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An Association Between Peptidoglycan Synthesis and Organization of the *Streptococcus pyogenes* ExPortal

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ABSTRACT The ExPortal of *Streptococcus pyogenes* is a focal microdomain of the cytoplasmic membrane that clusters the translocons of the general secretory pathway with accessory factors to facilitate the maturation of secreted polypeptides. While it is known that the ExPortal is enriched in anionic lipids, the mechanisms that organize the ExPortal are poorly understood. In the present study, we examined the role of the cell wall in organizing and maintaining the ExPortal. Removal of the cell wall resulted in a loss of ExPortal focal integrity accompanied by the circumferential redistribution of ExPortal lipid and protein components. