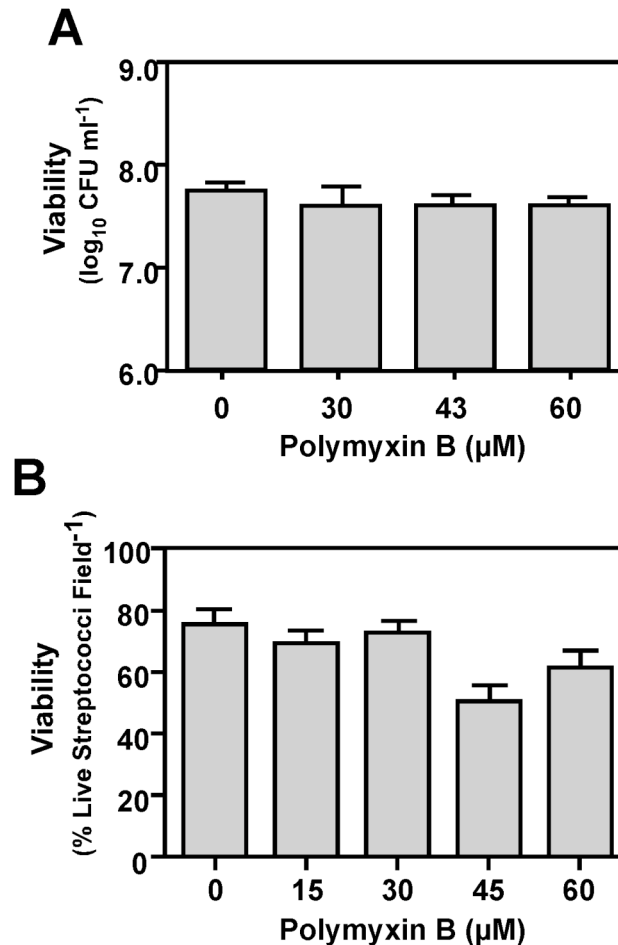


Figure S1. **Validation of sub-lethal polymyxin B challenge.** Cultures of *S. pyogenes* SF370 were treated with the indicated concentrations of polymyxin B in the challenge assay and viability assessed by (A) determining the number of colony forming units (CFU) following serial dilution and plating on C medium and (B) fluorescent microscopy of cells following staining with reagents that distinguish between cells with intact membranes and those whose membranes have become porated (LIVE/DEAD BacLight[™], Invitrogen). Samples for both viability assays were harvested following 2 hrs of treatment, and numbers of CFU determined following 24 hrs of incubation. Numbers of cells with porated membranes were derived from examination of at least 1000 stained cells. All data presented represents the mean and standard error of the mean derived from at least 3 experiments. Differences between mean values were evaluated for significance by a one-way ANOVA, which revealed no significant differences between any treated vs untreated cultures. Identical results were obtained from performing these same analyses on *S. pyogenes* strain HSC5 (data not shown).

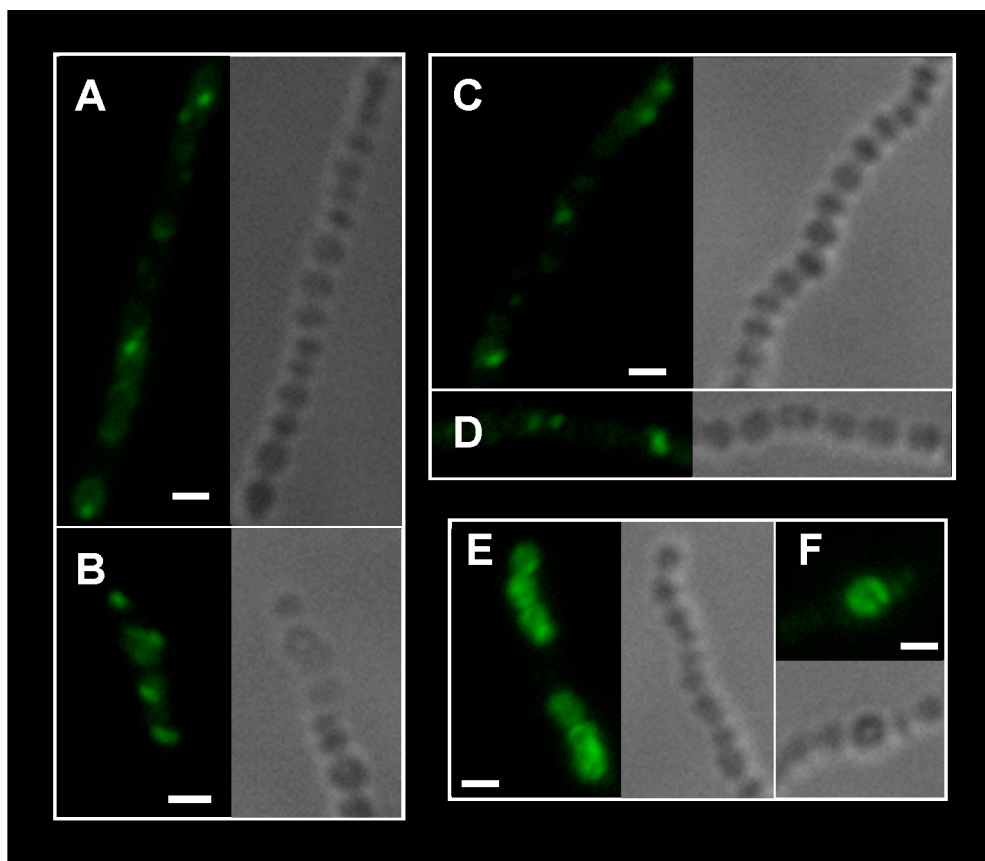


Figure S2. **Focal localization of anionic lipids is unaffected by heat stress.** Cultures of *S. pyogenes* HSC5 (A, C, E) and SF370 (B, D, F) were challenged in the standard assay as follows: Cultures were challenged with 10 μ M bodipy-labeled polymyxin B (Invitrogen) and incubated for 30 min at 37°C (A, B) or subjected to heat shock (42°C, 30 min.) (C, D) or challenged with an ExPortal-disrupting concentration of bodipy-labeled polymyxin B (45 μ M) (E, F). Samples were then analyzed by microscopy and each individual field was examined using fluorescence or phase contrast modes (left and right of each panel, respectively). Scale bar = 1 μ m.

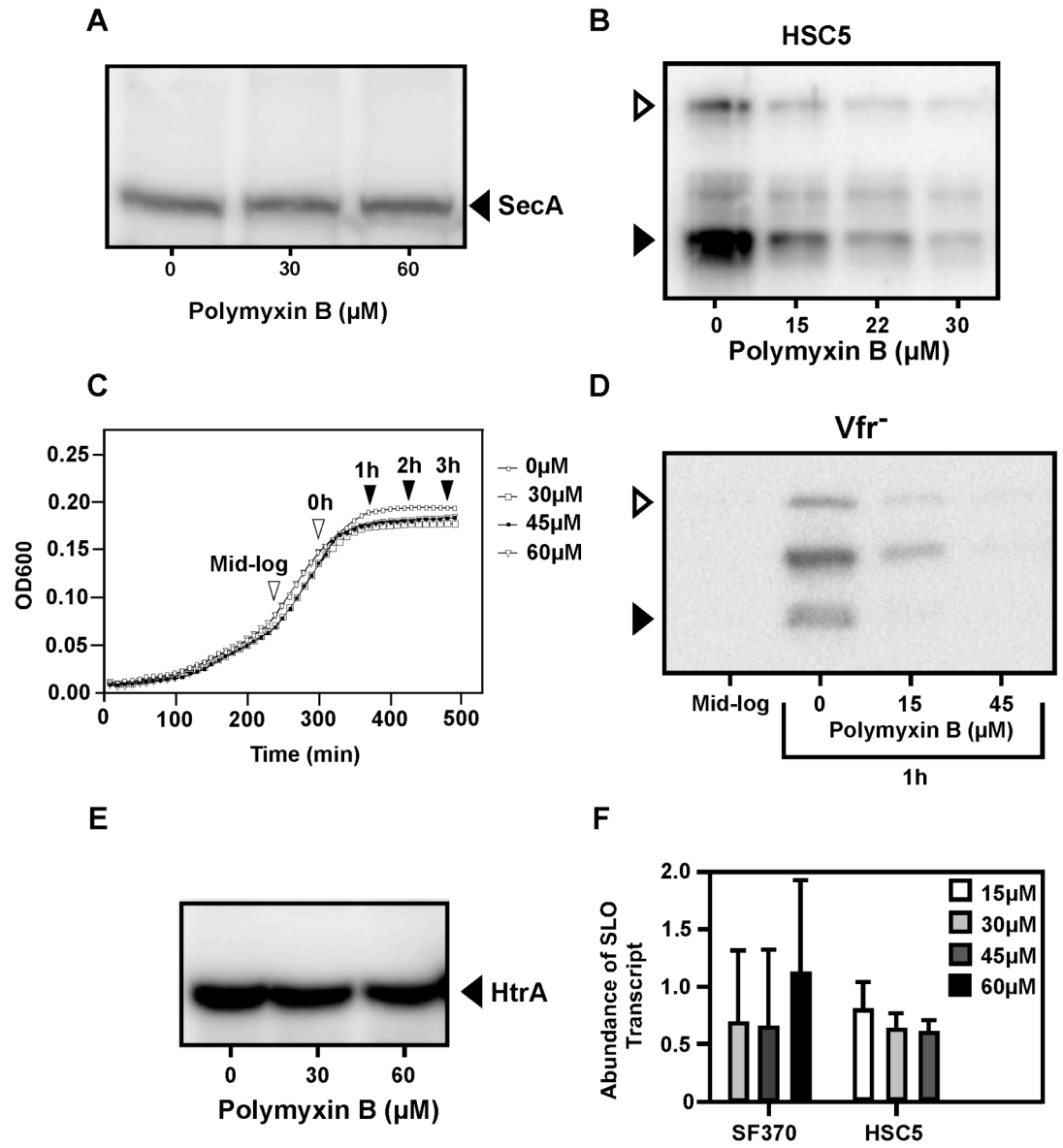


Figure S3. Validation of secretion-inhibitory effects in streptococci of sub-lethal polymyxin B levels. (A). Expression of the secretory translocon ATPase SecA in cultures of *S. pyogenes* SF370 was determined 2 hrs post-challenge with the concentrations of polymyxin B indicated in the Fig. A Western blot analysis of cell protoplasts is shown. (B) Western blot analysis of SpeB in culture supernatants of *S. pyogenes* HSC5 following sub-lethal polymyxin B challenge. Open and filled triangles indicate the migration of the zymogen and mature form of SpeB, respectively. (C). Growth of *S. pyogenes* in the absence or presence of the indicated amounts of polymyxin B. Arrows indicate the point at which cultures were challenged (0 h, Mid-log) and the subsequent time points where samples were harvested for the various analyses described in the text. (D). A Vfr⁻ mutant of HSC5 (GCP682) was cultured to the time of challenge (Mid-log) and then treated with the indicated concentrations of polymyxin B. Shown is a Western blot analysis of SpeB in culture supernatant. (E). A Western blot analysis of HtrA in lysates of *S. pyogenes* SF370 that were prepared 3 hrs post-challenge with the indicated concentrations of polymyxin B. (F). Real-time RT-PCR analysis of *slo* transcript abundance following challenge of *S. pyogenes* HSC5 with the indicated concentrations of polymyxin B. Data shown represent the mean and SEM derived from at least three independent experiments conducted on different days and each sample was analyzed in triplicate.

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Chapter III

The Streptococcal Cell Wall and Peptidoglycan Synthesis are Involved in ExPortal Organization

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The Streptococcal Cell Wall and Peptidoglycan Synthesis are Involved in ExPortal Organization

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ABSTRACT

S. pyogenes and other Gram-positive bacteria circumvent the lack of a specialized cellular compartment for folding secreted proteins following their translocation by clustering the Sec translocons at a defined microdomain of the cytoplasmic membrane that is also enriched in anionic lipids and accessory factors for protein biogenesis, referred to as the ExPortal. The function of this secretory organelle appears to be that of spatially coupling secretion with protein maturation, but the mechanisms underlying ExPortal organization and maintenance are still poorly understood. Emerging literature suggests that cell wall biogenesis components interact with cytoplasmic membrane structures and the cell division machinery to organize membrane localized processes. In the present study we examined the role the streptococcal cell wall and peptidoglycan synthesis in the cytoplasmic membrane play in organizing and maintaining the ExPortal and report that extracellular peptidoglycan is required for maintaining proper localization of both lipid and protein components of the ExPortal. Treatment with a lipid II-targeting lantibiotic disrupted localization of anionic lipids and ExPortal proteins, as well as secretion of ExPortal substrates in a manner similar to what was observed previously with cationic antimicrobial peptides at sub-lethal concentrations. Furthermore, proteins responsible for the membrane-associated steps of lipid II synthesis localize to foci in the streptococcal membrane that coincide with the anionic lipid microdomain and secretory translocons of the ExPortal, suggesting that localization of peptidoglycan synthesis coincides with the ExPortal and influences its organization.

INTRODUCTION

Streptococcus pyogenes is the causative agent of numerous diseases in humans that range from largely superficial infection of the skin and mucous membranes (impetigo, pharyngitis), to highly invasive and life-threatening diseases (necrotizing fasciitis), as well as, serious post-infection sequelae (rheumatic fever, glomerulonephritis, reviewed in [1]). A gram-positive pathogen, its ability to cause disease is dependent on the secretion of an extensive network of virulence proteins [1, 2]. In the absence of other known secretion systems, these virulence proteins are exported by the general secretory [3] pathway (for a review see [4]). *S. pyogenes* and other Gram-positive pathogens circumvent the lack of a specialized cellular compartment for folding secreted proteins following their translocation, a feature shared by all Gram-positive bacteria, by clustering the Sec translocons at a defined microdomain of the cytoplasmic membrane referred to as the ExPortal [5-9]. The ExPortal is also highly enriched with accessory factors for protein biogenesis at the extracellular leaflet of the membrane, including the sortases involved in covalent attachment of proteins to the cell wall, and the multi-function protease and chaperone HtrA that aids in the biogenesis of the active form of the SpeB protease [5-7, 10, 11]. These studies suggest the function of the ExPortal is to spatially couple secretion with protein maturation, but the mechanisms underlying the organization and maintenance of this secretory organelle are still poorly understood.

The ExPortal of *S. pyogenes* has been shown to have an asymmetric lipid content enriched in anionic phospholipids [10] that may contribute to the preferential retention of certain proteins at the ExPortal vs. the peripheral membrane [7]. Research into the interaction between *S. pyogenes* and cationic antimicrobial peptides (CAPs), which bind

to negatively charged membranes, showed that sub-lethal concentrations of certain peptides disrupt both localization of anionic lipids and retention of HtrA and the Sec translocon ATPase SecA at the ExPortal microdomain, and in doing so inhibit its secretory activity [12]. Interestingly, this study also revealed that one of these CAPs, human neutrophil peptide 1 (HNP-1), localizes to the bacterial cell surface which overlaps to regions of new cell wall synthesis in streptococci at the sub-lethal, ExPortal-disrupting concentrations tested. It has been previously demonstrated that HNP-1 targets the peptidoglycan precursor lipid II in bacterial membranes [13], much like other defensins for which a similar affinity for the lipid II molecule has been observed [14, 15]. This finding suggests that CAPs can interfere with ExPortal function by disruption of lipid II in addition to anionic lipids.

The best characterized lipid II-targeting molecules known belong to a class of small bacterial defense peptides commonly referred to as lantibiotics (for a review, see [16]). The best characterized of these, nisin, specifically binds lipid II in a defined stoichiometry in order to form pores that efficiently permeabilize bacterial membranes [17, 18]. More importantly however, non-porating variants of nisin have been shown to efficiently kill bacteria using an alternative mechanism by which lipid II is clustered into patches in the cytoplasmic membrane away from the regions where peptidoglycan synthesis occurs [19]. Other lantibiotics such as gallidermin, epidermin and mutacin have also been observed to act via this alternative mechanism [19-21], thus inhibiting cell wall synthesis and causing bacterial cell death without permeabilizing the cytoplasmic membrane. The fact that lipid II-targeting molecules can disrupt cell wall synthesis without compromising membrane permeability, combined with the observation that a

CAP shown to interact with lipid II can similarly target and disrupt the ExPortal hint at a possible link between cell wall biogenesis and organization of this secretory organelle.

The cell wall of Gram-positive pathogens is an extracellular organelle that plays a multitude of roles essential to both survival and virulence (for a review see [22]). A rigid exoskeleton made up of polymers of alternating N-acetylglucosamine (GlcNAc) and M-acetylmuramic acid (MurNAc) glycan units crosslinked via peptide bridges, its main purpose is to preserve cellular integrity against osmotic forces from without and within the bacterial cell [23]. The cell wall is also the attachment site for extracellular factors that interact with the host environment, which include surface-associated virulence proteins, lipoteichoic and teichoic acids. Since cell wall-anchored proteins are covalently attached by sortases to their peptidoglycan acceptor immediately following their secretion, most research into the relationship between gram-positive secretion and cell wall biogenesis, particularly in the case of streptococci, has focused on the characterization of sortase function (reviewed in [24]). In contrast, our present study corresponds to an emerging literature on how the organization of cell wall biogenesis relates to underlying cytoplasmic membrane structures and cell division machinery [25-28] by examining the possibility of a more direct relationship between protein secretion and cell wall synthesis in streptococci, particularly in terms of the organization of these processes. The final steps of cell wall peptidoglycan biogenesis occur at the inner leaflet of the cytoplasmic membrane. In gram-positive species including *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Enterococcus faecalis*, these involve cytosolic, as well as membrane-intrinsic and extrinsic proteins (for a review see [29]). The cellular localization of these proteins relative to the ExPortal microdomain of *Streptococcus*

pyogenes has not been documented and could reveal a great deal as to whether peptidoglycan biogenesis influences ExPortal organization. Thus, our current study also examined those proteins involved in the first and last of the membrane-associated steps of cell wall synthesis. MraY is a membrane intrinsic protein that carries out the first membrane-associated step in the synthesis of the lipid II precursor by catalyzing the transfer of the phospho-N-acetylmuramoyl(MurNAc)-pentapeptide from the soluble cytoplasmic UDP-MurNAc-pentapeptide substrate to the membrane embedded undecaprenyl-phosphate (C₅₅-P) acceptor, thus producing undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide (lipid I), the immediate precursor of lipid II. MurN, on the other hand, is a cytosolic polypeptide belonging to the family of FemABX peptidyl transferases that attach the amino acids constituting the peptide cross-links between peptidoglycan subunits in the cell wall structure of many gram-positive bacteria [30]. This is the final synthetic step occurring on the inner leaflet of the cytoplasmic membrane prior to translocation of lipid II across the membrane. In *S. aureus* synthesis of these peptide cross-links is required for the sortase-mediated attachment of surface virulence factors to the cell wall [31]. Moreover, evidence indicates that sorting of surface virulence factors occurs in the immediate vicinity of their site of secretion and coincides with the site of *de-novo* peptidoglycan synthesis [7, 9, 11, 32]. These data further suggest that peptidoglycan biogenesis and ExPortal-mediated secretion may be spatially coordinated in the streptococcal membrane.

Thus, in the present study we examined the role the streptococcal cell wall and peptidoglycan synthesis in the cytoplasmic membrane play in organizing and maintaining the ExPortal and report that cell wall peptidoglycan is required for maintaining proper

localization of both lipid and protein components of the ExPortal at their membrane microdomain. Treatment with a lipid II-targeting lantibiotic disrupted localization of anionic lipids and ExPortal proteins, as well as secretion of ExPortal substrates in a manner similar to what was observed previously with other CAPs at sub-lethal concentrations [12]. Furthermore, proteins responsible for the membrane-associated steps of lipid II synthesis localize to foci in the streptococcal membrane that coincide with the anionic lipid microdomain and secretory translocons of the ExPortal, suggesting that localization of peptidoglycan synthesis coincides with targeting and organization of the ExPortal.

RESULTS

Cell Wall Peptidoglycan is required for maintenance of the ExPortal anionic lipid microdomain. In order to characterize the involvement of cell wall biogenesis in ExPortal organization we first determined whether the cell wall is necessary to maintain ExPortal integrity. To that end we examined the localization of anionic lipids in the cytoplasmic membrane in protoplasts devoid of extracellular peptidoglycan. Treatment with purified PlyC bacteriophage lysin of logarithmic and stationary phase streptococcal cultures resuspended in either raffinose buffer or glycerol-supplemented media removed all surface peptidoglycan from the majority of streptococci, as monitored by the breakdown of streptococcal chains into individual protoplasts and the absence of either FL-Vancomycin or Wheat Germ Agglutinin (WGA) labeling on the protoplast surface (Fig. 1). The loss of cell-wall labeling in PlyC-treated streptococci coincided with a redistribution of labeled polymyxin B from unique foci to the entirety of the bacterial cell's surface, giving the protoplasts a "halo" appearance (Fig. 1A, B, H), distinct from the focal localization of the labeled peptide observed in intact streptococci (Fig. 1C and D) and PlyC-treated streptococci in which not all of the cell wall peptidoglycan was removed, as evidenced by the presence of FL-Vancomycin or WGA label on their surface (Fig. 1A, top of panel). The distribution of labeled polymyxin B in protoplasts also coincided with the distribution of neutral lipid-binding dye Nile Red (compare Fig. 1H and I), suggesting that in the absence of cell wall peptidoglycan, integrity of the anionic lipid microdomain of the ExPortal is lost and anionic lipids are uniformly distributed in the streptococcal membrane in a manner similar to neutral lipids. Staining streptococcal protoplasts with a fluorescent vital dye (LIVE/DEAD BacLight™) indicated that the

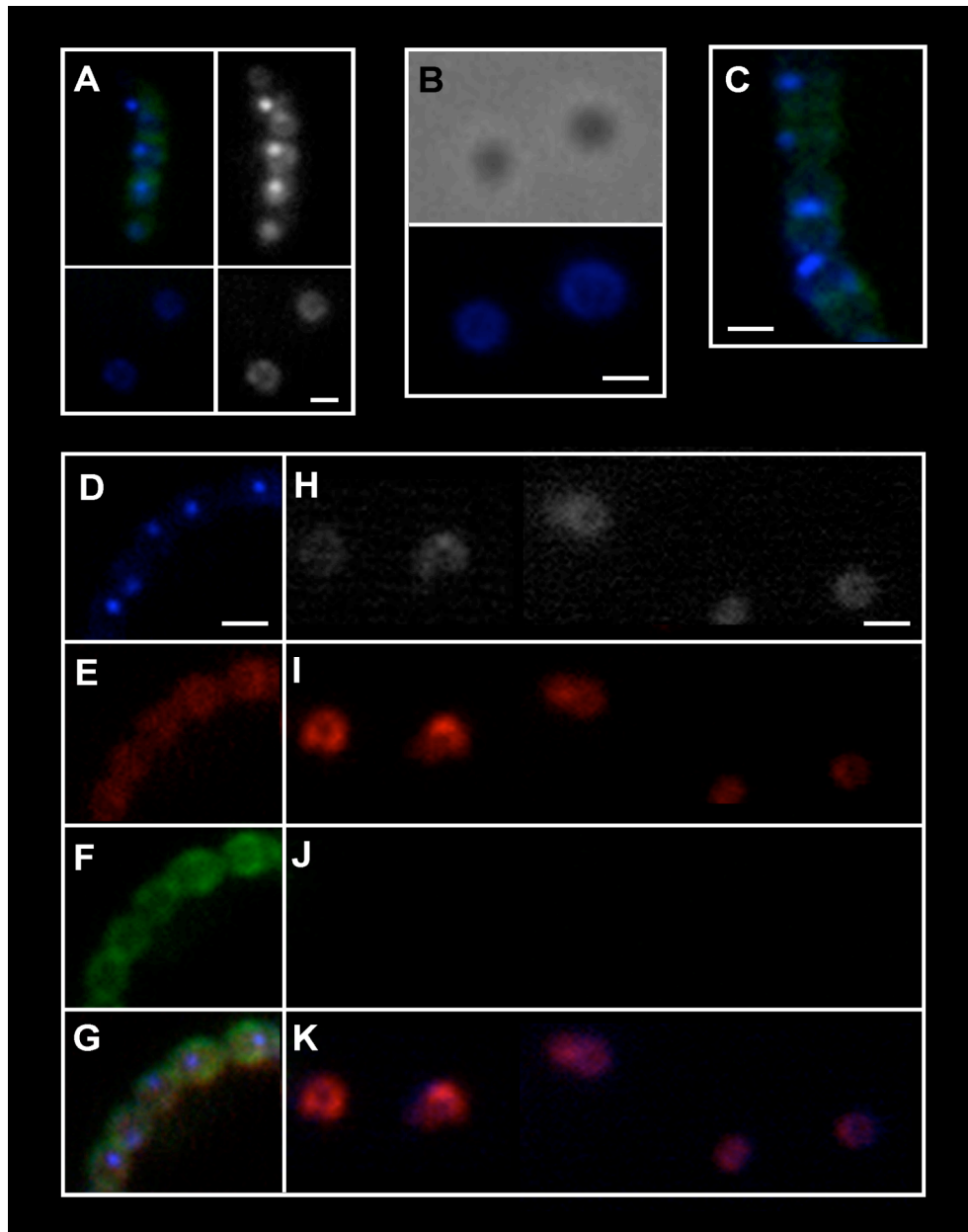


Figure 1. Surface peptidoglycan is required for maintenance of the ExPortal anionic lipid microdomain. The distribution of anionic lipids on the surface of *S. pyogenes* SF370 and HSC5 protoplasts was revealed by fluorescent microscopy. Loss of cell wall labeling by FL-vancomycin in SF370 protoplasts coincided with redistribution of anionic lipid labeling by dansyl-polymyxin B from focal sites in intact bacteria (**A, top**) to the entire protoplast membrane surface (**A, bottom**). Similar redistribution of dansyl-polymyxin B to the entire surface was observed in HSC5 protoplasts (**B**), compared to focal localization in intact bacteria (**C**). Localization of dansyl-polymyxin B (**D, H**) and neutral lipids (**E, I**) in WGA-stained (**F, J**) HSC5 intact cells (**D-G**) and protoplasts (**H-K**) further confirmed redistribution of anionic lipids to the entire membrane surface. Fluorescent (**A, right; D-F, H-J**), phase (**B, top**), and merged images (**A, left; B, C, G, K**) are shown. FL-vancomycin ($1\mu\text{g ml}^{-1}$, green), Dansyl-polymyxin B ($10\mu\text{M}$, blue), WGA ($5\mu\text{g ml}^{-1}$, green), Nile red ($2.5\mu\text{g ml}^{-1}$, red). Scale bar = $1\mu\text{m}$.

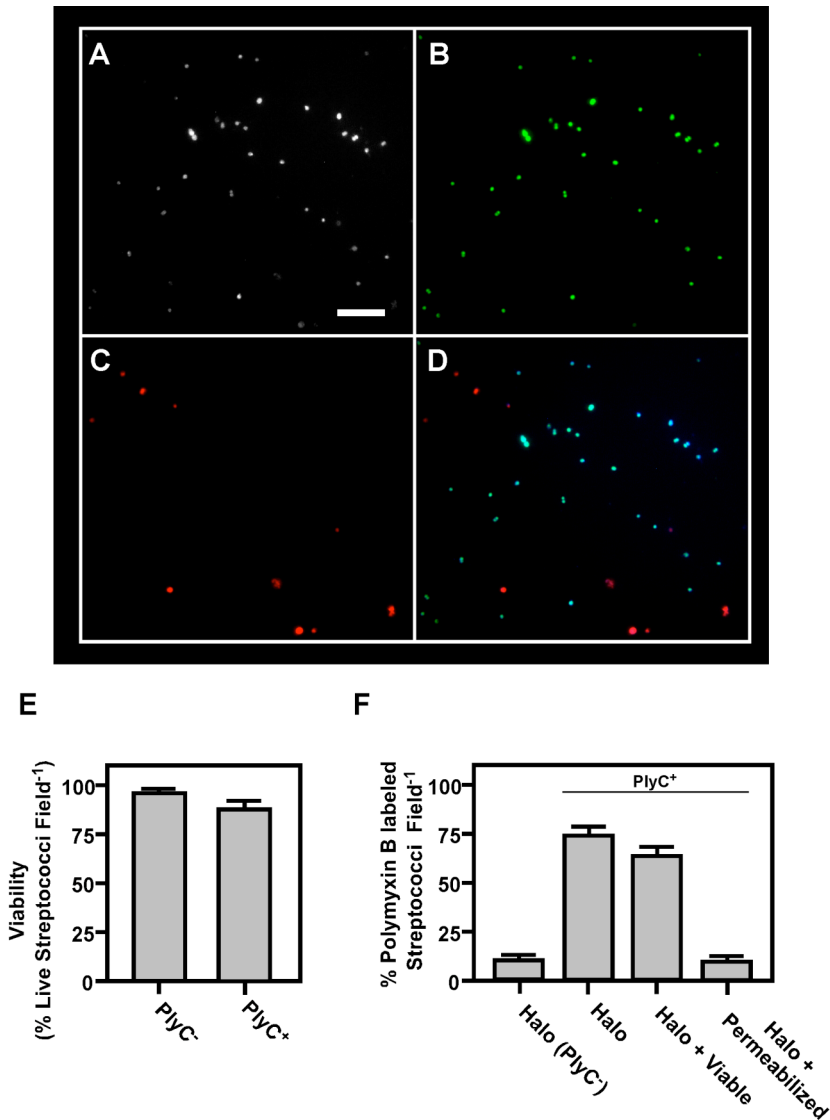


Figure 2. Loss of anionic lipid segregation at a microdomain is not due to membrane permeabilization. Membrane permeability of *S. pyogenes* protoplasts displaying altered anionic lipid distribution was assessed by fluorescent microscopy following staining with reagents that distinguish between protoplasts with intact membranes (viable) and those with permeabilized membranes (LIVE/DEAD BacLight™, Invitrogen). Fluorescent images of a representative field of HSC5 protoplasts displaying uniform membrane-staining with dansyl-polymyxin B (A), viable (B) and permeabilized cell staining (C), as well as corresponding merged image (D) are shown. Protoplasts generated by PlyC lysis treatment (PlyC⁺) have non-permeabilized membranes, indicated by viable cell staining similar to untreated (PlyC⁻) HSC5 cells (E). A significant number of protoplasts (PlyC⁺) displaying uniform membrane labeling with dansyl-polymyxin B (Halo) had intact membranes (Halo+viable) as opposed to permeabilized membranes (Halo+permeabilized)(F). Few intact cells display uniform membrane labeling by dansyl-polymyxin B (Halo PlyC⁻). Numbers of viable, permeabilized protoplasts and intact streptococcal cells were derived from examination of 1000 stained cells. Data presented represents the mean and standard error of the mean derived from at least 3 experiments. Differences between mean values were evaluated for significance by a one-way ANOVA ($P < 0.0001$). Scale bar = 10 μ m.

cytoplasmic membranes of the protoplasts examined are not permeabilized (Fig. 2A-D). Quantitation of protoplasts displaying the uniform membrane distribution of labeled polymyxin B described above revealed that a significantly large proportion of them had intact membranes (Fig. 2E and F, $P < 0.0001$). This suggests that the observed redistribution of anionic lipids resulting from the removal of surface peptidoglycan under our experimental conditions is not due to perturbation of membrane permeability.

Cell Wall Peptidoglycan is required for ExPortal protein targeting at the membrane.

Previous work demonstrated that localization of ExPortal-associated proteins such as SecA and HtrA coincided with the anionic lipid microdomain of the ExPortal [6, 10] and that disruption of the lipid microdomain by CAPs resulted in redistribution of these proteins in the streptococcal membrane [12]. Thus, we examined whether disruption of anionic lipid localization in the streptococcal membrane resulting from the absence of surface peptidoglycan affected the distribution of ExPortal-associated proteins. Immunofluorescence microscopy of streptococcal protoplasts revealed that the membrane-associated HtrA protease was distributed uniformly over the entirety of the membrane (Fig. 3A). Likewise, the translocon ATPase SecA was observed uniformly in the membrane for the most part, as protoplasts in which multiple foci of SecA labeling were also observed (Fig. 3B). Removal of cell wall peptidoglycan was confirmed by the absence of staining with WGA following PlyC treatment and fixation of streptococci. Again, streptococci in which surface peptidoglycan removal was incomplete following treatment displayed the unique foci of HtrA (Fig. 3C) and SecA (data not shown) localization that are commonly observed in streptococci with intact cell walls, consistent

with prior studies [6, 12]. The observed altered localization of HtrA and SecA further supports the hypothesis that integrity of an anionic lipid microdomain is central to targeting of ExPortal-related proteins in the membrane. Moreover, that localization of lipid and proteinaceous ExPortal components is altered in the absence of surface peptidoglycan suggests that an intact cell wall is necessary for ExPortal organization.

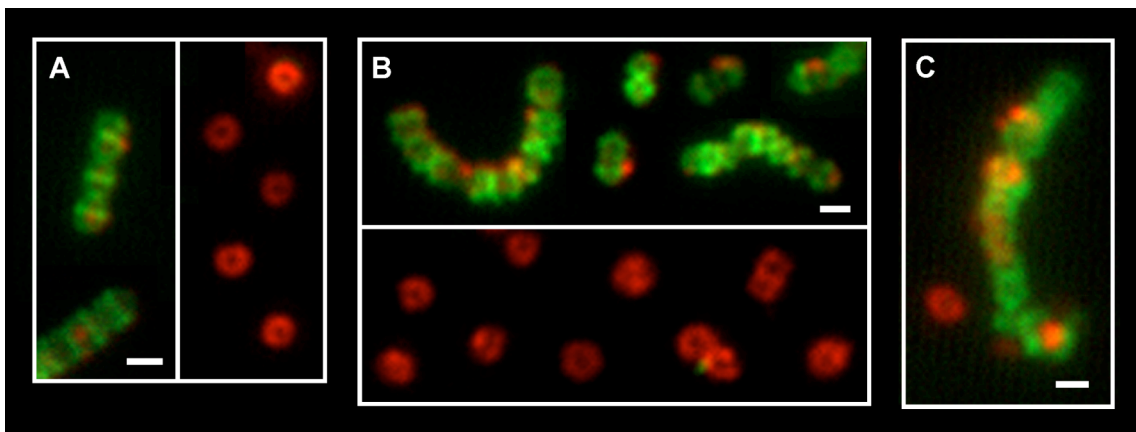


Figure 3. Redistribution of ExPortal proteins following removal of surface peptidoglycan. The distribution of HtrA (**A**) and SecA (**B**) in *S. pyogenes* HSC5 cells and protoplasts was assessed by immunofluorescence microscopy. Incomplete removal of surface peptidoglycan from streptococci following treatment with PlyC lysin does not alter focal localization of HtrA (**C**). Merged fluorescence images of intact streptococci (**A, left; B, top**) and protoplasts (**A, right; B, bottom**) are shown. WGA ($5\mu\text{g ml}^{-1}$, green), HtrA and SecA (red). Scale bar = $1\mu\text{m}$.

The lantibiotic gallidermin disrupts targeting of lipid and proteinaceous ExPortal components as well ExPortal-mediated secretion. The experimental results previously described suggest that beyond its role of protecting the membrane from damage from internal turgor pressure and maintaining cell shape, the streptococcal cell wall might also play a role in organizing or maintaining organization of the secretory organelle that is the ExPortal. Disruption of the ExPortal resulting from the loss of extracellular peptidoglycan does not appear to be due to compromised membrane permeability, indicating that the cell wall's involvement in organizing the ExPortal goes beyond that of maintaining membrane integrity. One possibility is that peptidoglycan synthesis and the ExPortal are linked, meaning the site of peptidoglycan production at the membrane coincides with and influences the localization of protein secretion and processing at the ExPortal. To test this hypothesis we first examined whether compounds known to be disruptive to cell wall synthesis affect ExPortal organization and function. As mentioned previously, lantibiotics such as nisin, gallidermin and mutacin have been demonstrated to possess a high affinity for the peptidoglycan precursor lipid II and to interact with it in order to porate membranes and disrupt cell wall synthesis. Thus, we monitored the localization of the anionic lipid microdomain of the ExPortal in the presence of lantibiotics at concentrations that were not lytic to streptococci. We assessed membrane permeability using a Live/Dead® stain to quantitate numbers of porated streptococci in lantibiotic treated cultures and determined lytic concentrations to be those that resulted <50% viable cells in observed microscopy fields. Examination of either HSC5 or SF370 strain cultures showed that gallidermin and nisin do not compromise streptococcal membrane integrity at concentrations lower than 2 μ M and 0.5 μ M, respectively (Fig. 4A

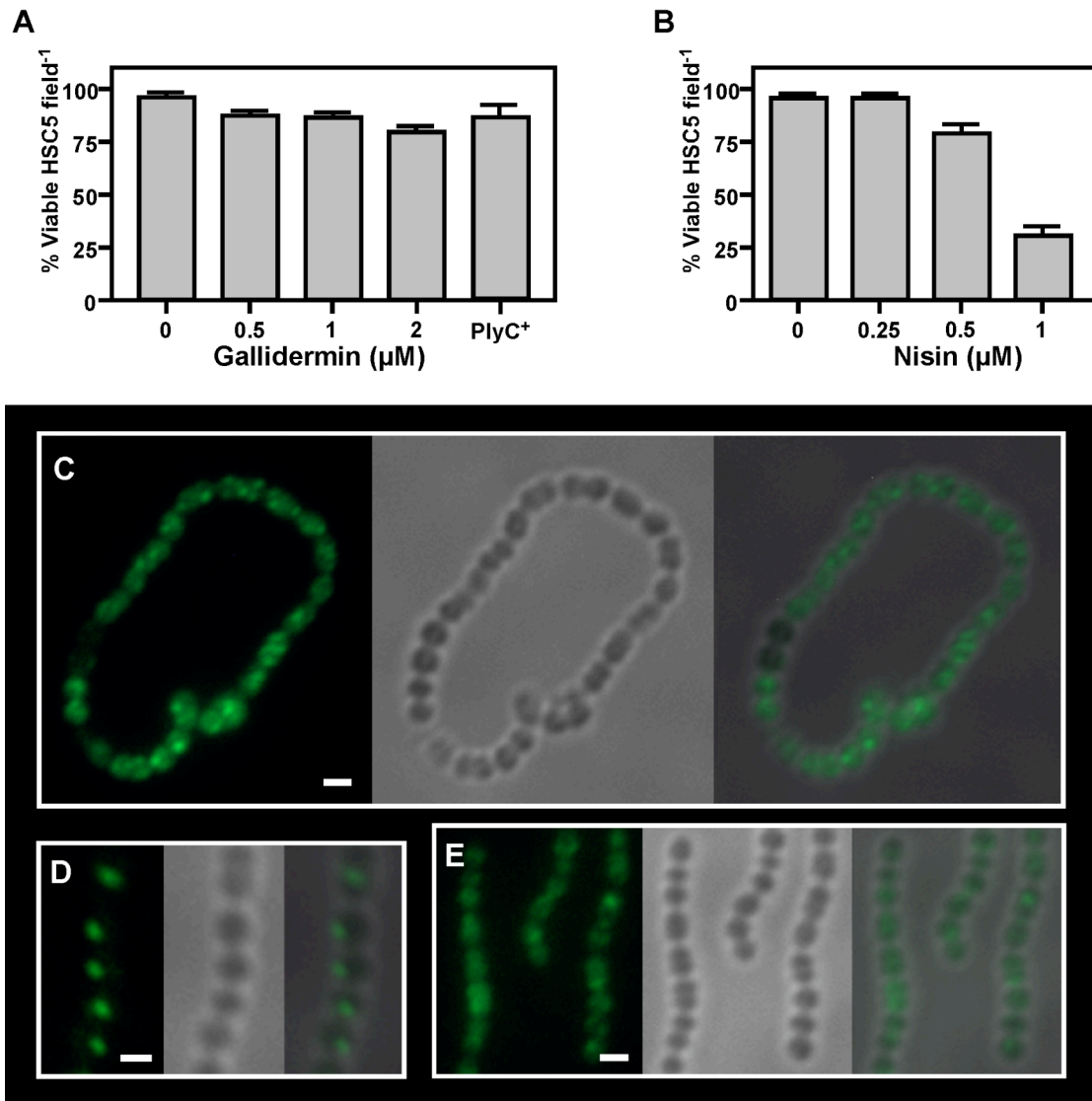


Figure 4. The lantibiotic gallidermin disrupts localization of anionic lipids at non-lytic concentrations. The localization of anionic lipids in *S. pyogenes* SF370 and HSC5 was assessed by fluorescence microscopy following challenge with a non-lytic concentration of gallidermin (1 μM). Non-lytic concentrations of the lantibiotics gallidermin (A) and nisin (B) were determined by fluorescent microscopy of cells following staining with LIVE/DEAD BacLight™ reagents (Invitrogen). Distribution of anionic lipids in the membrane was monitored by staining with sub-lethal levels of polymyxin B BODIPY®FL conjugate (10 μM) in gallidermin exposed (C) and untreated streptococci (D). Streptococci treated with anionic lipid microdomain-disrupting concentrations of polymyxin B BODIPY®FL conjugate (60 μM) are shown for comparison (E). Fluorescent, phase and merged images are shown. Scale bar = 1 μm. Samples for analysis of viability were harvested following 1 hr of lantibiotic treatment and numbers of cells with porated membranes were derived from examination of at least 1000 stained cells. All data presented represents the mean and standard error of the mean derived from at least 3 experiments. Numbers of viable protoplasts (PlyC⁺) are shown for comparison. Identical results were obtained from performing these same analyses on *S. pyogenes* strain SF370 (data not shown).

and B, data not shown). Fluorescent microscopy using bodipy-labeled polymyxin B to ascertain the localization of ExPortal-associated anionic lipids revealed that non-lytic concentrations of gallidermin disrupt the anionic lipid microdomain of the ExPortal (Fig. 4C) in a manner similar to high sub-lethal amounts of polymyxin B (compare, Fig. 4C, D and E), as observed by the localization of the labeled cationic peptide to multiple foci or in a more diffuse staining pattern than in untreated streptococci. Nisin had a disruptive effect on the localization of the fluorescent peptide to unique foci at concentrations determined to be lytic to streptococci in treated cultures (data not shown), which makes it difficult to discern whether this effect of nisin was due to its membrane-porating capacity or to its affinity for lipid II. However, given that the disruptive effect of gallidermin on anionic lipid localization in the membrane was observed with non-lytic amounts of the lantibiotic, we examined whether gallidermin also affected the localization of ExPortal-related proteins. Immunofluorescence microscopy revealed that gallidermin treatment of streptococcal cultures at a non-lytic concentration resulted in redistribution of HtrA to either multiple foci or, for the most part, in a diffuse pattern on the streptococcal surface (compare Fig. 5A and B). Co-labeling with fluorescent polymyxin B confirmed that the observed localization of HtrA corresponded with a re-distribution of ExPortal-associated anionic lipids, as identified by the labeled CAP.

Since gallidermin was observed to interfere with the localization of both lipid and proteinaceous components of the ExPortal, we examined whether the lantibiotic could inhibit translocation of proteins requiring an intact ExPortal for their secretion, as has been previously studied with cationic antimicrobial peptides [12]. Treatment of strain SF370 with a non-lytic concentration of gallidermin resulted in a reduction in the amount

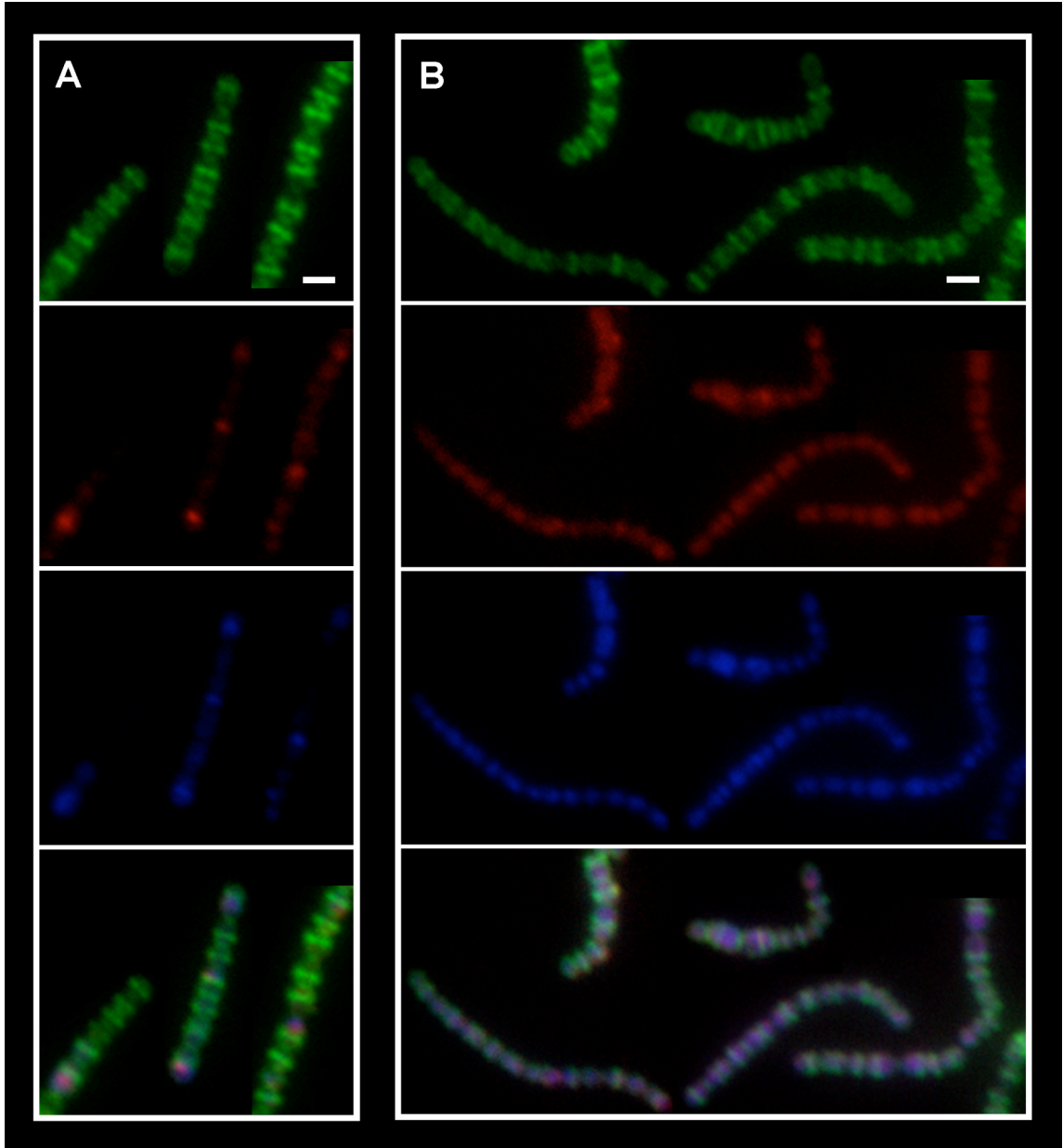


Figure 5. **Redistribution of HtrA following non-lytic gallidermin challenge.** The distribution HtrA on *S. pyogenes* HSC5 was assessed by immunofluorescent microscopy in the absence of (A) and following challenge with gallidermin at 1 μM (B). Fluorescent images of cell wall (WGA, 5 $\mu\text{g ml}^{-1}$, green), HtrA (red), anionic phospholipid (dansyl-polymyxin B, 10 μM , blue) labeling and merged fluorescent images (bottom panel) are shown. Scale bar = 1 μm .

of cysteine protease SpeB in culture supernatants as revealed by Western blot analysis (Fig. 6A). This reduction in the amount of secreted SpeB was similar to that observed in supernatants from cultures treated with high sub-lethal amounts of polymyxin B and was observed in supernatants from the HSC5 strain as well (Fig. 6B). Decreased levels of secreted Streptolysin O (SLO) cytolysin were also observed in gallidermin-treated cultures of both HSC5 and SF370 strains (Fig. 6 C and D), in the same manner as resulted from treatment with high sub-lethal amounts of polymyxin B. Protein synthesis and the capacity to translocate proteins appear to be unaffected as levels of SecA protein in gallidermin-exposed streptococci are unaltered relative to untreated bacteria as indicated by Western analysis of streptococcal lysates (Fig. 6E), suggesting the effect of gallidermin on secretion of ExPortal substrates is due to its disruption of ExPortal organization. Altogether, these results indicate that the lipid II-targeting activity of a lantibiotic disrupts the streptococcal ExPortal and support the hypothesis that synthesis of the peptidoglycan precursor at the membrane influences ExPortal organization.

Synthetic enzymes required for lipid II peptidoglycan precursor biogenesis co-localize with the ExPortal. As mentioned previously, the final steps of cell wall subunit biogenesis occur at the cytoplasmic membrane, meaning that lantibiotic interaction with lipid II likely occurs in the proximity of the membrane site where precursor synthesis takes place. The disruptive effect of gallidermin on ExPortal organization in the absence of changes in membrane permeability further supports the possibility that lipid II synthesis at the membrane affects maintenance of the ExPortal. If peptidoglycan precursor synthesis and the organization of ExPortal-mediated protein biogenesis and

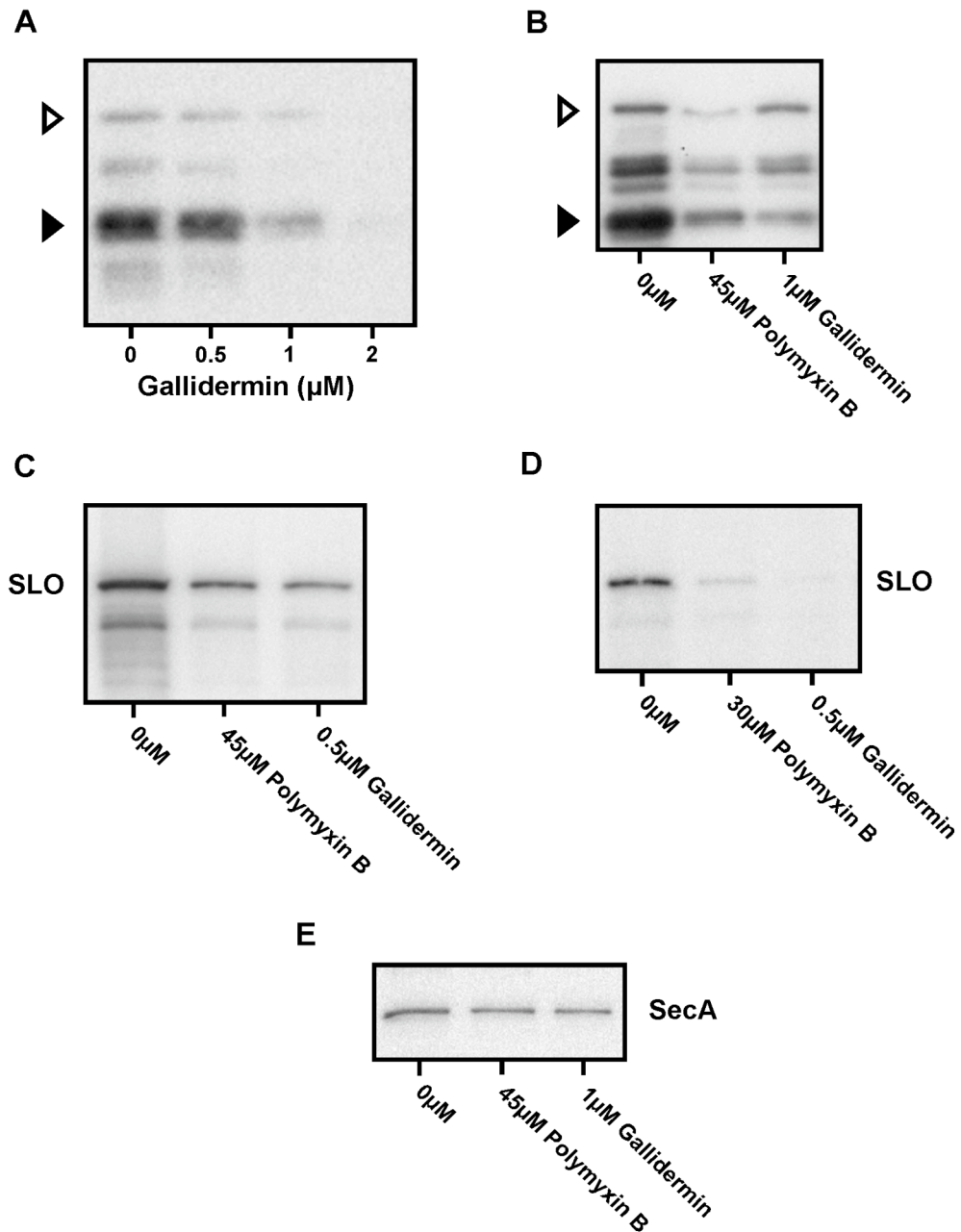


Figure 6. Non-lytic challenge with gallidermin inhibits secretion of SpeB and SLO. Secretion of the SpeB protease in cultures of *S. pyogenes* was determined following challenge with the indicated concentrations of gallidermin and polymyxin B by Western blot analysis of culture supernatant from SF370 (A) and HSC5 strains (B). Western blotting was also used to analyze the amount of SLO present in culture supernatant following challenge with the indicated concentrations of gallidermin and polymyxin B of SF370 (C) and HSC5 (D) cultures. Expression of SecA in HSC5 cultures was determined following challenge with the indicated concentrations of gallidermin by Western blot analysis of streptococcal lysates (E). All samples for SpeB analysis were harvested at 2 hrs post-challenge; samples for SLO and SecA were harvested 1hr post-challenge. Open and filled triangles indicate the migration of the zymogen and mature form of SpeB, respectively. Migration of the SLO and SecA polypeptides is also indicated.

translocation are linked in this manner, then lipid II synthesis and the ExPortal are likely to be spatially coordinated in the streptococcal membrane. To further examine this hypothesis we monitored the localization of two enzymes involved in the membrane-associated steps of lipid II synthesis, the membrane spanning MraY, which carries out the first membrane-associated step, and the cytosolic MurN transferase that catalyzes the final enzymatic step prior to translocation of the peptidoglycan subunits across the membrane. Both proteins were C-terminally tagged with superfolder GFP (sfGFP) and their expression examined by Western analysis, fluorescence and electron microscopy (Fig. 7). Peak expression of MraY and MurN occurred during log phase (Fig. 7A), which was not unexpected since most peptidoglycan synthesis occurs during cell division and growth. The membrane intrinsic MraY was observed to predominantly localize to unique foci in the streptococcal membrane (Fig. 7B-D). Localization of MraY to a membrane microdomain occurred under all experimental media conditions used in this study, observed most strongly during log-phase in both cultures grown in C media (Fig. 7B) and ThyB (Fig. 7C), as determined by fluorescence microscopy. Electron microscopy analysis of log-phase streptococci confirmed localization of the tagged protein to unique membrane microdomains, as evidenced by the clustering of immunogold particles at distinct sites (Fig. 6D). Cytosolic MurN was also found at foci in the vicinity of the streptococcal membrane (Fig. 7E-G), though some localization throughout the streptococcal cytoplasm was also observed (data not shown). Like MraY, MurN localized to foci at log phase (Fig. 7E and F) and was ascertained by electron microscopy as well (Fig. 7G).

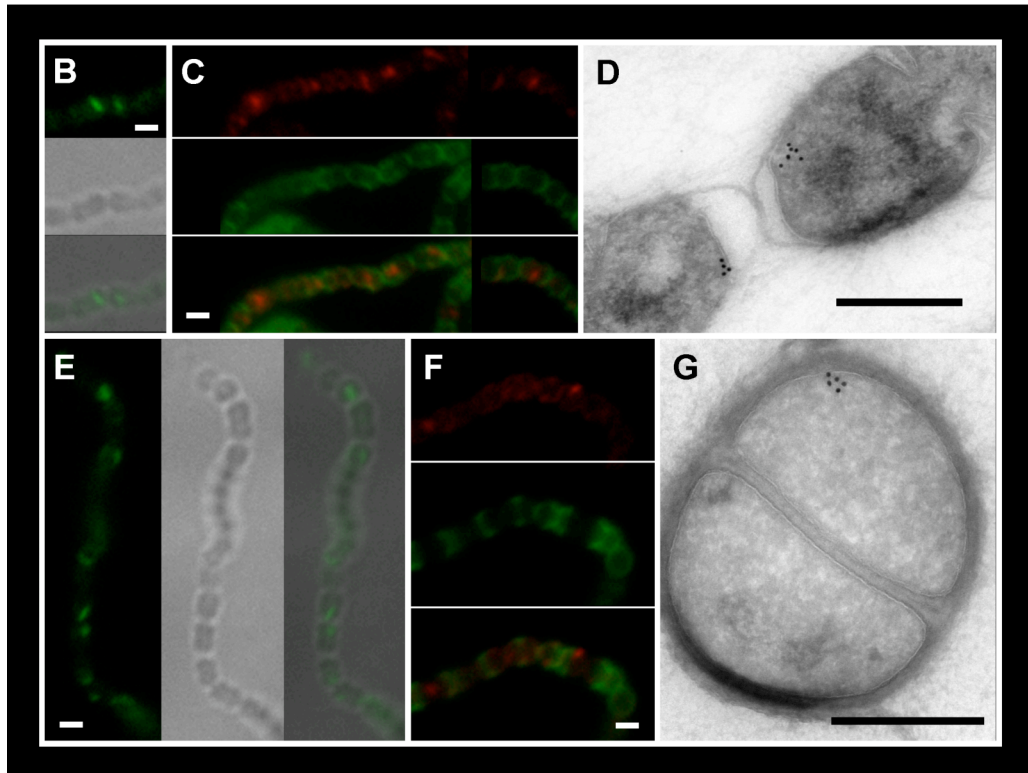
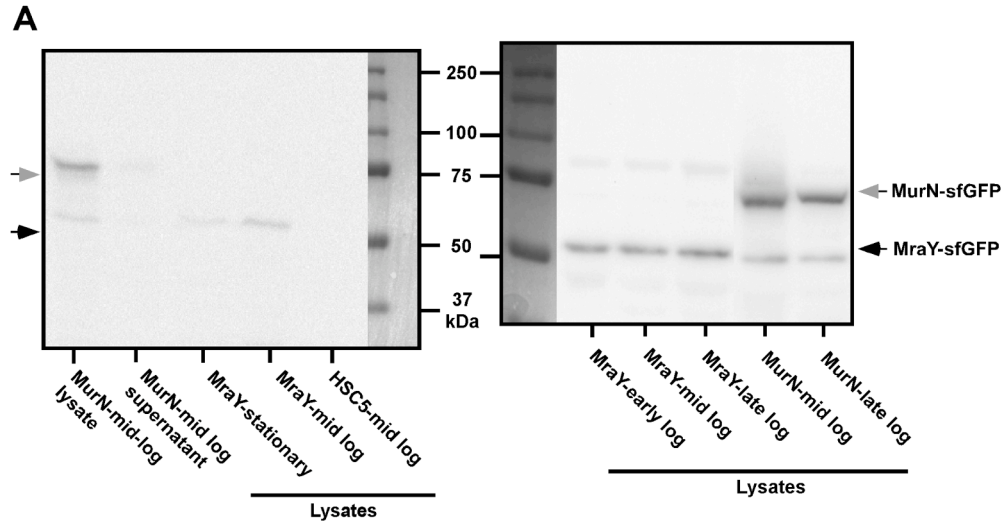


Figure 7. Focal localization of MraY and MurN at the *S. pyogenes* surface. The expression of C-terminally sfGFP-tagged MraY and MurN in *S. pyogenes* HSC5 was confirmed by Western blot analysis of the indicated streptococcal culture fractions (**A**). Arrows indicate the expected size of the fluorescent protein-tagged polypeptides (MraY-sfGFP: 63.5kDa; MurN-sfGFP: 74kDa). Localization of sfGFP-tagged MraY (**B-D**) and MurN (**E-G**) in *S. pyogenes* HSC5 was revealed by fluorescent microscopy (scale bar = 1 μm) (**B,C,E and F**) and immunogold electron microscopy using staining with a streptavidin-gold conjugate (scale bar = 500nm) (**D and G**). Real color, phase and merged images (**B,D**; sfGFP, green) are shown, as well as false color images (**C,F**; sfGFP red; WGA, 5 μg ml⁻¹, green).

To confirm that the observed localization of the tagged proteins corresponded to their native localization and not to any effects stemming from expression of the fluorescent protein tag, we also monitored the localization of C-terminally tagged YajC protein, which is maintained at the membrane by its single transmembrane segment. In other bacteria, YajC is considered an auxiliary subunit of the protein translocase by virtue of its co-expression and interaction with SecD and SecF proteins, but has been found to be non-essential for both viability and secretion [33]. Additionally, *S. pyogenes* does not express SecD and SecF [34] and thus the function of YajC in streptococci remains unknown. Unlike MraY and MurN, sfGFP-tagged YajC was observed over the entirety of the streptococcal membrane by both fluorescence (Fig. 8A and B) and electron microscopy (Fig. 8C) under all conditions tested, suggesting that the observed localization of MraY and MurN corresponds to their native cellular site.

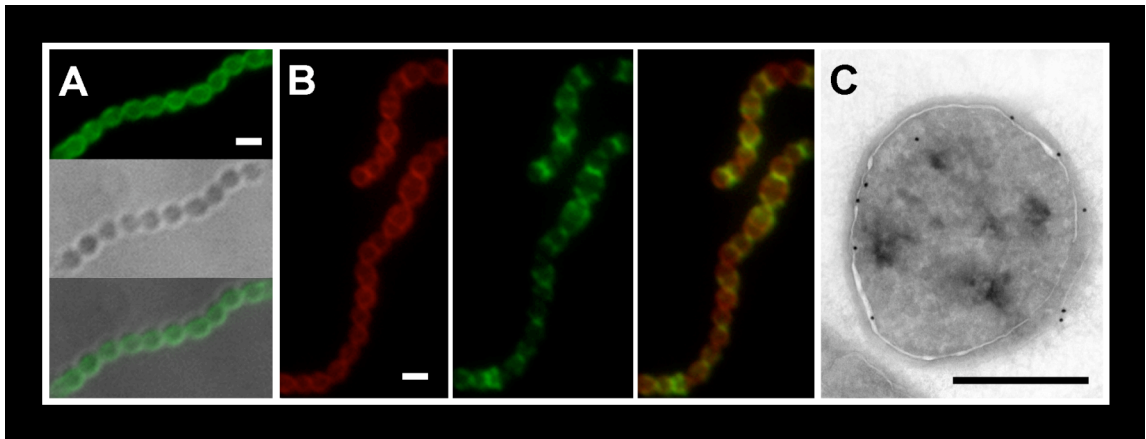


Figure 8. Distribution of YajC on the *S. pyogenes* surface. Localization of C-terminally sfGFP-tagged YajC in *S. pyogenes* HSC5 was revealed by fluorescent microscopy (scale bar = 1 μ m) (**A and B**) and immunogold electron microscopy using staining with a streptavidin-gold conjugate (scale bar = 500nm) (**C**). Real color, phase and merged images (**A**; sfGFP, green) are shown, as well as false color images (**B**; sfGFP red; WGA, 5 μ g ml⁻¹, green).

Next we determined whether focal localization of MraY and MurN corresponds to the site of the ExPortal. We have previously demonstrated that a fluorescently labeled cationic antimicrobial peptide preferentially targets to the site of the ExPortal lipid microdomain at low sub-lethal concentrations [12]. Fluorescence microscopy revealed that MraY (Fig. 9A and C) and MurN (Fig. 9B and D) localization predominantly coincides with that of labeling by the dansyl-polymyxin B probe, indicating that lipid II synthesis occurs at the anionic lipid microdomain of the ExPortal. Finally, we confirmed the targeting of lipid II synthetic machinery to the membrane region of the ExPortal by determining their localization relative to the Sec machinery translocons. Electron microscopy showed that immunogold labeling with antibodies against SecA and sfGFP-labeled MurN localized to the same focal site on the streptococcal surface (Fig. 9E), confirming that peptidoglycan precursor synthesis is spatially coordinated with the ExPortal.

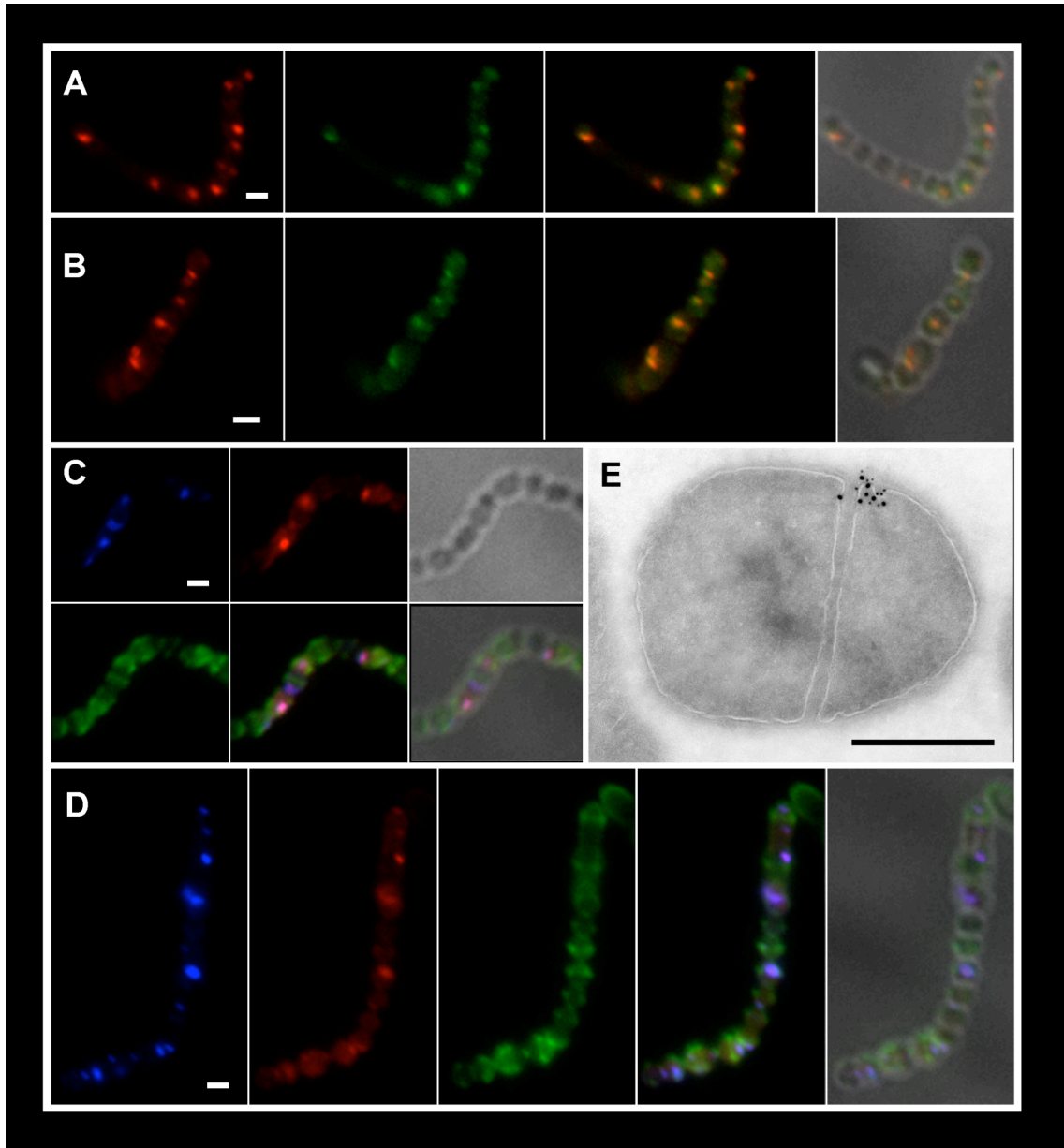


Figure 9. **The sites of MraY and MurN localization are coincident with the site of the ExPortal in the streptococcal membrane.** Localization of sfGFP-tagged MraY (A,C) and MurN (B,D) relative to the anionic lipid microdomain of the ExPortal in *S. pyogenes* HSC5 was revealed by fluorescent microscopy following staining by dansyl-polymyxin B ($10\mu\text{M}$; scale bar = $1\mu\text{m}$). False color, phase and merged images (A,B: dansyl-polymyxin B, red; sfGFP, green; C,D: dansyl-polymyxin B, blue; sfGFP, red; WGA, green) are shown. Co-localization of sfGFP-tagged MurN and SecA at the ExPortal was revealed by immunogold electron microscopy using staining with a streptavidin-gold conjugate (E). SecA was labeled with 12nm beads, MurN-sfGFP with 18nm gold-beads. Scale bar = 500nm.

DISCUSSION

Understanding the mechanisms involved in the biogenesis of secreted virulence factors in an organism whose pathogenicity depends highly on secreted effectors is of great importance to developing therapeutic strategies against infection by these pathogens. A great deal of research has been devoted to elucidating the regulation and expression of virulence proteins by *S. pyogenes* and other related Gram-positive pathogens [35]. However, knowledge of how these factors get translocated out of the streptococcal cell and processed into their biologically active forms is sparse and deserving of further investigation, especially given the emergence of resistance amongst gram-positive pathogens against clinically used antibiotics targeted at other cellular processes (e.g. macrolides which target protein synthesis; reviewed in [36]).

The data presented here indicate that biogenesis of the bacterial cell wall is not only necessary for protection from environmental insults and as a scaffold for the display of extracellular virulence effectors, but also for the maintenance of the secretory organelle that is the ExPortal. Examination of peptidoglycan involvement in the distribution of lipids in the underlying cytoplasmic membrane in *Bacillus subtilis* revealed that the cell wall is required for retention of anionic lipids at discrete microdomains [25]. In the investigation by Muchova *et al.* removal of surface peptidoglycan by lysozyme treatment produced protoplasts in which phosphatidylglycerol failed to localize in the spiral configuration that had been previously observed in intact cells using fluorescent lipid dyes [37, 38]. Most interestingly, inhibition of peptidoglycan production by depletion of the lipid II-synthesizing enzyme MurG disrupted localization of both phosphatidylglycerol and

cardiolipin in the membrane [25]. This suggests that peptidoglycan synthesis is necessary for the establishment of specific lipid domains in *B. subtilis* or that disruption of lipid II production causes disassembly of existing domains [25]. Our experimental data confirms that the presence of extracellular peptidoglycan is necessary for maintaining the asymmetric distribution of anionic lipids in the cytoplasmic membrane of a gram-positive pathogen. Additionally, disruption of anionic lipid localization in the membrane following cell wall removal in GAS resulted in mis-localization of ExPortal proteins, further supporting the hypothesis that segregation of anionic lipids at a particular microdomain constitutes a mechanism underlying ExPortal organization.

Our data and the results from Muchova *et al* indicate the gram-positive cell wall is involved in restricting lipids and proteins to specific regions of the cytoplasmic membrane. The bulk of each of the ExPortal proteins examined is found on opposite sides of the membrane, HtrA extracellularly and SecA intracellularly. Both, however, were mis-localized to a similar degree in the streptococcal cell membrane in the absence of the cell wall, even though SecA is unable to interact directly with extracellular peptidoglycan. Like HtrA in *S. pyogenes*, sortase localization in *E. faecalis* coordinates with that of the secretory translocons in the cytoplasmic membrane [7]. Most importantly, the cell wall sorting signal (CWS) of SrtC and SrtA sortases in *E. faecalis* contains a positively charged tail that was hypothesized to determine topology and mediate targeting and retention of the sortases within the membrane microdomains at which they were observed to localize [7]. Mutagenesis of the C-terminal CWS of SrtC to remove the positively charged tail or reduce its cationic character resulted in mis-localization of SrtC and reduction of its physiologic activity (pilus assembly), indicating that proper targeting

of the sortase to the membrane depended on the interaction of its cationic CWS with anionic components in the membrane [7]. Altogether, this data suggests that the retention of anionic phospholipids at specific, unique membrane sites constitutes the primary mechanism for targeting ExPortal proteins to their destination in the membrane, and that peptidoglycan mediates the site restriction of anionic phospholipids.

The non-membranolytic, lipid II-binding activity of gallidermin also disrupted localization of both lipid and proteinaceous elements of the ExPortal. The reported mechanism of lantibiotics like gallidermin that are bactericidal despite not permeabilizing cytoplasmic membranes is the sequestration of lipid II away from the sites of peptidoglycan synthesis [19, 20]. The effect of this can be considered to be two-fold: arresting further growth of the cell wall by starving penicillin binding proteins (PBPs) of substrate needed for peptidoglycan polymerization at the site of *de novo* cell wall synthesis, and depleting the lipid II content of the cell by preventing recycling of C₅₅-P lipid anchors needed for further lipid II production at the inner leaflet of the cytoplasmic membrane. The role of the cell wall in organizing the ExPortal may then have more to do with peptidoglycan synthesis, rather than with the peptidoglycan polymer itself, though it is apparent that integrity of this macromolecule is essential for lipid segregation in the membrane.

Previous research indicates that segregation of anionic lipids in bacterial membranes is also a mechanism for targeting the macromolecular complexes involved in cell wall synthesis and other essential cellular processes to the sites where their physiological activity is required. Lipid microdomains identified in *B. subtilis* can be isolated together with homologues of eukaryotic Flotillin1 and other proteins involved in

transport and signaling [39]. In *Caulobacter crescentus* the dependence of MurG localization on helical cables of the cytoskeleton protein MreB [40] and the co-localization of the division protein MinD with phosphatidylglycerol lipid spirals in the cytoplasmic membrane of *B. subtilis* [28] point to a link between the molecular machineries of peptidoglycan synthesis, cell division and membrane lipids. Most recently, examination of cell wall synthesis in growing *B. subtilis* cells found that MreB and its isoforms (Mbl, MreBH) assembled with components of the cell wall synthesis holoenzyme (MreC, MreD, PbpH, PBP2A and RodA) into discrete patches at the cytoplasmic membrane that moved processively along peripheral tracks perpendicular to the cell axis [26, 27]. MreC and MreD are transmembrane proteins reported to couple cytosolic MreB and its isoforms to the extracellular peptidoglycan synthesis machinery, and RodA an integral membrane protein linked to cell wall elongation and to the PBPs examined (PbpH and PBP2A; for a review see [41]). Motility of the observed MreB-associated peptidoglycan biosynthetic complexes was driven by cell wall synthesis and MreB polymers restricted localization of patch components in the membrane and directed their movement [26, 27]. One possible mechanism then by which peptidoglycan synthesis in GAS might affect lipid segregation in the cytoplasmic membrane is by association of cytoskeletal components inside the cell with cell wall synthesis outside the cell, placing constraints on the distribution of phospholipids in the intervening membrane, thus leading to the creation and stabilization of lipid domains. However, *S. pyogenes* lacks homologs to the cytoskeletal MreB proteins [34], and as a morphologically ovococoid bacterium, given its pattern of cell division in successive parallel planes perpendicular to the cell axis, *S. pyogenes* does not express the synthetic machinery required for cell elongation in

rod-shaped bacteria like *B. subtilis* (RodA, MreC, MreD; reviewed in [42]). Rather, the macromolecular complex directing cell division and growth in *S. pyogenes* consists of the tubulin homologue FtsZ, its accessory divisome proteins (i.e. FtsA, FtsW, EzrA, DivIB, DivIVA) and a set of high- and low-molecular weight PBPs common amongst enterococcal and streptococcal species [42]. Thus, although the relevance of peptidoglycan to the localization of membrane anionic lipids appears to be similar in *S. pyogenes* and *B. subtilis*, the mechanism underpinning this function of cell wall biogenesis may be different in each species and its elucidation requires characterization of the role divisome proteins of GAS play in organization of the ExPortal and cell wall synthesis.

In model membranes, lipids can adopt various fluid and liquid-ordered phases, characterized by the different spatial arrangement and motional freedom of each lipid molecule with respect to the surrounding molecules, and dependent on the composition, structure and environment of the membrane [43]. Different fluid phases can coexist within a single membrane plane delimited by a plane boundary, resulting in lateral phase segregation and contributing to the formation of lipid microdomains similar to those observed in bacterial cytoplasmic membranes. The properties of these lipid phases can determine the orientation, mobility and interaction of proteins and lipids contained therein and thus directly influence the biological functionality of the domains [44]. An alternative, or perhaps contributing mechanism underlying organization of the ExPortal by membrane lipid segregation may be localization of the C₅₅-P anchor of lipid II in the membrane. The membrane region at which C₅₅-P is localized, thereby determining the site at which lipid II synthesis occurs, is different in composition and environment from

the rest of the membrane and thus determines where anionic lipids segregate in the membrane. The data we present here demonstrate that in GAS, the membrane-associated steps of lipid II synthesis are restricted to a unique microdomain of the streptococcal membrane, since both an integral membrane transferase (MraY) and a cytoplasmic enzyme (MurN) required for the production of lipid II were predominantly localized at unique foci in streptococcal cells, as observed by both fluorescent and electron microscopy. Moreover, this site coincides with the localization of the anionic lipid microdomain and secretory translocons of the *S. pyogenes* ExPortal. Restriction of lipid II synthesis to the site C₅₅-P is found in the membrane could direct localization of phosphatidylglycerol, the predominant anionic lipid of GAS membranes. Alternative models in which anionic lipids maintain C₅₅-P localization in the membrane or in which C₅₅-P and phosphatidylglycerol are equally dependent on each other for localization cannot be ruled out. Regardless, both existing research and the data presented here are indicative of spatial coordination between molecules like C₅₅-P and anionic lipids. Previous work in *B. subtilis* suggests that the lipids associated with bacterial membrane microdomains include polyisoprenoids synthesized via pathways involving squalene synthases, as treatment with zaragozic acid, an inhibitor of squalene synthesis, prevented the formation of functional lipid microdomains in *B. subtilis* membranes [39]. Most interestingly, further characterization of squalene synthesis inhibition by inactivation of a gene involved in the pathway converting farnesyl pyrophosphate into squalene, inhibited protein secretion and biofilm formation, but not viability of the bacterium. The C₅₅-P membrane anchor of lipid II is also a polyisoprenoid synthesized from the condensation of eight isopentenyl pyrophosphate units to farnesyl pyrophosphate, catalyzed by

undecaprenyl pyrophosphate synthase (UppS) [45, 46]. Therefore, production of the essential C₅₅-P isoprenoid could be the key factor for localization of both cell wall synthesis and the ExPortal. This does not however exclude the possibility that targeting of C₅₅-P in the cytoplasmic membrane depends on the localization of the UppS transferase responsible for its production, which in turn may involve the action of divisome proteins, as these are involved in determining the site of cell wall growth and septation necessary for the genesis of two daughter cells with their own cell walls from a single parent cell.

Further characterization of the ExPortal organization will require examining the involvement of proteins known to coordinate cell division with cell wall synthesis in other gram-positive bacteria. It will also be useful to examine the effect of compounds that target different aspects of peptidoglycan and lipid II synthesis on organization and function of the ExPortal. Recent work revealed that the secretory activity of the streptococcal ExPortal and its organization are particularly sensitive to the action of CAPs [12]. Since CAPs are a class of antimicrobial molecules against which bacterial pathogens have developed limited resistance, it is important to understand how these peptides target the ExPortal in order to develop novel and more effective therapeutics against GAS. Conversely, characterizing the mechanisms and specific factors supporting organization and function of the ExPortal can reveal novel targets to be exploited against *S. pyogenes* and other gram-positive pathogens with similar secretory mechanisms.

EXPERIMENTAL PROCEDURES

Strains, media and growth conditions: As indicated, experiments utilized *S. pyogenes* HSC5 [47] or SF370 [34]. Localization of HtrA was analyzed in HSC5 following transformation by pHtrA-HA, which expresses a HA-tagged derivative of HtrA [6]. Localization of MraY, MurN, YajC was analyzed in HSC5 strain streptococci expressing C-terminal SuperFolder GFP protein fusions generated by chromosomal integration of pLAV5912, pLAV51012 and pGCP477. Routine culture was at 37°C in Todd-Hewitt broth (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium). Functional assays utilized cultures grown in unmodified C medium as described previously [48], supplemented with spectinomycin (100ug ml⁻¹, Sigma #S4014) when indicated. To produce solid media, Bacto Agar (Difco) was added to a final concentration of 1.4%. Liquid cultures were grown without agitation in closed containers and solid cultures were incubated under anaerobic conditions using a commercial gas generator (GasPak, cat. #260678, BBL) as described [48]. SpeB protease activity was inhibited in cultures used to analyze the abundance of secreted streptococcal proteins by including the cysteine protease inhibitor E64 (Sigma #E3132) [49]. In selected experiments, media were supplemented with the cationic antimicrobial peptide polymyxin B (cat. #P0972-50MU, Sigma), the lantibiotics nisin (Sigma #N5764), or gallidermin (a generous gift from Dr. James Smith, Texas A&M University). Protoplasts were generated by treating streptococci with PlyC lysin (5U ml⁻¹, 10 minutes) in 30% (wt/vol) raffinose solution supplemented with 5mM EDTA. For visualization of lipid and protein localization, protoplasts were resuspended in either ThyB or C media supplemented with 5-8% (v/v) glycerol, as indicated.

DNA techniques: Plasmid DNA was isolated via standard techniques and used to transform *S. pyogenes* as described previously [50]. Restriction endonucleases, ligases and polymerases were used according to manufacturer's recommendations.

Fluorescent protein-tagged constructs: All fluorescent-tagged proteins examined in this study were expressed in the HSC5 strain by chromosomal integration of the C-terminal tags. Cloning primers are detailed in Table 1. To generate GFP-tagged constructs, the coding sequence for SuperFolder GFP (sfGFP) was first amplified from pET28sfGFP (a kind gift from Geoff Waldo at Los Alamos National Laboratory <http://www.lanl.gov/projects/gfp/index.shtml> and <http://www.theranostech.com/products.htm>) using primers GP526 and GP527. The amplified sfGFP sequence was then directly cloned by overlap extension PCR cloning using Phusion DNA polymerase (Finnzymes) as previously described [51] into pSPC18, a derivative of the pUC18 vector with the ampicillin resistance gene *bla* replaced by the spectinomycin resistance gene *aad9* [52, 53]. The final product (pGCP458) contains a promoter-less sfGFP lacking the start ATG and a 5' BamHI restriction site for direct cloning of the 3' ends of ORFs to create C-terminal tag protein fusions upon chromosomal integration. Amplification of 300 nucleotide C-terminal fragments, absent the stop codon, from *mraY*, *murN* and *yajC* using the primer pairs MraY2 Fwd/MraY2-Rev, MurN Fwd/MurN Rev and GP532/GP533 respectively, followed by double restriction digest (BamHI/PstI) and ligation generated pSPC18:MraY-sfGFP (pLAV5912) and pSPC18:MurN-sfGFP (pLAV51012) and pSPC18:YajC-sfGFP (pGCP477).

PlyC expression and purification: Recombinant PlyC, a lysin produced by streptococcal phage C1, was expressed and purified as previously described [54, 55], with certain modifications. Both subunits of the lysin (PlyCA and PlyCB) were 6-X-His C-terminally tagged, and co-purified on Talon® metal affinity resin (Cat#: 635503, Clontech) from lysates of separate strains (pPlyCA, pPlyCB). Specific activity of the purified enzyme was measured according to established protocols [55].

Analysis of Viability: Viability was assessed by staining with a fluorescent vital dye (LIVE/DEAD®, BacLight™) as recommended by the manufacturer (Invitrogen). Viability was quantitated by examination using a fluorescent microscope (Leica model DM IRE 2) with enumeration of the percentage of live bacteria in randomly chosen microscopic fields totaling >1000 cells for each condition examined. Data reported represent the mean and standard error of the mean derived from a minimum of 3 independent experiments. Differences between calculated means were evaluated for significance using a one-way Analysis of Variance (ANOVA).

Cellular staining and fluorescent microscopy: Streptococcal cultures were challenged with polymyxin B or lantibiotics as described above, stained with various fluorescent reagents and then analyzed by fluorescent microscopy as follows: Analysis of the location and integrity of a membrane microdomain enriched in anionic phospholipids was assessed by staining with sub-lethal concentrations of fluorescent polymyxin B derivatives including dansyl-polymyxin B (10µM, cat. #P13238, Invitrogen) and polymyxin B BODIPY®FL conjugate (10µM and 60µM, cat. #P13235, Invitrogen).

Samples were examined using a Leica model DM IRE 2 fluorescent microscope and images captured using a QImaging Retiga 1350 EX charged-coupled device camera and Openlab software (Improvision). Where indicated, simultaneous treatment with 2 reagents was conducted in order to assess co-localization of staining. In these experiments streptococcal cells walls were visualized by staining with FL-vancomycin (1 $\mu\text{g ml}^{-1}$, cat. #V34850, Invitrogen), or Wheat Germ Agglutinin (WGA) Alexa Fluor 488 or 350 conjugates (5 $\mu\text{g ml}^{-1}$, cat. #W11261, #W11263 Invitrogen) and neutral membrane lipids were visualized by staining with Nile Red (2.5 $\mu\text{g ml}^{-1}$, cat. #N1142, Invitrogen). Images were processed for publication using Adobe Photoshop CS3.

Immunofluorescent microscopy: The localization of SecA and HA-tagged HtrA protein was detected by immunofluorescent microscopy, as described [6]. Aliquots from cultures of intact cells, protoplasts, as well as untreated or lantibiotic-treated (1 μM gallidermin) challenge assay cultures (see above) were treated and fixed according to the method of Raz and Fischetti [11]. Antisera used included a polyclonal rabbit anti-SecA antiserum (a generous gift from Donald Oliver) used at a dilution of 1:100 that was detected using an AlexaFluor 488-labeled goat anti-rabbit IgG (Invitrogen) at 1:500 and an AlexaFluor 594-conjugated rabbit IgG anti-HA epitope antiserum (Invitrogen) used at 1:500. Wheat germ agglutinin (WGA)-AlexaFluor 350 (Invitrogen) at a final concentration of 5 $\mu\text{g ml}^{-1}$ was used to visualize the streptococcal cell walls, and polymyxin B BODIPY®FL conjugate (10 μM) was used to visualize anionic lipids. Slides were mounted in an anti-fade reagent (Prolong Gold, Invitrogen) and images captured.

Immunogold electron microscopy: Localization of sfGFP-tagged MraY and MurN and YajC along with polymyxin B binding was examined by electron microscopy, as follows: Cultures were grown as indicated above, aliquots were removed and prepared for immunoelectron microscopy as described [5, 6]. Sections were stained using a streptavidin-gold conjugate (20nm, BBI International) and examined by electron microscopy as detailed [5, 6]. Localization of MraY and MurN and YajC was determined at early- and mid-logarithmic phase [5, 6]. Co-localization of MurN and SecA was assessed by staining using streptavidin-gold conjugated beads of different sizes (12nM and 18nM, BBI International).

Analysis of protein expression and secretion: Supernatant and lysate fractions from cultures challenged with gallidermin and polymyxin B were prepared and analyzed for the presence of SpeB, SLO and SecA by Western blotting as described [56]. Lysate fractions from strains expressing sfGFP-tagged MraY, MurN and YajC were similarly examined for the presence of the sfGFP tag. Blots were developed using a Chemidoc XRS imager (BioRad) and relative protein concentrations determined using Quantity One software (BioRad, version 4.6.7).

Table 1. Primers utilized for construction of fluorescent protein tagged constructs.

Primer	Sequence ¹
MraY2 Fwd	CCCCTGCAGCTTGGAGCTATGTTGGCTGC
MraY2 Rev	CCCGGATCCGAAGACGTATAAGATAGCTAAAAGTAGTAAACTAGC
MurN Fwd	CCCCTGCAGTTTATCTATAACCGAACAAGAGGCC
MurN Rev	CCCGGATCC ATAGCGGTTTAATAGCTTTTTTGC
GP526	gcaggtcgactctaga <u>ggatcc</u> AGCAAAGGAGAAGAAGACTTTTCAC
GP527	cacacaggaacagctatgacat <u>CCCGGG</u> TTATTTGTAGAGCTCATCCATGC
GP532	GATACTGCAGTGGTTTATGCAACGTCAAC
GP533	GATAGGATCCATGGCTTTCAATGGCAC

¹Restriction sites underlined, and for GP526, GP527 lower case denotes pSPC18 sequence and the uppercase denotes sfGFP sequence.

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Chapter IV

Work In Progress: Characterizing ExPortal Substrates and Factors Contributing to its Organization

ABSTRACT

Further characterization of ExPortal organization will entail multiple genetic and molecular approaches to isolate the precise factors involved in its establishment and maintenance. These approaches include molecular screens to identify substrates in addition to SpeB and SLO requiring the spatial coordination of secretory translocons and accessory factors for efficient biogenesis and export, as well as targeted mutagenesis of additional cell wall synthesis proteins (i.e. transglycosylases, transpeptidases and cell wall hydrolases) to characterize their involvement in ExPortal maintenance. Preliminary data resulting from such efforts is presented here. A proteomics screen for polypeptides other than SpeB or SLO with altered secretion profiles in response to CAP exposure identified the virulence protein EndoS as a potential ExPortal substrate. Identification of putative cell wall hydrolases encoded in the *Streptococcus pyogenes* genome and initial characterization of their involvement in ExPortal maintenance by mutagenic approaches revealed that though the Isp polypeptide co-localizes on the streptococcal surface with the anionic lipid microdomain of the ExPortal, Isp and its homolog Isp2 are not required for maintenance of said microdomain. Additionally Isp is unaffected in its expression or localization following exposure of streptococci to polymyxin B, indicating further work is necessary to determine what role, if any, these putative hydrolases have in ExPortal organization.

INTRODUCTION

One approach to elucidating the mechanism(s) by which the ExPortal coordinates protein secretion and processing is to identify and characterize its secreted substrates. Understanding how these substrates are expressed and what post-translational modifications they undergo in order to be processed into their biologically active forms can reveal a great deal about ExPortal function. Not all proteins secreted by streptococci require the spatial coordination of accessory factors and the secretory translocons, as indicated by the effect exposure to sub-lethal levels of CAPs had on ExPortal organization and protein secretion. Since sub-lethal polymyxin B concentrations selectively inhibited secretion of those proteins requiring an intact ExPortal, I undertook a preliminary analysis of what other secreted proteins of streptococci are affected in their biogenesis by exposure to sub-lethal levels of a CAP. This analysis identified EndoS, a secreted 108kDa protein with specific endoglycosidase activity that cleaves human Immunoglobulin G [1], as a potential ExPortal substrate affected by CAP-mediated disruption of its secretory activity.

Data presented in Chapter III demonstrates that cell wall biogenesis is involved in organizing the ExPortal and suggests localization of the secretory organelle is coordinated with the site of peptidoglycan precursor (lipid II) synthesis. In coccoid bacteria like *S. pyogenes*, cell wall synthesis primarily occurs at the site of cell division and involves the action of transglycosylases, transpeptidases and cell wall hydrolases [2]. Activity and regulation of these may be important to ExPortal organization as well as cell growth and division. Eukaryotic-type Ser/Thr kinases (STK) reported in bacteria, including GAS, can regulate cell division, growth, and virulence. Recent research

demonstrated that *S. pyogenes* STK -controlled cell division is mediated under the positive regulation of a secreted protein termed CdhA that possesses a cysteine and histidine-dependent aminohydrolase/peptidase (CHAP) domain with functional cell wall hydrolase activity [3]. Disruption of the CdhA-encoding gene resulted in severe cell division and growth defects, while a mutant expressing CdhA absent the CHAP domain was solely attenuated for virulence and highly susceptible to cell wall-acting antibiotics[3]. CdhA was thus characterized as a multifunctional protein with a N-terminal region involved in cell division plane-recognition and a C-terminal CHAP domain involved in virulence[3]. Characterization of CdhA suggests that localization of the macromolecular machineries involved in cell division and cell wall synthesis at their site of action in streptococci requires accessory proteins. CdhA and/or related proteins then may also act directly or indirectly, through their involvement in regulation of cell wall synthesis, on ExPortal organization. Thus, I hypothesize that CdhA or orthologous proteins associated with cell wall assembly and involved in determining the plane of cell division in GAS, may also play a role in ExPortal organization.

The work of Pancholi et al indicates the functional CHAP domain of CdhA is not involved in the protein's role of cell division plane-recognition, given that the absence of this domain does not produce the same division and growth defects that disrupting expression of the whole CdhA polypeptide does. Additional cell wall hydrolases may compensate for the absence of CdhA enzymatic activity and the role of recognizing the plane of cell division could involve interaction of CdhA with these hydrolases in the cell wall or with other factors at the cytoplasmic membrane. Two additional proteins of GAS contain a C-terminal CHAP domain closely resembling that of CdhA. Identified as Isp

(Immunogenic secreted protein) and Isp2, they are highly homologous to one another, are encoded as part of the *mga* regulon, but have no assigned cellular function [4, 5]. Since the CHAP domain of CdhA was shown to still be important for virulence and antibiotic resistance, I hypothesize Isp and Isp2 function in conjunction with CdhA to modulate cell wall assembly, while targeting to their site of action by CdhA influences localization of the cell division plane and the ExPortal. Thus, I proceeded to characterize the function of Isp, Isp2 and CdhA in cell division and ExPortal organization. Preliminary data shows that lack of *isp* alone, or in combination with the absence of *isp2*, does not recapitulate the effects on cell division and morphology observed by disruption of CdhA. The absence of *isp* and *isp2* also did not affect localization of the ExPortal anionic lipid microdomain nor secretion of the SpeB protease. Attempts to generate an in-frame deletion of CdhA or its CHAP domain in the strains lacking *isp* and/or *isp2* have thus far proven unsuccessful, which hints at a dependence of these factors on each other for cell viability.

RESULTS

EndoS. Proteins in supernatants harvested from stationary phase streptococcal cultures that were treated with a sub-lethal concentration of polymyxin B were compared to those from untreated cultures. Proteins found in both samples were isolated by gel electrophoresis and subjected to trypsinization and subsequent analysis by mass spectrometry at the Siteman Cancer Center Proteomics Core Laboratory (Barnes-Jewish Hospital/Washington University School of Medicine, http://proteomics.wustl.edu/siteman/Proteomics_page3_new.htm). The results obtained were examined using ScaffoldTM 2 Proteome software (ScaffoldTM Version_2.1.03, Proteome Software Inc.). Spectrometry analysis confirmed that SpeB was absent from supernatants of streptococcal cultures exposed to polymyxin B and identified additional proteins absent or in lower relative amounts in treated cultures. Among these, one protein of interest for which no trace was detected in peptide treated samples, was the EndoS. Further characterization of EndoS as an ExPortal substrate was conducted by examining expression of the endoglycosidase in the presence of polymyxin B. Previous research indicates that, like SpeB, EndoS expression is growth phase regulated, with peak expression occurring in stationary phase [6]. Western analysis of supernatants from CAP-treated and untreated cultures harvested under the same conditions as indicated for SpeB in Chapter I, revealed a dose-dependent reduction in the amount of secreted EndoS protein resulting from exposure to polymyxin B (Fig. 1A) and HNP-1 (Fig. 1B). This effect on levels of secreted EndoS was observed in both HSC5 and SF370 (Fig. 1C, D) strains. Transcriptional analysis however, showed that levels of EndoS transcript were

also affected by exposure to polymyxin B (Fig. 1E). These results suggest that disruption of the ExPortal can also affect transcription of streptococcal virulence proteins.

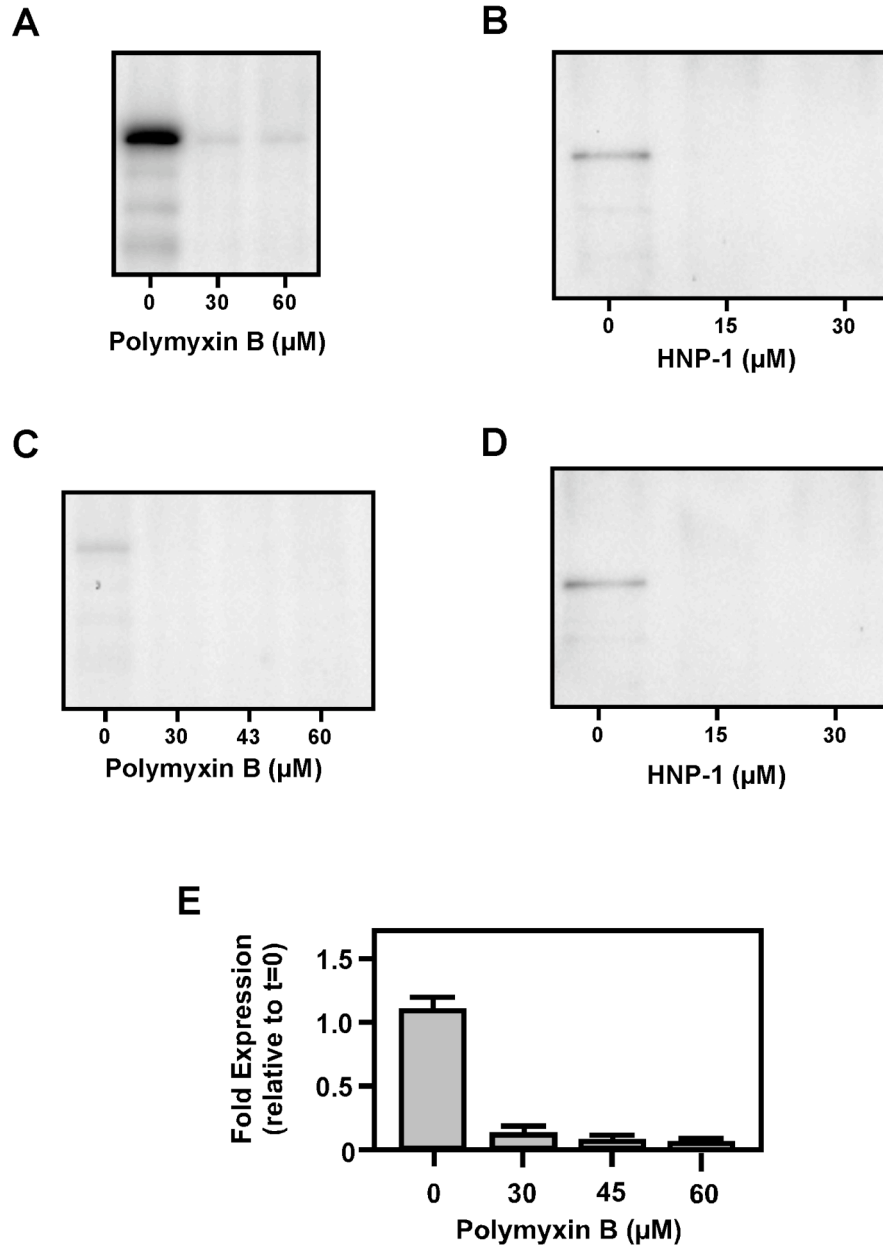


Figure 1. **High sub-lethal challenge with polymyxin B and HNP-1 inhibits secretion of EndoS.** Expression of the EndoS endoglycosidase in cultures of *S. pyogenes* HSC5 (A, B) and SF370 (C, D) was determined following challenge with the indicated concentrations of polymyxin B and HNP-1 by Western blot analysis of culture supernatant (A-D) and by real-time RT-PCR analysis of *endoS* transcript abundance (E). All samples for EndoS analysis were harvested at 2 hrs post-challenge.

CdhA, Isp/Isp2. Penicillin binding protein (PBP) transglycosylases, transpeptidases and cell wall hydrolases are hypothesized to primarily localize as a multienzyme complex or holoenzyme to the sites of peptidoglycan synthesis in bacteria (for a review see [7]). Cell wall hydrolase activity serves to remodel peptidoglycan strands as new subunits are added to the polymer during growth, cell separation following division and cell wall turnover [8-10]. CdhA was demonstrated to have functional hydrolase activity, and given that Isp contains a C-terminal CHAP domain similar to that of CdhA, Isp is potentially a previously unidentified streptococcal cell wall hydrolase. Thus in order to further characterize the role of peptidoglycan biogenesis in ExPortal organization, I examined how Isp localizes in the streptococcal cell. Isp localization was monitored by C-terminally tagging Isp using a hemagglutinin (HA)-mCherry tandem tag. The purpose of the tandem tag was to examine localization of the protein by both fluorescent and immunofluorescent microscopy as well as expression by Western analysis. Fluorescent microscopy revealed that Isp localizes to discrete regions on the streptococcal surface in both HSC5 (Fig. 2A) and SF370 strains (Fig. 2B). These regions are observed as either unique foci or as hemispherical domains between cells, mirroring the observed localization of HNP-1 in GAS (see Fig. 6, Chapter II).

The disruption of anionic lipid microdomains by CAPs does not appear to disrupt cell growth and division, as indicated by data presented in Chapter I. The converse, that disruption of the cell wall and of peptidoglycan synthesis affects anionic lipid microdomain localization, appears to be the case, as supported by data described in Chapter III. I examined then whether exposure to a CAP alters localization of Isp. Neither low nor high sub-lethal concentrations of polymyxin B appear to affect localization of Isp

(Fig. 2C, D). The lack of an effect on Isp localization as a result of exposure to an ExPortal-disrupting CAP was observed in both the HSC5 (Fig. 2C, D) and SF370 strain (data not shown). Next I determined whether loss of Isp expression affects localization of the ExPortal anionic lipid microdomain. Low sub-lethal amounts of a fluorescently labeled polymyxin B derivative (dansyl-polymyxin B) shown to target the ExPortal (see Fig., Chapter I) in wild-type streptococci similarly localized to unique foci in a mutant strain containing an in-frame deletion of Isp (Fig. 2E). Deletion of both Isp and its homolog Isp2 did not alter localization of dansyl-polymyxin B either (Fig. 2F), suggesting that individually or together these putative cell wall hydrolases do not influence ExPortal localization. Lastly I examined the localization of Isp on the streptococcal cell surface relative to the ExPortal. Fluorescence microscopy shows that the fluorescently-labeled Isp polypeptide and the anionic lipid microdomain of the ExPortal labeled with polymyxin B localize adjacent to one another (Fig. 2G, H). This could be the result of both targeting to sites of active peptidoglycan synthesis and does not imply that localization of one is dependent on the other given the previous data described.

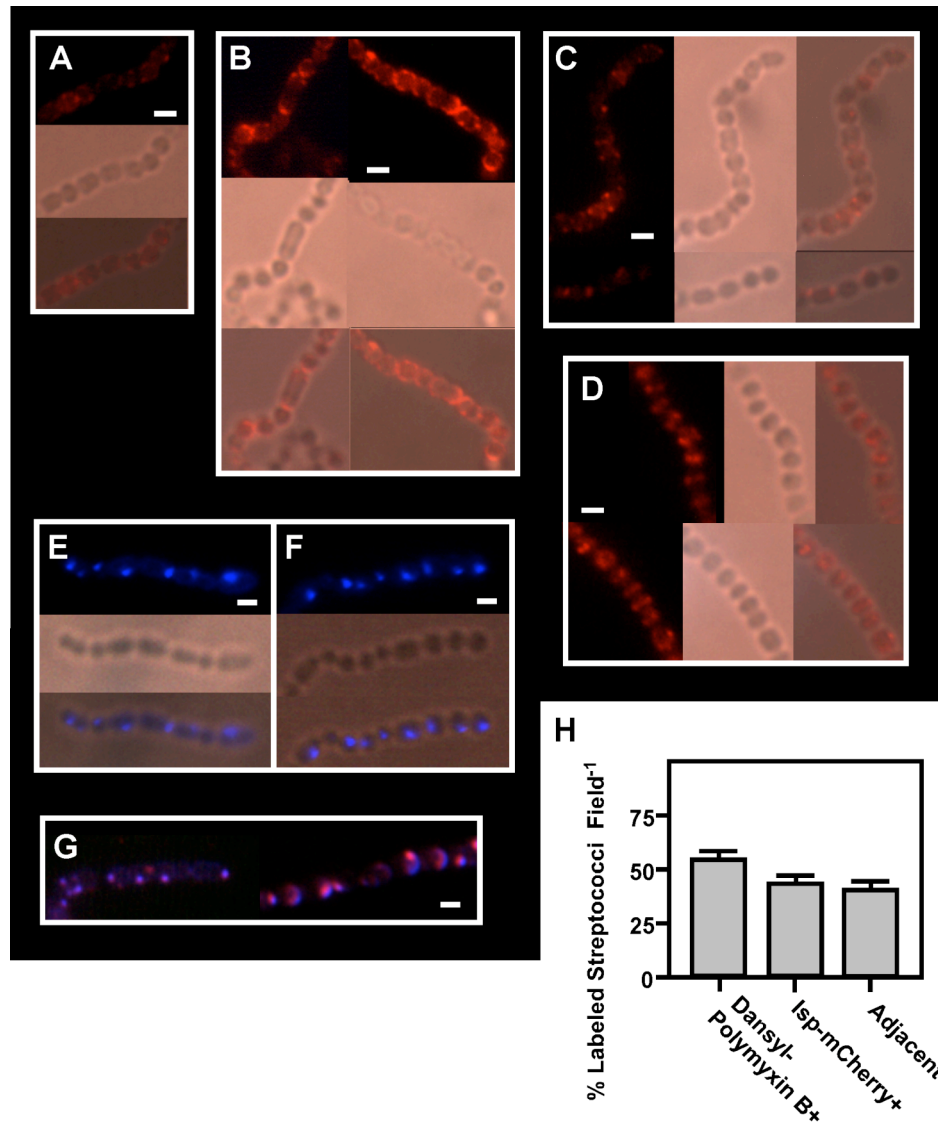


Figure 2. Characterization of Isp and Isp2 putative cell wall hydrolases and their role in ExPortal organization. Localization of Isp and Isp2 on the streptococcal surface and their involvement in organization of the ExPortal was examined by fluorescence microscopy. Localization of C-terminally mCherry-tagged Isp protein was revealed by fluorescence microscopy in HSC5 (A) and SF370 (B) strains. Localization of Isp-mCherry was unaltered by polymyxin B challenge of streptococci with low (C) and high (D) sub-lethal levels of the cationic peptide. Localization of anionic lipids was examined by fluorescence microscopy using dansyl-polymyxin B to label membranes of HSC5 mutant strains containing in-frame deletions of *isp* (E) and both *isp* and *isp2* (F). Co-localization of the ExPortal anionic lipid microdomain and Isp protein was determined by fluorescence microscopy in dansyl-polymyxin B-stained (blue) HSC5 strains expressing mCherry-tagged Isp (red) (G) and quantitated (H). Fluorescence, phase and merged images are shown. Scale bar = 1 μ m. Numbers of polymyxin B and mCherry fluorescence-labeled streptococci were derived from examination of at least 1000 stained cells. All data presented represents the mean and standard error of the mean derived from at least 3 experiments.

DISCUSSION

The observed effects of polymyxin B on expression of EndoS indicate that disruption of ExPortal integrity by the CAP can also alter expression of streptococcal virulence factors at the level of gene transcription. This suggests that localization of anionic membrane lipids and associated ExPortal proteins also affects gene regulation. One possible mechanism by which polymyxin B may be affecting EndoS expression is disruption of one of the multiple two-component signal transduction systems identified in GAS that regulate virulence gene expression in response to environmental cues [11]. The CAP itself could be interacting with such a two component regulator, as is hypothesized in the case of cathelicidin induced stimulation of the *hasABC* operon described in Chapter I [12, 13]. Another possibility is that ExPortal disruption inhibits the function of an accessory factor required for activation or de-repression of EndoS transcription. Function of such a factor could be dependent on lipid-protein or protein-protein interactions at the site of protein secretion, similar to how phosphatidylglycerol has been shown to be involved in optimal protein translocation via the Sec pathway by influencing interaction of SecA with the SecYEG translocon [14, 15]. Whatever the case, the identification of EndoS as a virulence factor of streptococci affected by CAP activity on the ExPortal opens up an additional avenue of investigation into the role of ExPortal function in streptococcal virulence.

Further characterization of Isp, Isp2 and CdhA localization and activity is evidently necessary in order to determine whether these are all indeed functional cell wall hydrolases involved in peptidoglycan synthesis and if and how their activity affects ExPortal organization. The experimental data published by Pancholi et. al. strongly

suggest that CdhA does play a role in proper cell wall assembly during cell division. If Isp and Isp2 have functional cell wall hydrolase activity, then the phenotype of the $\Delta isp/\Delta isp2$ suggests a functional CHAP domain in CdhA is necessary and sufficient for cell division and growth. It will be most interesting to examine whether deletion of the CHAP domain of CdhA in an $\Delta isp/\Delta isp2$ background recapitulates the $\Delta CdhA$ phenotype, as this would strongly indicate that interaction of all three CHAP-domain proteins is required or the division-plane localizing role of CdhA, with the N-terminal domain of CdhA acting as the targeting factor for all three proteins and the associated Isp and Isp2 activity involved in the role of CdhA in virulence and beta-lactam resistance. Other as of yet unidentified factors may also be involved, highlighting the importance and novelty of this research. Characterization of the ExPortal in the CdhA-disrupted strain is pending collaboration with Pancholi et al, as my attempts to generate a CdhA null mutant have been unsuccessful. Further mutagenesis and characterization of CdhA, Isp, Isp2 and other components of cell wall synthesis is needed to assess their involvement in ExPortal organization.

EXPERIMENTAL PROCEDURES

Strains, media and growth conditions: As indicated, experiments utilized *S. pyogenes* HSC5 [16] or SF370 [17] and all references to genomic loci are based on the genome of SF370 [17]. Localization of Isp was analyzed in HSC5 following transformation by pIsp-HA-mCherry (pLAV41312), which expresses a Hemagglutinin (HA)-mCherry-tandem-tag derivative of Isp. Localization and integrity of the ExPortal anionic lipid microdomain was assessed in HSC5 following in-frame deletion of *isp* and *isp2* coding regions. Routine culture was at 37°C in Todd-Hewitt broth (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium). Localization and functional assays utilized cultures grown in unmodified C medium as described previously [18], supplemented with spectinomycin (100ug ml⁻¹, Sigma #S4014) when indicated. To produce solid media, Bacto Agar (Difco) was added to a final concentration of 1.4%. Liquid cultures were grown without agitation in closed containers and solid cultures were incubated under anaerobic conditions using a commercial gas generator (GasPak, cat. #260678, BBL) as described [18]. In selected experiments, media were supplemented with the cationic antimicrobial peptide polymyxin B (cat. #P0972-50MU, Sigma).

DNA techniques: Plasmid DNA was isolated via standard techniques and used to transform *S. pyogenes* as described previously [19]. Restriction endonucleases, ligases and polymerases were used according to manufacturer's recommendations.

Construction of *S. pyogenes* deletion mutants: All references to genomic loci are based on the genome of *S. pyogenes* SF370 [17]. The construction of mutants containing in-frame deletions in *isp* (spy2025) and *isp2* (spy1801) was performed by allelic replacement [20] using the primers listed in Table 1. The in-frame deletions were constructed as follows. Flanking regions of *isp* and *isp2* were amplified from HSC5 genomic DNA using the corresponding primers indicated in Table 1 to generate fragments 500 nucleotides in length with a 5' XhoI upstream restriction site, an SphI restriction site in common at the 3' end of the upstream flanking region and 5' end of the downstream flanking region and an XbaI downstream restriction site at the 3' end of the downstream flanking region. The amplified flanking regions were ligated following restriction digestion of the shared SphI site. The resulting products were amplified using the 5' upstream and 3' downstream primer pairs (Spy_2026/Isp4 Fwd-Isp/Spy_2023 Rev and Spy_1802/Isp2 Fwd- Isp2/Spy_1798 Rev) to generate fragments containing in-frame deletions of the regions of *isp* and *isp2* encoding L11-P542 and M11-N493 respectively. These deletion fragments were cloned into the pJRS233 shuttle vector using the XhoI and XbaI restriction sites to generate pJRS233- Δ Isp (pLAV4910) and pJRS233- Δ Isp2 (pLAV11510). HSC5 strain streptococci were transformed as previously described [20] with pLAV4910 to generate HSC5: Δ *isp*, which was subsequently transformed using pLAV11510 to generate HSC5: Δ *isp*/ Δ *isp2*. In frame deletions were confirmed by PCR and sequence analysis using the appropriate primers.

Epitope/fluorescent protein-tagged constructs: Epitope and fluorescent-tagged proteins examined in this study were expressed in the HSC5 strain by chromosomal integration of

the C-terminal tags. Cloning primers are detailed in Table 1. To generate the HA-mCherry-tandem-tagged constructs, the coding sequence for mCherry was first amplified from pmCherry-ATG5 (Addgene) using primers IspmCherry2 Fwd/Rev. The amplified mCherry sequence was then directly cloned using BamHI/SacI restriction sites into pSPC18, a derivative of the pUC18 vector with the ampicillin resistance gene *bla* replaced by the spectinomycin resistance gene *aad9* [21, 22]. The final product (pLAV8510) contains a promoter-less mCherry lacking the start ATG and a 5' BamHI restriction site for direct cloning of the 3' ends of ORFs to create C-terminal tag protein fusions upon chromosomal integration. Amplification of a 500 nucleotide C-terminal fragment from *isp*, absent the stop codon and including a 3' HA-tag nucleotide sequence, using the primer pair Isp-HA Fwd/Rev followed by double restriction digest (BamHI/PstI) and ligation generated pSPC18:Isp-HA-mCherry (pLAV41312).

Cellular staining and fluorescent microscopy: Analysis of the location and integrity of a membrane microdomain enriched in anionic phospholipids was assessed by staining with sub-lethal concentrations of a fluorescent polymyxin B derivative (dansyl-polymyxin B, 10 μ M, cat. #P13238, Invitrogen). Samples were examined using a Leica model DM IRE 2 fluorescent microscope and images captured using a QImaging Retiga 1350 EX charged-coupled device camera and Openlab software (Improvision). Co-localization of Isp with fluorescent polymyxin B derivatives was quantitated as the percentage of polymyxin B -stained cells that exhibited focal staining with each individual reagent where the two foci were superimposable. Data presented for each condition represents the mean and standard error of the mean derived from at least 3 independent experiments and

examination of a minimum of 1000 stained cells. Images were processed for publication using Adobe Photoshop CS3.

Analysis of protein expression and secretion: Supernatant fractions from cultures challenged with polymyxin B were prepared and analyzed for the presence of EndoS by Western blotting as described [23]. Blots were developed using a Chemidoc XRS imager (BioRad) and relative protein concentrations determined using Quantity One software (BioRad, version 4.6.7). Antibody against EndoS was a generous gift from Mattias Collin (Lund University, Sweden).

Table 1. Primers utilized for construction of epitope/fluorescent-tagged constructs and in-frame deletions.

Primer	Sequence ¹	Description
IspmCherry2 Fwd	<u>CCCGGATCC</u> GTGAGCAAGGGCG AGGAG	5' amplification primer for mCherry
IspmCherry2 Rev	<u>CCC</u> GAGCTCCTACTTGTACAGCT CGTCCATGC	3' amplification primer for mCherry
Isp-HA Fwd	<u>CCCCTGCAGATAT</u> CATGACCAAA CTAGACCAAT	5' primer for insertion of HA epitope tag
Isp-HA Rev	<u>CCCGCATGC</u> <i>TTAAGCATAATCTGG</i> <i>AACATCATATGGATATGGTCTTGG</i> GAGTTTGTCCCC	3' primer for insertion of HA epitope tag
Spy_2026/Isp4 Fwd	<u>CCCCTCGAGTATCAAT</u> TCTCGAAG AACAATTCGTCAA	5' primer for amplifying the upstream flanking region of <i>isp</i>
Spy_2026/Isp Rev	<u>CCCGCATGC</u> TGTTACTGCTAACAATTCCTTTT CTTCAT	3' primer for amplifying the upstream flanking region of <i>isp</i>
Isp/Spy_2023 Fwd	<u>CCCGCATGCGT</u> CGTAGGGGACAA ACTCCCAAGACCATAA	5' primer for amplifying the downstream flanking region of <i>isp</i>
Isp/Spy_2023 Rev	<u>CCCTCTAGAT</u> GAGAAAACAGGGC TTTGGGTTCTATTTTG	3' primer for amplifying the downstream flanking region of <i>isp</i>
Spy_1802/Isp2 Fwd	<u>CCCCTCGAGGGT</u> TCTGATCTAAG CTTACCATTTC	5' primer for amplifying the upstream flanking region of <i>isp2</i>
Spy_1802/Isp2 Rev	<u>CCCGCATGCGG</u> CAACTCTTAATA GTTTGTTTTTATT	3' primer for amplifying the upstream flanking region of <i>isp2</i>
Isp2/Spy_1798 Fwd	<u>CCCGCATGC</u> AACATATGTTAT TGGCAAGAGTAAAAA	5' primer for amplifying the downstream flanking region of <i>isp2</i>
Isp2/Spy_1798 Rev	<u>CCCTCTAGAT</u> CTATCACTTTG TGATTATTTTGTCAA	3' primer for amplifying the downstream flanking region of <i>isp2</i>

¹Restriction sites underlined, Hemagglutinin (HA) tag italicized.

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Chapter V

**An Emerging Model for Spatial Coordination of Protein Biogenesis and Secretion:
the Role of Lipid Segregation and Cell Wall Synthesis in ExPortal Organization**

INTRODUCTION

Previous work discovered the co-localization of secretory translocons with an accessory factor for protein biogenesis at an anionic lipid microdomain, which defined the ExPortal as a secretory organelle dedicated to coordinating protein processing with secretion. My thesis research sought to further understanding of how such spatial coordination in the membrane is achieved and maintained, and what its relevance is to streptococcal pathogenesis. The experimental data resulting from this research constitutes a meaningful and exciting contribution to both of these aims.

Investigation of cationic antimicrobial peptide (CAP) interaction with the ExPortal increased knowledge of an aspect of streptococcal pathogenesis that is not often examined. As effectors of innate immunity, CAPs have traditionally been considered to primarily act as membrane permeabilizing factors against which bacteria have little resistance. However, the work presented here demonstrates in a physiological model what has, for the most part, been investigated in model lipid membranes. That is, CAPs interact with the cytoplasmic membranes of bacteria via mechanisms that do not result in membrane permeabilization, but can have substantial effects on cell viability nonetheless. In *Streptococcus pyogenes*, CAPs influenced the secretion of virulence factors that have been demonstrated to be of great importance to pathogenicity, opening up a new area for investigation into how CAPs may participate in colonization of a human host by GAS.

The results presented also support a tentative model for ExPortal organization. According to this model, anionic lipids in the streptococcal membrane play a central role in the localization of secretory translocons and accessory factors in the membrane.

Segregation of these charged lipids to a microdomain in the membrane appears to be mediated by, or at least involve, interaction with the cell wall and with peptidoglycan production. Further testing of this model will include characterization of the involvement in ExPortal organization and function of cell division proteins required for directing cell wall synthesis and growth. This makes *S. pyogenes* an emerging model organism for examining the intersection of multiple cellular processes in the membrane of Gram-positive pathogens.

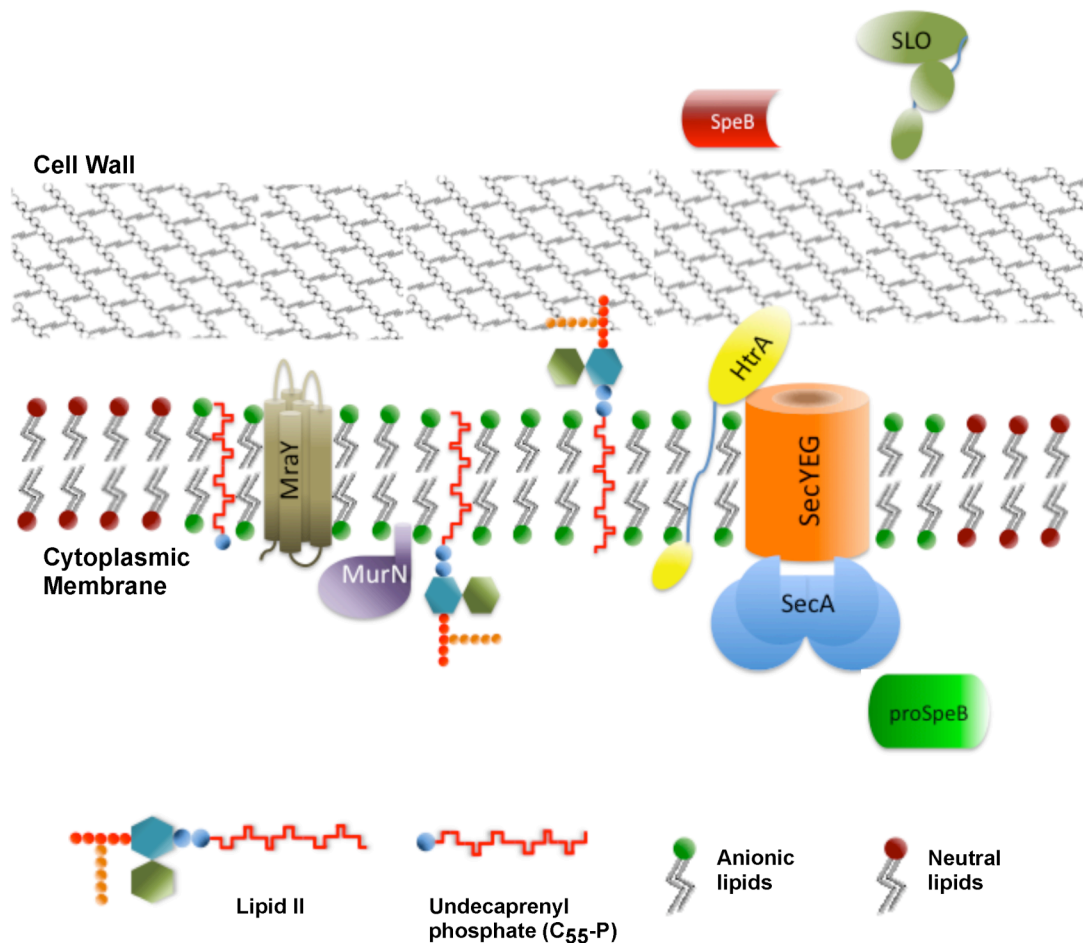


Figure. 1 **An emerging model of ExPortal organization.** Spatial coordination of anionic lipids, peptidoglycan biogenesis proteins and secretory translocons in the ExPortal to effect efficient secretion and processing of streptococcal virulence factors.

CONCLUSIONS

The model of ExPortal organization and maintenance supported by the experimental results presented can be described as follows and is schematically represented (Fig. 1). Interaction of anionic lipids with the Sec translocons, accessory factors (HtrA), and secreted substrates requiring post-translational processing (SpeB, SLO) appears to target and maintain these polypeptides at the ExPortal. I have shown that without segregation of anionic lipids to unique microdomains in the streptococcal membrane, targeting and maintenance of these ExPortal proteins at their cellular site is lost, meaning lipid segregation in the membrane is central to ExPortal organization. Thus segregation of anionic lipids, mainly phosphatidylglycerol, constitutes the primary mechanism for targeting protein components to the site of the ExPortal. Identification of the peptide sequences involved in the lipid-protein interactions localizing these proteins is therefore an area of interest for further research. The establishment and maintenance of anionic lipid microdomains is in turn dependent on the presence of extracellular peptidoglycan and its biogenesis. Production of the peptidoglycan precursor lipid II is localized to the same membrane site as the ExPortal, and loss of peptidoglycan disrupts anionic lipid and ExPortal protein localization in the membrane. Lipid II production at the site of the ExPortal may also be dependent on the presence of an anionic lipid microdomain to which membrane-associated lipid II-synthetic proteins are targeted. However, the observation that a lipid II-binding lantibiotic (gallidermin) disrupts ExPortal lipid and protein localization favors a mechanism whereby targeting of the peptidoglycan precursor influences lipid segregation in the membrane, rather than the converse. The experimental results presented support a model in which cell wall outside

the membrane restricts localization of anionic lipids by determining the site of lipid II synthesis. Without an intact cell wall in which PBPs assemble peptidoglycan, lipid II in the outer leaflet of the cytoplasmic membrane is deprived of acceptors for its glycopeptide cargo. This can prevent recycling of its prenyl-phosphate anchor and inhibit further lipid II production, thus disrupting ExPortal organization and inducing morphological defects if peptidoglycan synthesis is completely arrested (see Fig. 3 Chapter I). There is experimental precedent that supports such a model in the substrate recognition mechanism of PBP recruitment to division septa. A D-Ala-D-Ala carboxypeptidase (PBP3) of *S. pneumoniae*, encoded by the *dacA* gene also present in GAS, degrades the substrate of PBP transpeptidases by trimming the lipid II peptapeptide [1-3]. *S.pneumoniae* mutants disrupted in PBP3 display defects in division [4] and in co-localization of PBPs with the FtsZ ring [2]. PBP3 appears distributed over the whole cell surface except the mid-cell region before division starts [2], meaning pentapeptides are only available at equatorial peptidoglycan, where PBPs would be recruited to by affinity for their substrate. This cooperation between PBP substrate affinity and availability lends support to the hypothesis that the cell wall restricts localization of peptidoglycan synthesis through the activity of PBPs and the availability of sites for peptidoglycan polymerization in intact cell wall. According to this model then, the site at which cell wall synthesis primarily occurs determines where lipid II is produced and in so doing affects ExPortal localization. In summary, my thesis work has expanded our model of ExPortal organization, by demonstrating that anionic lipid segregation is central to targeting ExPortal proteins to their membrane site and that this lipid microdomain is maintained by the cell wall and depends on lipid II biogenesis localized at the ExPortal.

This model of ExPortal organization proposes that spatial coordination of protein secretion and processing results from cooperation of multiple cellular mechanisms in a highly interdependent manner. The individual contributions cell wall biogenesis, lipid segregation and protein interaction make are each necessary but not sufficient by themselves to either establish or maintain the spatial coordination of secretory translocons and accessory factors. This suggests further dissection of the specific roles each plays in ExPortal organization may prove difficult. However, it is that much more important for the discovery of highly effective antimicrobials, as it makes the ExPortal a useful target for the development of novel antibiotics that can disrupt multiple cellular processes.

Mechanism of CAP activity on the ExPortal. One aim of my thesis research was to investigate involvement of the ExPortal in streptococcal pathogenesis. Previous research suggested that CAP activity is an aspect of host immunity that would interact directly with the ExPortal. As described in Chapter I, the principal mechanism of CAP activity is the permeabilization of the cytoplasmic lipid membrane. Interaction of membrane lipids with multimeric protein complexes is central to the biological processes of membrane biogenesis, cell wall synthesis, and responding to environmental challenges (i.e. cell signaling, secretion of virulence factors). Integrity of the cytoplasmic membrane is therefore crucial to bacterial survival. Hence the prominence and effectiveness of CAPs as antimicrobials against which bacterial pathogens have limited resistance. The experimental data presented here indicate that CAPs affect their bacterial target in a manner other than by altering membrane permeability. A readily available CAP

(polymyxin B) and a physiologically important one (HNP-1) were shown to interact directly with the ExPortal and disrupt its organization and function in the absence of membranolytic activity. These findings contribute novel observations in a physiological model to emerging research that is elucidating the mechanisms of non-membranolytic interaction by which CAPs influence pathogens.

Several factors modulate how CAPs partition into membranes, the most important of which are membrane electrical potential, curvature strain of the membrane and hydrophobic interactions with the lipid acyl chains [5]. Electrical potential is dictated by the electrostatic interactions between the lipid headgroups and the peptides' charged residues. Cell membranes rich in lipids with a net negative charge at physiological pH, like phosphatidylglycerol (PG), cardiolipin (CL) or phosphatidylserine [6], tend to be highly electronegative, making them preferentially targeted for interaction with CAPs. Unlike unsaturated lipids that promote negative curvature strain in monolayers, PG generates no such strain given its cylindrical geometry. Interaction of CAPs with the acyl chains of lipid molecules occurs via van der Waals and hydrophobic interactions that can frustrate lipid packing in the membrane, inducing membrane thinning and/or lipid phase segregation. These properties of PG/CAP interaction explain the preferential localization of CAPs like polymyxin B and HNP-1 at the ExPortal. The electronegativity of an anionic lipid microdomain such as that of the ExPortal promotes interaction with the CAPs, and the reduced membrane strain in a region enriched for the cylindrical topology of PG facilitates insertion of the peptides into the bilayer and interaction with the hydrocarbon acyl chains.

Hydrophobicity and a net cationic nature are common to all CAPs however, but not all CAPs tested showed a propensity to affect ExPortal secretory function. One explanation is that varying degrees of hydrophobicity and net charge have great influence on the affinity of CAPs for membranes of varying composition. A study of diastereomeric peptides composed of varying ratios of lysine and leucine revealed that highly hydrophobic peptides had lytic activity against anionic and zwitterionic phospholipid vesicles alongside hemolytic and antibacterial activities, whereas highly cationic peptides only permeabilized acidic phospholipid vesicles and lysed bacteria [7]. Though composed of the same two amino acids in different proportions, these proportions had a marked effect on the activity of the synthetic CAPs. Given the enormous diversity in the amino acid sequences of CAPs, not all will target the ExPortal as effectively.

Binding to the streptococcal membrane was not directly examined in all the CAPs studied, thus it is possible that those CAPs that did not affect ExPortal secretory function do bind anionic lipids at the ExPortal, but their initial interaction with the membrane surface did not have any appreciable disruptive effects on its organization. This could be because these peptides do not exert any effects beyond a membranolytic activity on their bacterial targets or that the sub-lethal concentrations tested don't reflect the threshold concentration of the peptides under the experimental conditions used. Below this so-called threshold concentration CAPs fold and remain adsorbed parallel to the bilayer [8-11]. As the ratio of peptides to lipid increases, peptides orient perpendicular to the membrane [12], insert and partition into the hydrophobic core of the bilayer. Aspects influencing partitioning are peptide concentration, the propensity of a CAP to

oligomerize, composition of the membrane, fluidity, chemistry and size of the phospholipid headgroups, transmembrane electrical potential and pH [8, 11, 13-15]. Thus, CAPs that did not have disruptive effects on the ExPortal might not have achieved the threshold concentrations necessary to influence its organization or they had a propensity to only adsorb at the surface unless they achieved the threshold concentration that induces permeabilization of the membrane by one of the mechanisms described in Chapter I.

As described in chapter III, different lipid phases can coexist within a single membrane plane, delimited by a plane boundary, giving rise to lateral phase segregation and formation of the lipid microdomains observed in bacterial cytoplasmic membranes. The properties of these lipid phases determine the orientation, mobility and interaction of proteins and lipids contained therein and thus directly influence the biological functionality of the domains. Research using synthetic amphipathic α -helical peptides showed that formation of lipid phases can be influenced by peptides of this kind, which also exhibited antimicrobial activity [16]. Peptides affecting lipid phases have been observed to perturb the membrane by forming specific lipid-peptide domains, by lateral phase segregation of zwitterionic from anionic phospholipids and by inducing non-lamellar phases at physiologically relevant concentrations [5]. One demonstration of such modes of action was the observation of anionic phospholipids de-mixing in Gram-positive model membranes exposed to peptides with this kind of activity [17]. Peptide induced lipid segregation then appears to be the mechanism employed by those CAPs that have disruptive effects on the ExPortal. The activity of CAPs like polymyxin B and HNP-1, which target PG and/or lipid II localized in an anionic lipid microdomain of the

streptococcal membrane, most likely disrupts the physiological role of the ExPortal by causing lipid phase boundary defects through peptide-induced reorganization of membrane lipids. The possibility that polymyxin B and HNP-1 act on other or additional polypeptide targets to influence ExPortal organization and biogenesis of secreted factors requires further investigation as previously described in Chapter I and therefore cannot be entirely ruled out. However, the data presented here as well as investigation conducted in other bacterial organisms strongly suggests that lipid segregation by CAPs promotes dissolution of lipid-protein complexes in the membrane and abrogates their functionality.

Challenges to the ExPortal model of coordinated protein secretion and processing. A

great deal of data, published and unpublished, has been presented here to support a mechanism of localized protein secretion and maturation in *S. pyogenes*. However, experimental data has been published that disputes the spatial coordination of secretory translocons and accessory factors at a unique site in the streptococcal membrane. One study indicated that M protein and Protein F (SfbI) localized at the cell surface of GAS at distinct and separate sub-cellular regions: M protein directed at septa and SfbI at polar domains [18]. Swapping of N-terminal signal sequence domains between the two proteins switched their localization patterns, with SfbI observed at septa and M protein at polar domains [18], suggesting that differences in signal sequences is determinant in targeting of streptococcal factors to their extracellular site. Analysis of sortase-mediated anchoring of M protein and SfbI to their respective cellular surface locations revealed that the two proteins are anchored simultaneously throughout the cell cycle, with M protein rapidly anchored at the septum and SfbI accumulating gradually on peripheral peptidoglycan,

culminating in mainly polar localization at the old cell poles [19]. However, disruption of sortase function did not impede the appearance of M protein and SfbI at their respective cellular sites, suggesting that these secreted factors were not translocated at a shared, unique microdomain and then sorted to their extracellular site, but directly translocated at their site of localization [19]. Though these data appear to call into question the existence of an ExPortal for coordinated protein secretion and biogenesis, alternative mechanisms for this differential targeting of secreted proteins in GAS should be considered and investigated. For example, the data indicates that these proteins target differently on the streptococcal surface, whether by sortase-mediated anchoring or immediately following translocation. It is possible that the signal sequences, which as demonstrated by Carlsson et. al. are the determinants of localization, serve to segregate the secreted proteins in the membrane immediately following translocation and prior to the action of sortase-mediated anchoring to the cell wall, in order to efficiently distribute M protein and SfbI to their final surface destination. One experimental approach to determine if this is the case would be to swap signal sequence domains of M protein and SfbI with those of known ExPortal substrates (SpeB and SLO) to assess localization upon translocation. Efforts to directly tag the SecYEG translocon in the streptococcal membrane have thus far proven unsuccessful, but if accomplished, direct labeling of SecYEG in the cytoplasmic membrane will greatly help resolve these conflicting results.

FUTURE DIRECTIONS

Further characterization of ExPortal organization and refinement of this model will include examination of the role cytoskeletal and cell wall synthesis proteins play in ExPortal maintenance. Due to their ellipsoid shape and pattern of division in successive parallel planes perpendicular to the long cell axis, GAS are morphologically described as ovococci along with other streptococci and enterococci [20]. Ovococci synthesize the cell wall primarily at the division site, with new hemispheres of the daughter cells synthesized between the old hemispheres of the parent cell, as demonstrated by labeling of *S. pneumoniae* cells mostly at mid-cell with fluorescent-vancomycin [21]. Ultrastructural, mutational and drug treatment studies in streptococci indicate that PBPs are arranged into two synthetic machineries co-localized at a single cellular site [22, 23]. One machinery synthesizes peptidoglycan at mid-cell on the inner face of the cell wall concomitant with splitting of the new wall, resulting in the observed initial phase of longitudinal growth. [20]. This is followed by activity of the second PBP machinery which builds the septal cross-wall ahead of septal splitting of the wall in order to produce the cross-wall observed in ovococci. High molecular weight (HMW) PBPs localize mid-cell at the site of FtsZ ring formation at the onset of cell division, suggesting a mechanism of FtsZ triggered cell wall synthesis [20]. Thus, most activity of PBPs in ovococci involves polymerization of peptidoglycan during cell division-associated growth.

One hypothesis of interest for future work to test then, is that the multimeric cell division machinery (divisome) of streptococci is involved in localizing the ExPortal, most likely by determining the site of peptidoglycan synthesis. As mentioned in Chapter III, GAS lack cell wall elongation-associated divisome proteins (RodA, MinCD), but express

other peptidoglycan synthesis-related divisome factors: DivIB, DivIC, DivIVA, and EzrA. DivIB and DivIC are bitopic membrane proteins with major extracellular domains that in other ovococci are often encoded in operons along with genes involved in lipid II synthesis [20]. In *S. pyogenes* DivIB is encoded directly downstream of a lipid II synthase gene (*murG*) and immediately upstream of a Z-ring accessory factor gene (*ftsA*), while DivIVA is encoded a not very far downstream of the latter [24]. In *S. pneumoniae* and *E. faecalis*, loss of DivIVA expression produces aberrant cell shapes, incomplete septa and cells devoid of nucleoid [25, 26] and in *S. aureus* DivIVA has been shown to primarily localize at division septa [27]. The integral membrane protein EzrA regulates timing and positioning of FtsZ rings in *B. subtilis* [28] and can directly bind to and regulate assembly of FtsZ polymers *in vitro* [29]. EzrA is also required for recruitment of PBP1 to the cell division site in *B. subtilis* as part of the septal wall synthesis machinery [30]. While not essential in *S. aureus*, EzrA is important for regulation of cell size and is likewise required for localization of PBP1 [31].

Given all this experimental evidence of the close relationship between divisome proteins and cell wall synthesis in ovococci like GAS, one approach to examine the role of said proteins in ExPortal organization would be to generate in *S. pyogenes* mutants disrupted in DivIB, DivIVA and EzrA. Attempts to generate in-frame deletion mutants as well as targeted disruptions of DivIB, EzrA and its associated PBP (PBP1) have thus far been unsuccessful. A method of targeted gene disruption using synthetic inducible riboswitches, developed by Topp *et al.* [32] and applied in GAS by Bugrysheva *et al.* in controlling expression of an essential gene [33], is an alternative approach to consider for generating the desired disruption of EzrA, DivIB and PBP1. Examining localization of

these proteins and the effect of their disruption on the distribution of lipid and proteinaceous ExPortal components constitutes an important next step in understanding organization of this secretory organelle.

Given the diversity and ubiquity of CAPs throughout all realms of life, it should come as no surprise that bacterial pathogens have developed resistance mechanisms against them. As described in Chapter I, a great deal of research has gone into identifying and characterizing these mechanisms in order to develop therapeutic strategies that enhance the inherent ability and efficiency of CAPs at killing pathogens. One avenue of research that resulted from the investigation of CAP interaction with the ExPortal undertaken in this thesis work was the development of a screen to isolate spontaneous mutations rendering *S. pyogenes* impervious to the inhibitory effect of CAPs on ExPortal-mediated protein secretion. The aims of such a screen are to further understanding of host-pathogen interactions by dissecting how GAS coordinate the protein secretion and processing that is central to their pathogenesis and to gain insight into how streptococci might overcome the action of host antimicrobials. Initial characterization of mutants isolated in this screen has validated the experimental approach by identifying mutants encoding between 1 and 4 single nucleotide polymorphisms that map to molecular pathways involved in secretion, lipid biosynthesis and protein processing. Efforts to recreate the identified mutations by directed mutagenesis and characterize their phenotypes are currently underway and show great promise.

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APPENDIX

(Research published as part of the co-authored manuscript: Rosch, J.W., Vega, L.A., Beyer, J., Lin, A., Caparon, M.G., *The signal recognition particle pathway is required for virulence in Streptococcus pyogenes*. **Infect Immun**, 2008. 76(6): p. 2612-9)

DISSERTATION AUTHOR'S CONTRIBUTION

My contribution to this manuscript included the following. I aided in assessing the requirement of the SRP for the secretion of streptococcal virulence factors (M protein, SPN, SLO and SpeB), the results of which are published as Figure 3. I also determined the expression levels of the virulence factors examined above by real time RT-PCR, which are detailed in Table 1. I carried out analysis of the medium-dependent SpeB secretion defect in the SRP mutant illustrated in Figure 4 and undertook characterization of the mutant strain's pathogenicity in the murine subcutaneous ulcer model, the results of which are published as Figure 6. Finally I aided in writing and editing the manuscript for publication. Aside from page numbering and margins, formatting of this manuscript was not altered from the published version for the purpose of inclusion in this dissertation.

**The Signal Recognition Particle Pathway is Required
for Virulence in *Streptococcus pyogenes***

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Running title: SRP pathway protein secretion and streptococcal virulence

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ABSTRACT

The signal recognition particle (SRP) pathway is a universally conserved pathway for targeting polypeptides for secretion via the co-translational pathway. In particular, the SRP pathway is thought to be the main mechanism for targeting polypeptides in Gram-positive bacteria, including a number of important human pathogens. Though widely considered to be an essential cellular component, recent advances have indicated this pathway may be dispensable in Gram-positive bacteria of the genus *Streptococcus* under *in vitro* conditions. However, its importance for the pathogenesis of streptococcal disease is unknown. In this study, we investigated the importance of the SRP pathway for virulence factor secretion in the human pathogen *Streptococcus pyogenes*. While not essential for viability *in vitro*, SRP mutants demonstrated a carbohydrate-specific growth defect. We also observed that a distinct subset of virulence factors were dependent upon the SRP pathway for secretion, whereas others were completely independent of this pathway. Significantly, deletion of the SRP pathway resulted in mutants that were highly attenuated in both a zebrafish model of necrotic myositis and a murine subcutaneous ulcer model, highlighting the importance of this pathway *in vivo*. These studies emphasize the importance of the SRP pathway for the *in vivo* survival and pathogenesis of *S. pyogenes*.

INTRODUCTION

The important human pathogen *Streptococcus pyogenes* is able to establish infection and cause disease in a wide variety of tissues in the host including the pharynx (eg. “strep throat”) and the epidermis (eg. impetigo, erysipelas), as well as deeper tissues like the dermis, fascia and muscle (eg. cellulitis, necrotizing fasciitis, myositis). Crucial to the establishment of infection is the production of a multitude of virulence factors that are secreted across the single cellular membrane of this Gram-positive pathogen (14). Understanding how these various factors are trafficked to their appropriate destinations, including the streptococcal cell membrane, its cell surface, the extracellular spaces and host cells, will be important for elucidating the role of protein secretion in streptococcal virulence. An unexplored question is how intracellular routing of a nascent polypeptide contributes to its trafficking fate.

Protein secretion by Gram-positive bacteria has recently been revealed to be a complex, organized process with specific domains dedicated to protein secretion and folding (11, 42, 43). However, the pathways used to route presecretory proteins to the sites of translocation across the membrane are not well understood. As Gram-positive bacteria lack a discernable homolog of SecB, a chaperone involved in the post-translation targeting pathway, most polypeptides are presumably targeted co-translationally via the signal recognition particle (SRP) pathway (45). This is supported by the fact that most Gram-positive signal sequences tend to be longer and more hydrophobic than their Gram-negative counterparts, which are features thought to be important for efficient routing of the presecretory protein to the SRP pathway (12). Many studies in model bacterial species, including *Escherichia coli* and *Bacillus subtilis*, have highlighted the essential

nature of the SRP pathway for bacterial viability (25, 41). However, more recent studies of bacteria in the genus *Streptococcus* have indicated that SRP essentiality may not be a universal characteristic of all bacteria (23).

The SRP itself is a ribonucleotide-protein complex composed of a protein, the fifty-four homolog (Ffh) and an RNA known as the small cytoplasmic RNA (scRNA)(45). The SRP binds to signal sequences as they exit the ribosome and targets them to the bacterial membrane by binding the membrane-associated receptor, FtsY, which then releases the signal peptide to the Sec translocon (30). Recent cryo-electron micrographs revealed the details behind this process whereby the interaction between FtsY with the ribosome and Ffh results in a conformational change in the ribosome to promote binding to the translocation machinery (21). Crucial for proper function of SRP is the GTPase activity of both Ffh and FtsY, which is shared in a common catalytic chamber of this heterodimeric complex (17). The SRP receptor interacts directly with the SecYEG translocon, highlighting the close association between these two protein complexes (3).

Interestingly, deletion of the genes encoding Ffh, FtsY and scRNA singly or in combination in *Streptococcus mutans* is not a lethal event for growth on complex media (23). Under non-stress conditions, the SRP mutants have growth yields similar to wild type, although with somewhat longer doubling times during logarithmic growth (23). The SRP mutants do have a reduced capacity to resist certain stresses, most prominently osmotic and acid shock (20) and fail to form biofilm (24). However, a decreased resistance to stress is not a global phenotype as the mutants do retain an ability to adapt to acidic environments under conditions that more gradually expose them to decreased pH

(13). Also, their growth patterns are not altered in response to many other stresses including high and low temperature (23). The importance of the SRP for the pathogenesis of *S. mutans* disease remains to be determined.

Studies in *E. coli* have suggested that the SRP pathway is the major pathway for targeting secretion of integral membrane proteins, which typically lack cleavable signal sequences (44). Similarly, analysis of membranes of *S. mutans* SRP⁻ mutants demonstrated that approximately 17 proteins were either absent or were present at significantly reduced levels (24). This latter class included the β -subunit of the F₁F₀ ATPase, which is known to be a SRP substrate in other bacterial species (47). Since this proton pump plays a central role in the ability to adapt to acidic conditions (5), this defect may explain the enhanced sensitivity of these mutants to acid stress. Other proteins with altered membrane abundance included several glycolytic enzymes and LuxS, which is responsible for synthesis of an autoinducer known to be important for formation of streptococcal biofilm (46, 48). However, most of these proteins lack clearly identifiable signal sequences, so the global importance of the SRP for the trafficking of polypeptides with both cleavable and non-cleavable signal sequences in the streptococci is unknown.

The objectives of the present study were to examine whether the non-essentiality of the SRP can be generalized to other pathogenic streptococcal species, and if so, what role the SRP pathway may play in the secretion of virulence factors and whether the SRP is required for virulence. Our analysis of an Ffh-deficient mutant of *S. pyogenes* revealed that a distinct subset of virulence factors with cleavable signal sequences was dependent upon the SRP pathway for secretion. Furthermore, SRP⁻ mutants were highly attenuated in animal models of necrotic myositis and subcutaneous infection. These data provide

insight into the conservation of the SRP pathway function in the streptococci, and suggest that while it is not essential for growth, it is essential for virulence.

MATERIALS AND METHODS

Strains, media, culture conditions. Strains used included, *Escherichia coli* DH5 α and *S. pyogenes* HSC5 (22). Routine culture of *S. pyogenes* was at 37°C and employed Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco). Culture of *E. coli* utilized Luria-Bertanini (LB) broth at 37°C with shaking. Proteolytic assays employed strains grown in C-medium that were cultured under the conditions previously described (32). When required, antibiotics were used at the following concentrations: erythromycin, 750 μ g/ml for *E. coli* and 1 μ g/ml for *S. pyogenes*.

DNA and computational techniques. Plasmid DNA was isolated via standard techniques and used to transform *S. pyogenes* as described previously (10). Restriction endonucleases, ligases, and polymerases were used according to manufacturer's recommendations. The fidelity of all constructs derived by PCR were confirmed by DNA sequencing analyses. All references to genomic loci are based on the genome of *S. pyogenes* strain SF370 (15). Gene assignments were based on the information available in the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp), and were supported by subsequent interrogation of the SF370 genome using BLAST (2) and query sequences derived from *Bacillus subtilis* gene products with experimentally confirmed activities, as noted in the text.

Construction of Mutants. An in-frame deletion was constructed by an inverse PCR technique (31) to delete an internal fragment encompassing 500bp of the central portion of *ffh* (Spy1200). Allelic replacement proceeded as described previously (31), with confirmation of mutant genome structure by PCR using primers of the appropriate sequences. Primers for the deletion included Ffhdeletion1 AAC CTA GGG GGC CTA

TGG AAG ACC TCT TGA AAA TGA TTC CAG G and Ffh deletion 2 AAC CTA GGC AAA CCT TTT CTG ACA ATA TCG ACT GC. The resulting fragment of *ffh* has a deletion from amino acids spanning positions 180 to 340.

Cellular fractionation and preparation of membranes. Protoplasts were prepared as previously described (40) and then lysed by multiple freeze-thaw cycles (-80°C/37°C) followed by agitation with glass beads (106 µm, Sigma) using a reciprocating shaking device (FastPrep®, Q.biogene) at a speed setting of 4.5 for 45 seconds, repeated 4-5 times. Membranes were collected by centrifugation (120,000xg, 4°C, 60 minutes) and resuspended in distilled water. SDS sample buffer was added to the membranes followed by a 10 minute boil. Membrane fractions were then separated by SDS-PAGE, transferred to Immobilon-PSQ membrane, and stained with Coomassie Brilliant blue R. Protein identification was performed by N-terminal sequencing of Coomassie-stained bands (Midwest Analytical, St. Louis, MO).

Analysis and identification of proteins. Cell wall fraction, membrane, and cytosolic fractions were prepared from cultures as previously described (43). Cell free supernatants were subjected to TCA precipitation for protein concentration. Cell wall fractions from protoplast preparations were utilized for M protein analysis while culture supernatants were utilized for SpeB, SLO, and SPN. Other fractions were consistently negative for the presence of these proteins. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes. Blocking and antibody binding was done in PBS-T with 5% non-fat dry milk. Antisera against SLO and SpeB have been described previously (31, 35). An anti-peptide antiserum that recognizes multiple serotypes of M protein was generated as described (16). An antiserum that specifically reacts against SPN was generated using

purified SPN (C. Smith, M. Caparon, S. Hultgren; unpublished). Analyses of culture supernatant fluids for SpeB proteolytic activity and SLO hemolytic activity were conducted as described (32, 35).

Analysis of transcription. RNA from various streptococcal strains was isolated and analyzed as described previously (7, 29). Briefly, overnight growth in THY medium was diluted 1:100 in fresh THY medium followed by growth at 37°C to mid-logarithmic phase ($OD_{600} = 0.500$). RNA was then isolated by using glass beads (Lysing Matrix A, Q/BioGene) and a high-speed reciprocating shaking device (FP-120, Savant Instruments). RNA was further purified (RNeasy Mini Kit, Qiagen) and contaminating DNA was removed by DNase treatments according to the manufacturers instructions (RNase free DNase set, QIAGEN and DNase I, amplification grade, Invitrogen). The A_{260}/A_{280} ratio was used to determine the RNA concentration and purity. For cDNA synthesis, 5 μ g total RNA was treated with 200 U Superscript II Reverse transcriptase (Invitrogen) using 250 ng of oligonucleotide random primers (Invitrogen). Real time RT-PCR was performed employed an iCycler thermocycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) and the methods and primers described previously (29). Transcript abundance was normalized to *recA* as previously described (29) and data presented represent the mean and standard deviation derived from at least two independent experiments that were performed on different days, with each individual sample analyzed in triplicate.

Zebrafish infections. Bacterial strains were back-diluted 1:100 from overnight culture and grown in THY broth until $OD_{600}=0.30$. Bacteria were then subjected to brief sonication to disrupt bacterial chains and used for intramuscular injection of zebrafish, as described previously (39). Experimental groups consisted of 10 zebrafish, each of which

was injected with 10^5 CFU in a volume of 10 μ l or with an equivalent volume of sterile medium alone in the case of Mock infection. Survival was monitored for 5 days and any differences in survival between groups infected with wild type or mutant strains tested for significance as described (7). Data presented are pooled from 3 independent experiments, each of which was conducted on a different days.

Mouse subcutaneous infections. Bacterial strains were grown and prepared as outlined above, then used to infect mice subcutaneously as previously described (8). Experimental groups consisted of 5 mice, each of which received a subcutaneous injection into the right flank of 10^8 CFU in a volume of 100 μ l. Lesion development was monitored over a period of 96 hours and recorded as described elsewhere (8). Differences between groups in lesion area and in the number of mice developing an ulcer were tested for significance by the Mann-Whitney U test and the Chi-square test with Yates' correction, respectively (8). For all test statistics, the null hypothesis was rejected when $P < 0.05$. Data presented are pooled from two independent experiments that were conducted on different days.

RESULTS

Ffh is not essential in *Streptococcus pyogenes*. Disruption of the SRP pathway leads to a variety of phenotypes in the various bacterial species examined. In *E. coli* and *B. subtilis* deletion of Ffh is lethal (41). Furthermore, conditional depletion of Ffh and FtsY in *B. subtilis* results in a marked impairment of protein secretion (25), although the effect on the secretome may be more modest when the bacteria are cultured in rich media (49). In *Streptococcus mutans*, disruption of Ffh was first revealed in a screen for acid-sensitive mutants (19). It was subsequently shown that all components of the SRP pathway could be extensively disrupted in *S. mutans* with few phenotypic consequences apart from sensitivity to certain stresses (13). Interestingly, disruption of both the SRP and one of its two homologs of YidC (YidC2) resulted in *S. mutans* cells which were severely impaired in growth (23). We sought to ascertain whether SRP was also dispensable for the growth of *S. pyogenes* and the effect of SRP disruption on the secretion of several important virulence factors. An in-frame deletion allele of the gene encoding Ffh was constructed to remove 500 bp from the central portion of the Ffh open reading frame, which includes the region encoding conserved residues necessary for GTPase activity that are crucial for function (17). The resulting allele (*ffh*_{Δ180-340}) was successfully used to replace the wild type gene in *S. pyogenes* HSC5 (Fig. 1A) and the resulting mutants grew normally and produced colonies on THY agar plates that were indistinguishable from wild-type. Thus, similar to *S. mutans*, the SRP pathway is dispensable for viability of *S. pyogenes*.

A medium-specific growth defect. The method for allelic replacement generates a tandem duplication of wild type and mutant alleles that resolves by homologous recombination to produce strains carrying either the wild type or mutant allele (9). One Ffh^- mutant (FFH1) was chosen for further analysis and its ability to grow in liquid media under various nutrient conditions was examined when compared with a matched wild type revertant (WT_{rev}). When grown in standard THY broth, a complex medium rich in glucose (18) the mutant showed a modest defect in its growth rate, but obtained an equivalent growth yield as compared to wild type on the basis of culture densities (Fig. 1B). When grown in C-medium, a more minimal medium rich in peptides but low in glucose, the Ffh^- mutant presented a pronounced defect in both growth rate and yield, with cultures reaching a final density that was less than 50% of that obtained with a strain with the wild type gene (Fig. 1C). However, this defect could be rescued by the addition of additional glucose to C-medium, which produced a growth profile that was indistinguishable from that of THY medium (Fig. 1, compare 1D to 1B). Addition of NaCl (up to 150 mM) did not restore growth; however, buffering the pH of the medium to between pH 6.0 to pH 6.5 had the same effect as the addition of glucose, and restored growth of the mutant to levels identical to THY medium (data not shown).

Stress Response Activated. Membrane protein profiles of the wild type revertant and Ffh^- mutant were compared. Consistent with the relatively normal growth characteristics of the mutant in THY broth medium, these profiles were remarkably similar (Fig. 2). An exception was the presence of two bands that were consistently more intense in membranes of the mutant when compared with the wild type revertant. Subjecting these

bands to N-terminal sequencing revealed them to be DnaK and GroEL (Fig. 2). These proteins play prominent roles in several stress responses, particularly under conditions where misfolded proteins accumulate (38, 49). This observation suggests that despite near normal growth characteristics in this medium, that the mutant is accumulating some mis-folded proteins.

Selective defects in secretion of proteins with cleavable signal sequences. A prominent characteristic of *S. pyogenes* is its ability to secrete a large number of proteins during growth in culture and in tissue that have cleavable signal sequences. The fate of a subset of these proteins, including the M protein, is to become covalently associated with the cell wall (34). Others have well-characterized toxic activities and are known to have distinctly different trafficking fates when the bacterium is growing while attached to surface of host cells. For example, many secreted proteins, such as the SpeB cysteine protease, are trafficked into the extracellular spaces. At least one, the *S. pyogenes* NAD-glycohydrolase (known as Nga or SPN), is translocated across the host cell membrane into its cytosol by a process that involves a second secreted translocator protein, streptolysin O (known as SLO), that is itself delivered to the host cell membrane (33, 35, 37). Since M protein, SpeB, SPN and SLO have different trafficking fates, the consequence of the loss of Ffh on their secretion was assessed. Following its secretion via the Sec pathway, the signal sequence of the M protein is cleaved and then it is subsequently processed at a site towards its carboxy-terminal end and covalently cross-linked to peptidoglycan by the enzyme sortase (4). Examination of cell walls revealed that the amount of M protein in the mutant was as similar or lower than the amount of M

protein in the wild type parental strain (Fig. 3) indicating that Ffh is not essential, for the secretion of M protein and its delivery to the sortase pathway. In contrast, the loss of Ffh had marked effects on the secretion of SPN and SLO. Analysis of culture supernatants revealed that SPN and SLO were undetectable even after the proteins in these supernatants were concentrated by TCA precipitation (Fig 3). Additionally, culture supernatants from Ffh⁻ mutants lacked detectable SLO hemolytic activity and SPN NAD glycohydrolase activity (Table 1), confirming a defect for secretion of these proteins. The genes encoding SPN and SLO are co-transcribed from a common promoter (36) and the failure to observe the secreted forms of either protein was not due to a defect in transcription from this promoter as analysis of the *slo* and *spn* transcript by real time RT-PCR at the time of maximal expression in logarithmically-growing cultures indicated that it was as abundant in the mutant as it was in the wild type revertant (Table 1). A similar analysis of the transcript levels of the gene encoding M protein at a time point corresponding to the period of its maximal expression also showed no differences between the Ffh⁻ mutant strain in comparison with the wild type revertant (Table 1). No intracellular pool of SLO was detected when the mutant was subjected to lysis at this time point (data not shown), suggesting that the polypeptide was rapidly degraded in the absence of secretion.). Taken together, these data show that the SRP pathway does make a critical contribution to secretion of at least a subset of the exported virulence factors of *S. pyogenes* that possess cleavable signal sequences.

Medium-dependent effect on secretion of SpeB. The absence of Ffh had a different effect on secretion of SpeB, as it was observed that the Ffh⁻ mutant secreted the SpeB

polypeptide at levels higher than those observed for the wild type revertant during growth in THY medium (Fig. 3). However, this increased level of SpeB protein did not result in greater expression of protease activity in the Ffh⁻ mutant, as SpeB-dependent cysteine protease activity in culture supernatant from the mutant was somewhat reduced from levels observed for the wild type revertant (Table 1). This phenotype was medium-dependent, as the supernatant fluids from the Ffh⁻ mutant lacked both detectable cysteine protease activity (data not shown) and detectable levels of the SpeB polypeptide following culture in the glucose-deficient C medium, which is a medium that supports robust expression of SpeB by the wild type revertant (Fig. 4). This defect was not due to a failure of the mutant to express SpeB, as the intracellular fraction of the mutant contained detectable levels of the SpeB polypeptide (cytoplasmic, Fig. 4). Glucose has a repressive effect on expression of SpeB (28, 29) and as expected, supplementing C media with glucose resulted in repression of SpeB expression in the wild type revertant (compare WT_{rev} supernatant, C medium + Glc to C medium, Fig. 4). However, the addition of glucose resulted in increased SpeB expression in the Ffh⁻ mutant, as levels in the supernatant fraction for both the SpeB polypeptide (C medium + Glc, Fig. 4) and cysteine protease activity (data not shown) were elevated to the levels observed in the wild type revertant in unsupplemented C medium. Similar to their effects on growth in C medium (see above), buffering to pH 6.5, but not the addition of NaCl, resulted in enhanced levels of SpeB in the supernatant fluids of the mutant (data not shown).

The SRP pathway is required for pathogenesis in a zebrafish model of necrotic myositis. Since the Ffh⁻ mutant had a both a medium-specific growth defect and a

selective deficiency in its ability to export several toxins, it was of interest to determine if the mutant was also altered in its ability to cause disease. The virulence of the Ffh⁻ mutant was evaluated in a zebrafish model of necrotic myositis that reproduces several features commonly observed in streptococcal infection of human muscle, including local growth in muscle tissue, extensive necrosis of the infected tissue and diminished inflammation (39). Infection of zebrafish with the wild type revertant at approximately 10-fold the LD₅₀ produced characteristic survival curves in which greater than 90% of infected zebrafish did not survive past day 3 post-infection (Fig. 5). Also, all fish infected with the wild type revertant demonstrated a characteristic discolored lesion at the site of infection apparent 24 hrs post-infection that reflects the extent of the underlying necrosis in muscle (39) and all zebrafish that developed a lesion did not survive. In contrast, greater than 95% of zebrafish infected with the Ffh⁻ mutant were still viable at Day 3 (Fig. 5) demonstrating that the loss of the SRP rendered *S. pyogenes* significantly less virulent ($p < 0.0001$). Although not as intense or of equivalent area as in zebrafish infected with the wild type revertant, approximately 25% of the zebrafish infected with the mutant did develop lesions by Day 2, and these animals typically survived and their lesions resolved. Lesion development had not been associated with any of the attenuated mutants analyzed previously (8, 39).

The SRP pathway is required for pathogenesis in a murine model of subcutaneous infection. Virulence of the Ffh⁻ mutant was also examined in a murine model of subcutaneous infection, in which challenge with *S. pyogenes* HSC5 has been observed to cause visible ulcer-like lesions following injection into subcutaneous tissue (8).

Consistent with previous reports, all mice injected with the wild type revertant had visible lesions by 48 hrs post-infection, while most mice infected with the Ffh- mutant failed to develop any evidence of a visible ulcer even following 72 (Fig. 6) and 96 hours of examination (data not shown), demonstrating that the mutant was significantly less virulent ($P < 0.02$). A subset of mice infected with the mutant did develop ulcers (Fig. 6). However, the wild type revertant caused lesions that were significantly larger in size compared with the mutant when compared at a time when lesion formation by the wild type revertant had reached a maximum (72 hrs, $P < 0.005$, Fig. 6) and at subsequent time points over the duration of the experiment (96 hrs). These data provide additional evidence to suggest that Ffh play an important role in *S. pyogenes* virulence.

DISCUSSION

These data suggest that the non-essentiality of the SRP pathway may be a general feature of the streptococci. However, the SRP does contribute to a number of important functions of *S. pyogenes*, including the ability to efficiently utilize carbohydrates for growth, to secrete several proteins involved in host cell-pathogen interactions, and to cause disease in multiple animal models of streptococcal infection. The fact that the SRP is not required for growth should allow more refined molecular studies of this important pathway for targeting presecretory proteins that should provide insight into the pathway in other bacterial species where the SRP is essential. Further analyses of the SRP pathway in *S. pyogenes* should also be useful for understanding how targeting pathways may influence the trafficking fate of secreted proteins during infection.

While it is clear that the signal sequence is important for recognition by the SRP, the specific features of the signal sequence that dictate whether the nascent presecretory protein will be routed to the SRP pathway or to another pathway are not clear. Signal sequences themselves canonically have 3 regions, including an amino-terminal region enriched in positively charged residues (N region), a central region consisting of hydrophobic residues (H region) and moderately polar carboxy-terminal domain (C region) (Fig. 5). Studies in *E. coli* have suggested that while it is not an exclusive signal, that the probability of SRP recognition of any one signal peptide does correlate with its degree of hydrophobicity (26, 27). Preliminary results from our analysis using the computational method of Huber et al (26) failed to identify any correspondence between hydrophobicity and whether the signal sequences of the 4 protein analyzed in this study were routed to the SRP pathway. A complication is that the longer hydrophobic regions

typical of Gram-positive signal sequence generally renders them quite hydrophobic overall. The signal sequences of SLO, SPN, M protein and SpeB are unremarkable in this regard and have H regions encompassing 17-18 residues (Fig. 5). It has also been suggested that the presence of glycine residues in the H region can destabilize formation of an alpha-helix that results in an inability to interact with the SRP (1). An examination of the four streptococcal signal sequences shows their H regions all contain multiple glycine residues (Fig. 5). Thus, there are no obvious characteristics of these signal sequences that distinguish between their routing pathway.

Evidence suggests that an important function for the SRP pathway is in the insertion of integral membrane proteins (44). Since nutrient transporters typically include an integral membrane component, the loss of a transporter could explain the medium-specific growth defect of the *S. pyogenes* Ffh⁻ mutant. As the growth and SpeB secretion defect could be reversed by the addition of glucose, it is possible that an important peptide transporter requires the SRP for its insertion into the membrane, making the mutant more dependent on carbohydrates for growth and the secretion of virulence factors. A differential dependence of nutrient transporters on the SRP pathway may also explain recent studies that shown that depletion of the SRP in *B. subtilis* results in a pronounced secretion defect when cells were grown in minimal media that was much less severe when cell were grown under rich media conditions (49). Likewise, the viable SRP null mutants of *S. mutans* are typically grown in complex rich media (23). An increased requirement for glucose could help to explain the *S. pyogenes* Ffh⁻ mutant's attenuated ability to cause disease. Analysis of the transcriptome during infection has shown that *S. pyogenes* experiences a significant starvation for carbohydrates in well-developed

necrotic lesions (29). This idea is supported by evidence that suggests that the nutritional environment experienced by *S. pyogenes* in a necrotic lesion is similar to the nutritional landscape presented by unsupplemented C medium during growth in culture (29) and this medium had the most restrictive effect on the Ffh⁻ mutant's growth and secretion of the several media evaluated.

Alternatively, the Ffh⁻ mutant's requirement for glucose for growth and for secretion of SpeB in C medium may reflect the complex transcriptional regulatory networks that control expression of SpeB. It is known that transcription of the gene that encodes this protease is highly regulated and sensitive to several different regulatory pathways, including those that respond to nutritional cues (29). For example, transcription of *speB* is repressed by a culture pH higher than pH 6.5, and glucose and NaCl concentrations of 100 mM and 140 mM or greater, respectively (29). Interestingly, the Ffh⁻ mutant expressed higher levels of SpeB, and hyperexpression was not sensitive to repression by glucose or pH. Also, while the mutant grew poorly in unsupplemented C medium, it did express SpeB, although failed to secrete the protease. A failure to secrete the protease could be a factor that contributes to poor growth in unsupplemented medium as SpeB proteolytic activity in the cytoplasmic compartment of the cell would likely be deleterious. In addition, since the effect of supplementation does not appear to be a repression of *speB* expression, but rather, a rescue of the secretion defect, it is possible that there is an accessory secretion factor required for high level secretion that is regulated by pH and glucose. Accessory factors secretion factors that promote high levels of secretion have been described for other Gram-positive bacterial species (for review, see Ref # 45).

Analysis of the trafficking of thioredoxin in *E. coli* has suggested that proteins exported by SRP-dependent signal sequences are those that have the ability to fold rapidly in the cytoplasm (26). If allowed to accumulate in the cytoplasm for any length of time, it is likely that these proteins would jam the translocons if an attempt was made to secrete them in a partially-folded state, a situation that can be by-passed by co-translational secretion (26). Support for this function for the SRP pathway comes from the observation that SPN depends on the SRP for secretion. As an NAD glycohydrolase, SPN is indiscriminate in its source of substrate and is toxic to both streptococcal and host cells. In fact, the ability of *S. pyogenes* to produce SPN is absolutely dependent on co-expression of an immunity factor known as IFS (36). This immunity factor acts as a competitive inhibitor of SPN's NAD substrate to ameliorate its toxicity (36). Interestingly, IFS resides exclusively in the streptococcal cytoplasm, suggesting that even if a few molecules of SPN go off-pathway and fold rapidly in the cytoplasm, the result is an inability to sustain viability. Secretory stress induced by partially folded proteins could also explain the fact that even under conditions where it is growing robustly, that the mutant appeared to be experiencing a stress response as evidenced by the enhanced association of DnaK and GroEL with membranes. A similar stress response has been observed in Ffh-depleted *B. subtilis* (49) and in *S. mutans* mutants (24).

Our analysis has contributed to the emerging picture that protein secretion by *S. pyogenes* and other Gram-positive bacteria is a complex and organized process. For example, *S. pyogenes* has been shown to have a distinct membrane microdomain dedicated to protein secretion known as the ExPortal (42), which may serve as a protein folding organelle by promoting the interaction of unfolded nascent secretory proteins

with accessory maturation factors (43). Secreted proteins that are substrates of the sortase pathway are not distributed randomly in the cell wall. Rather, the M protein first appears linked to the cell wall predominantly along septum, while Protein F first appears at higher concentrations at the cell poles (11). These events imply that there is considerable cooperation between the protein export and cell wall synthesis machineries and imply that intracellular routing signals play an important role in dictating trafficking fate. This notion is supported by the observation that exchange of signal sequences between M protein and Protein F alters how the proteins appear in the wall (11). Thus, while the SRP appears dispensable for growth of various species of streptococci, understanding how the SRP pathway contributes to the secretion process will be essential for understanding how protein secretion contributes to the pathogenic mechanisms by which this group of bacteria cause disease.

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FIGURES

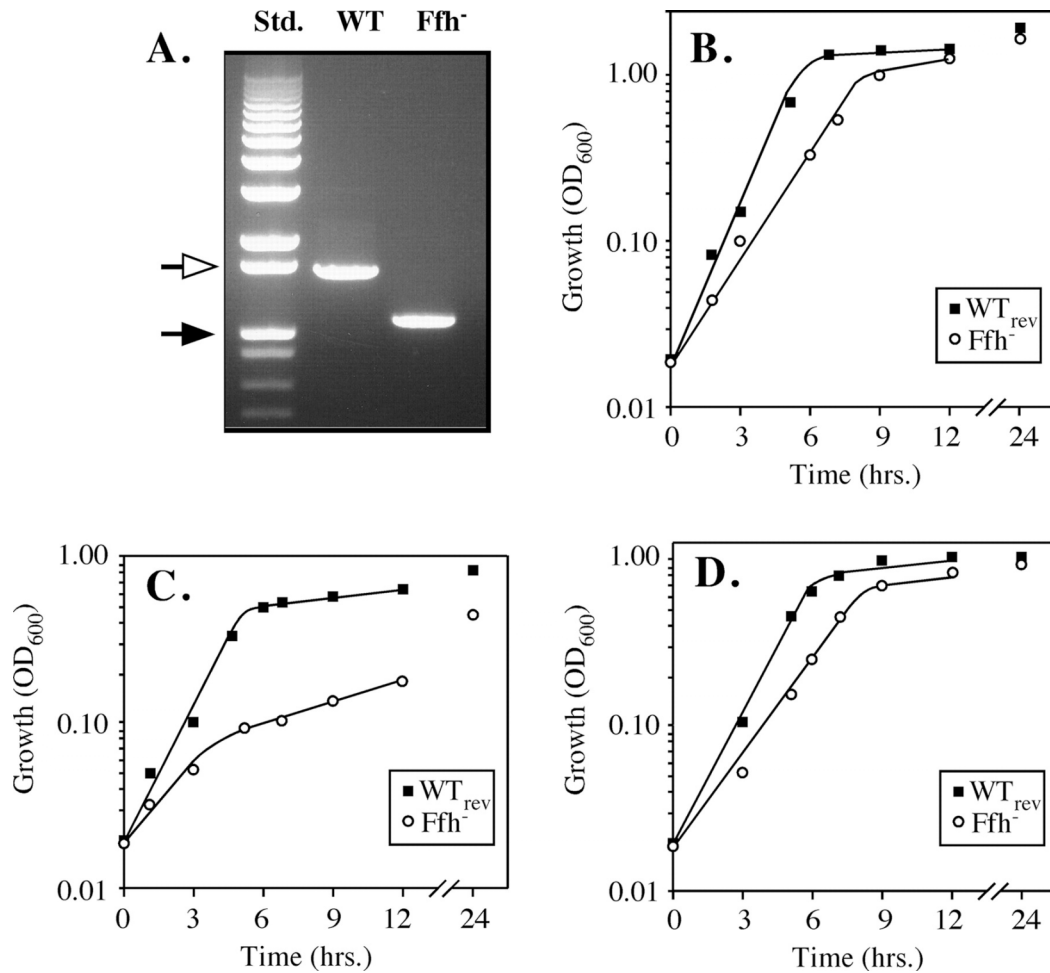


Figure 1. Deletion of *ffh* results in specific nutritional requirements. Successful replacement of *ffh* (*spy1200*) in the wild type strain with a deletion allele (*ffh*_{Δ180-340}) produced Ffh⁻ strain FFH1. (A) Analysis of PCR products generated using *ffh*-specific primers by agarose gel electrophoresis revealed bands of the expected sizes for the wild type (WT) and mutant (Ffh⁻) strains as shown. Open and closed arrows at the left of the DNA size standards (Std.) (TrackIt[™] DNA ladder, Invitrogen) indicate the 1650 and 1000 bp bands, respectively. The growth of the mutant (Ffh⁻) in liquid media was compared to a matched wild type revertant (WT_{rev}) using THY medium (B), C-medium (C) and C-medium supplemented with glucose (D). Data shown are from a single experiment representative of a minimum of three independent experiments, each of which was conducted on different days.

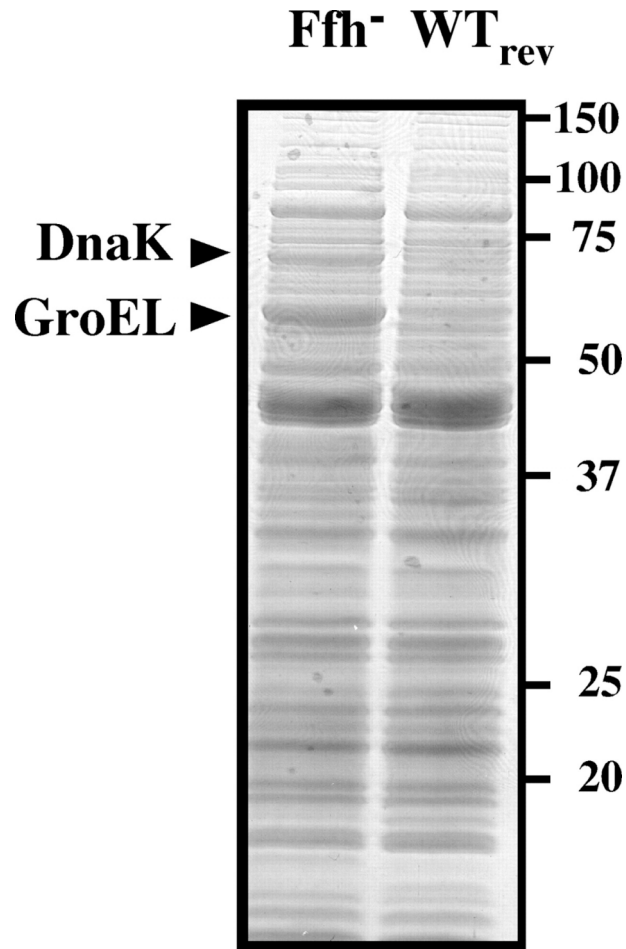


Figure 2. The majority of membrane proteins are correctly targeted in the absence of the SRP. Membranes purified from mutant (Ffh⁻) and a matched wild type revertant (WT_{rev}) were analyzed by SDS-PAGE and staining with Coomassie blue as shown. The identities of the bands labeled on the left of the Figure were determined by N-terminal amino-acid sequencing. The migration of several size standards is indicated to the right of the Figure.

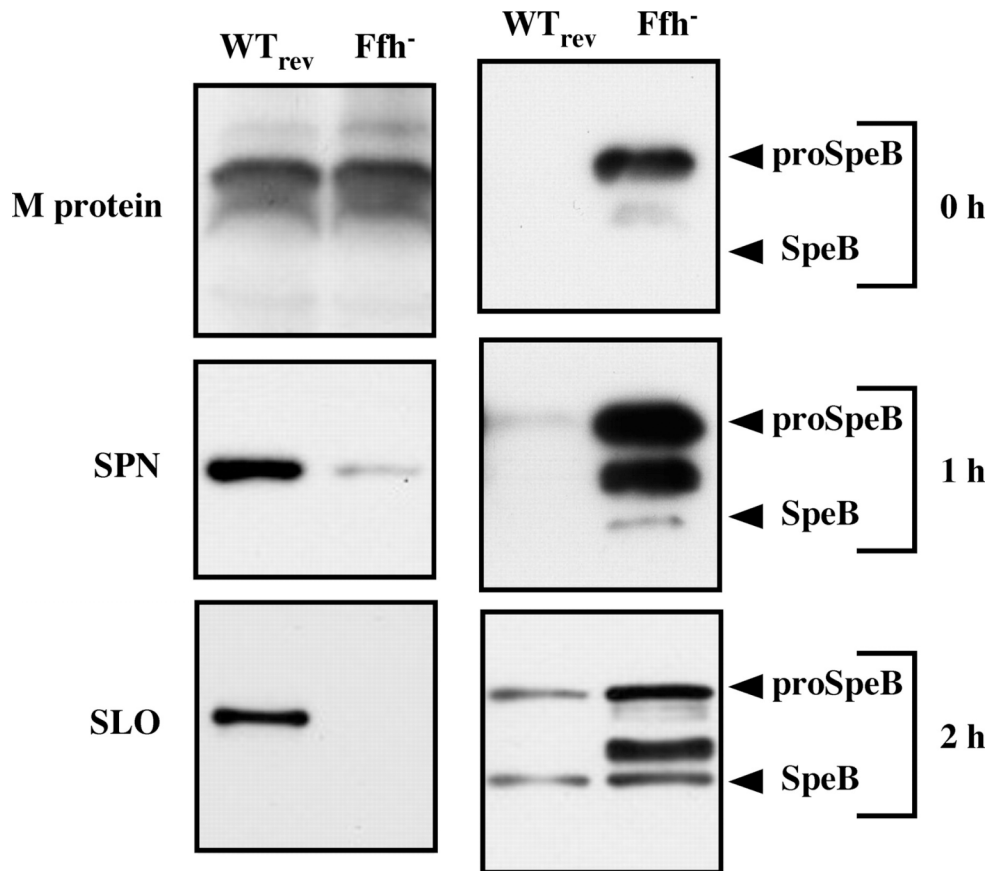


Figure 3. The SRP is required for the secretion of a distinct subset of virulence factors.

Analysis of the secretion of a selection of virulence factors that have both cleavable signal sequences and distinct trafficking fates during infection is shown. Included are the sortase pathway substrate the M protein, the SpeB cysteine protease that is released into the extracellular compartment, SLO that is trafficked to the host cell membrane, and SPN that is translocated into the host cell cytosol. Compared by Western blot analyses are the mutant (Ffh⁻) and a matched wild type revertant (WT_{rev}) using antisera specific for each indicated protein. Samples analyzed for M protein were cell walls from protoplast preparations, cell-free overnight culture supernatant fluids for SpeB, and cell-free overnight supernatant fluids that were concentrated by TCA precipitation for SLO and SPN. The migration of the zymogen (proSpeB) and mature (SpeB) forms of SpeB are indicated to the right of the SpeB panel.

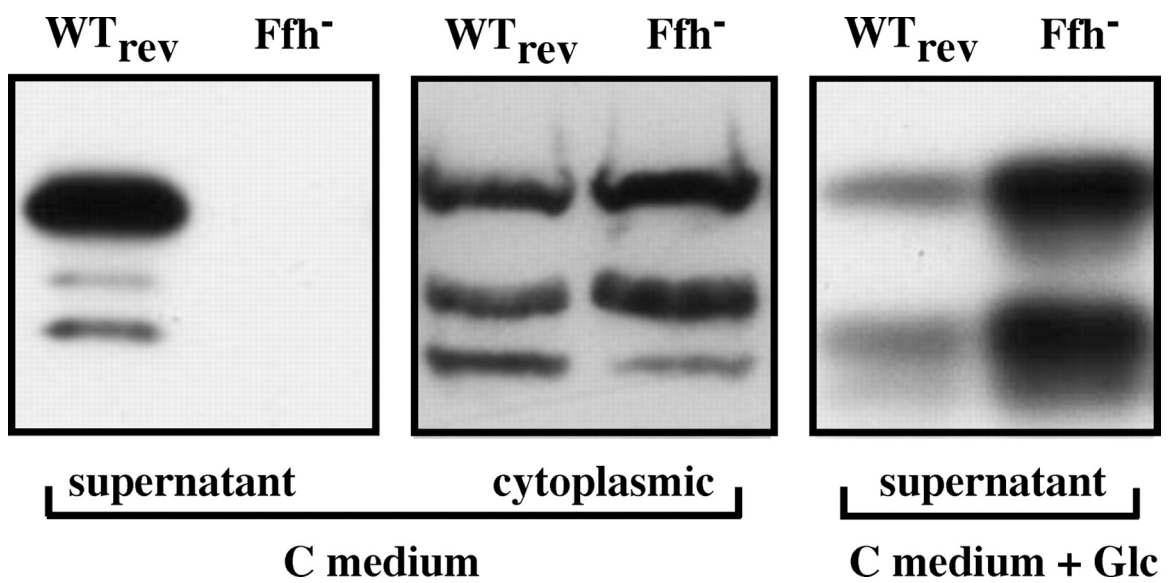


Figure 4. A medium-dependent secretion defect. A Western blot analysis of SpeB expression from mutant (Ffh⁻) and wild type revertant (WT_{rev}) following culture in C medium or C medium supplemented with glucose (C medium + Glc) is shown. As indicated in the Figure, both supernatant and cytoplasmic fractions were analyzed.

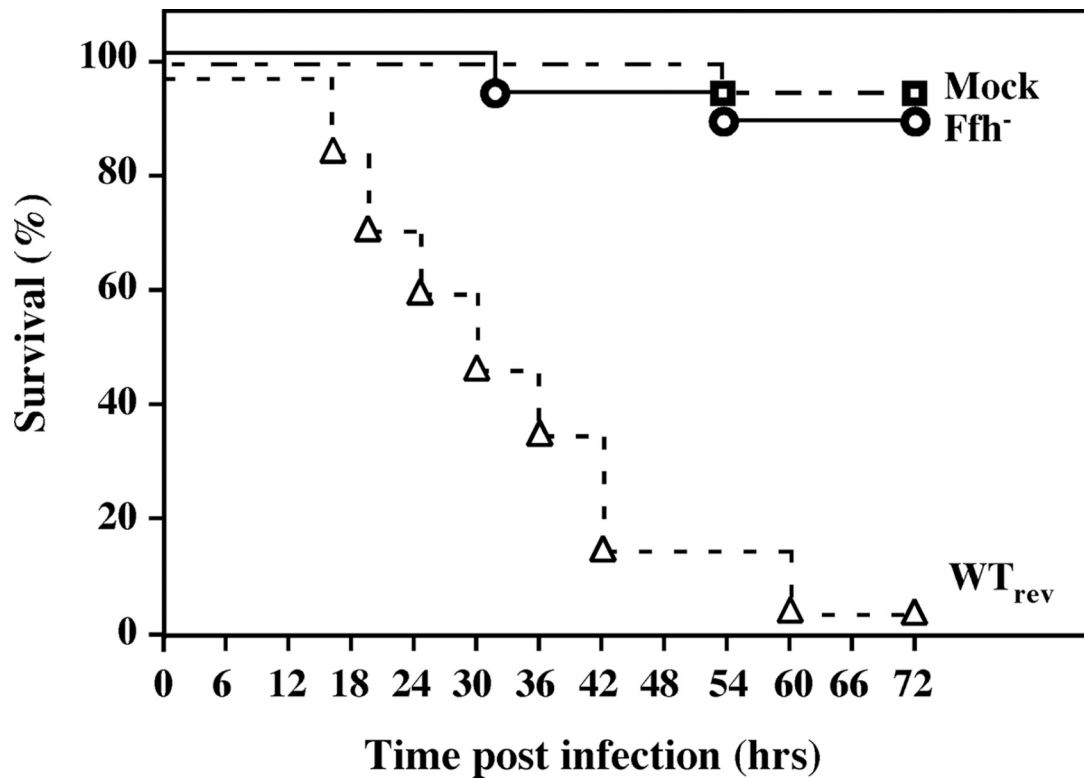


Figure 5. The SRP is required for virulence in an animal model of necrotic myositis. Groups of zebrafish were challenged intramuscularly with 1×10^5 CFU of mutant (Ffh⁻), a matched wild type revertant (WT_{rev}) or were injected with sterile media alone (Mock). Survival was monitored for 72 hours and the data presented as a Kaplan-Meier plot. Data are pooled from three independent experiments, each of which was conducted using 10 zebrafish per group. These data indicate that the mutant was significantly less lethal than the wild type revertant ($P < 0.0001$).

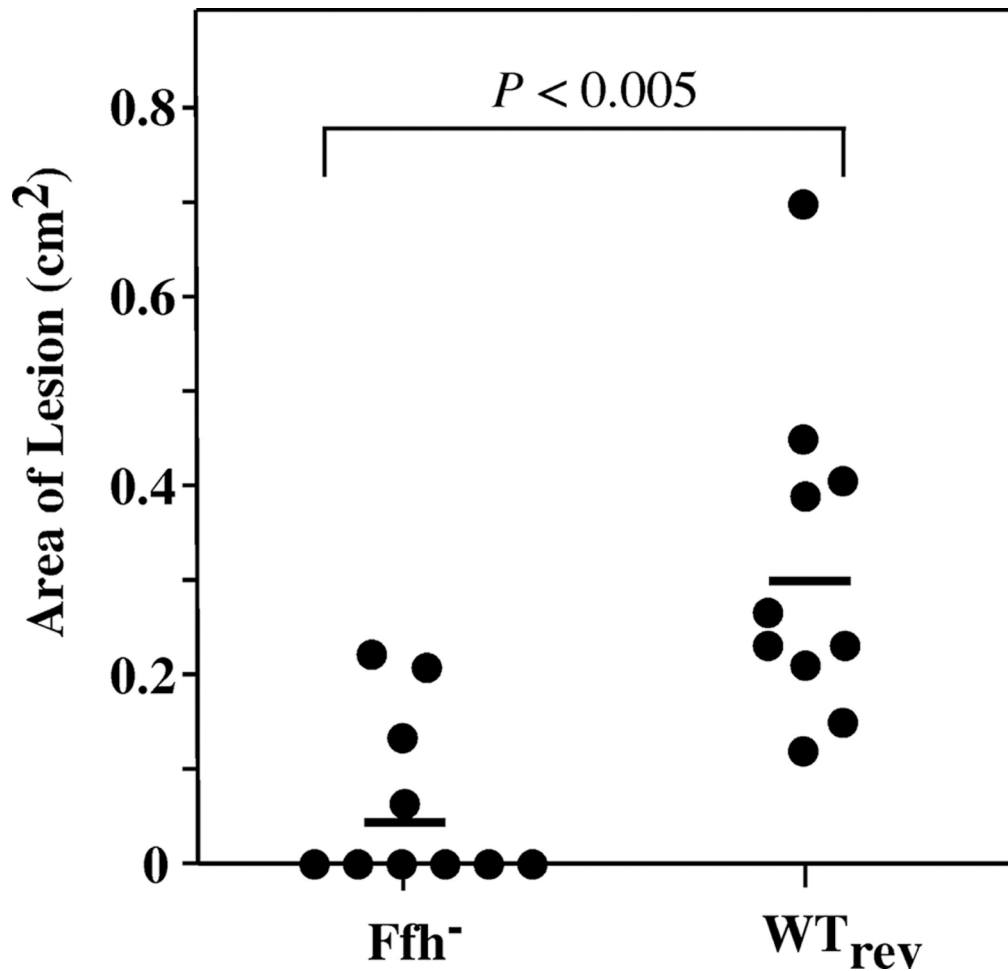


Figure 6. The SRP is required for virulence in the murine subcutaneous ulcer model. Groups of female SKH1 hairless mice received a subcutaneous injection of 10^7 CFU of mutant (Ffh⁻) or a matched wild type revertant (WT_{rev}) into the hind flank. Following 72 hrs, the area of any visible ulcer that formed at the site of injection was determined as described (8). Data shown are pooled from two independent experiments. Each symbol represents the area of the ulcer observed in an individual mouse, and the horizontal bar indicates the mean value obtained for the pooled set of 10 mice. As indicated in the Figure, lesions caused by the WT_{rev} were significantly larger than those caused by the Ffh⁻ mutant.

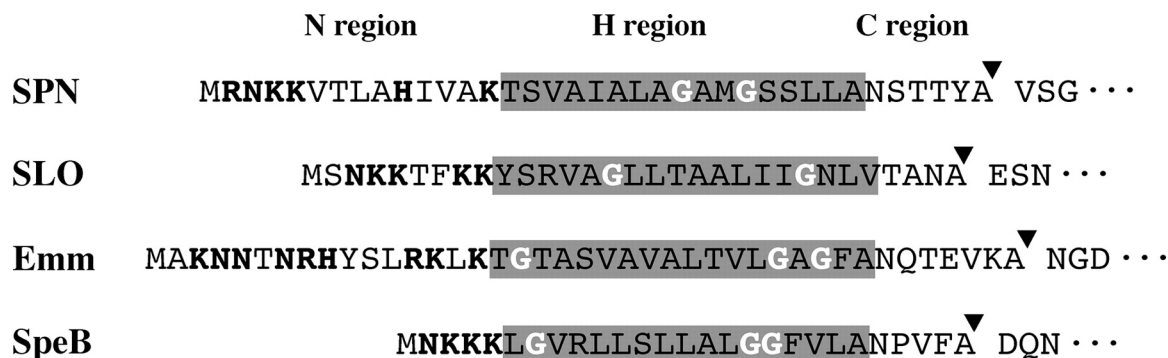


Figure 7. Comparison of virulence factor signal sequences. The N, H and C regions of the signal peptides of SPN (Spy0165), SLO (Spy0167), Emm1 (Spy2018) and SpeB (Spy2038) are shown. Positively-charged residues in the N region and the residues of the hydrophobic H region are indicated in bold type and by grey highlighting, respectively. Helix disrupting glycine residues in the H regions are indicated in white type. The signal peptidase cleavage site (indicated by the arrow) predicted computationally by SignalP, version 3.0 (6) agreed with the mature N-termini that have been experimentally determined and reported for each protein.

TABLE 1. Comparison of virulence factor expression levels between wild-type and Ffh⁻ *S. pyogenes* strains

Virulence factor	Growth phase ^a	Gene transcript abundance (ratio) ^b	Protein expression (%) ^c	Activity (%) ^d
M protein	Late log	0.78 ± 0.20	101 ± 22	NT ^e
SpeB	Late log	97.3 ± 84	ND ^f	NT
	Stat (<i>t</i> = 0 h)	2.0 ± 0.4	1,000 ^g	NT
	Stat (<i>t</i> = 1 h)	1.4 ± 0.8	1,464 ± 395	NT
	Stat (<i>t</i> = 2 h)	0.19 ± 0.1	928 ± 22	64 ± 3.4
	Late log	1.02 ± 0.12	ND	ND
SLO	Late log	1.02 ± 0.12	ND	ND
SPN	Late log	0.80 ± 0.24	11 ± 8.7	ND

^a Growth in THY broth was monitored by OD₆₀₀ for analysis at the stages noted, including the onset of stationary (Stat) phase (*t* = 0 h) and several subsequent time points.

^b Relative transcript abundance of the genes encoding the listed virulence factors was determined by real-time RT-PCR and is expressed as the ratio of Ffh⁻/WT_{rev}.

^c Relative protein expression was determined by densitometric analyses of Western blots and is expressed as the percentage of Ffh⁻ versus WT_{rev}.

^d Activities tested were as follows: for SpeB, cysteine protease activity; for SLO, hemolytic activity; and for SPN, NAD glycohydrolase activity.

^e NT, not tested.

^f ND, not detected.

^g The protein level in WT_{rev} was undetectable. A minimum estimate is presented.

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Publications:

Ullmann, BD, Myers H, Chiranand W, Lazzell AL, Zhao Q, **Vega LA**, Lopez-Ribot JL, Gardner PR, Gustin, MC. 2004. Inducible defense mechanism against nitric oxide in *Candida albicans*. *Eukaryotic Cell* 3, 715-23.

Chávez V, Mohri-Shiomi A, Maadani A, **Vega LA**, Garsin DA. 2007. Oxidative stress enzymes are required for DAF-16-mediated immunity due to generation of reactive oxygen species by *Caenorhabditis elegans*. *Genetics* 176, 1567-77.

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Vega LA, Caparon MG. 2012. Cationic antimicrobial peptides disrupt the *Streptococcus pyogenes* ExPortal. *Molecular Microbiology*. Epub ahead of print 2012 Jul 11.

Vega LA, Port GC, Caparon MG. 2012. The Streptococcal Cell Wall and Peptidoglycan Synthesis are Involved in ExPortal Organization. In preparation.

Awards and Honors:

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Grants and Fellowships

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Meetings and Symposia

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St. Jude Children's Research Hospital National Graduate Student Symposium (NGSS), Memphis, TN (March 20-23, 2012)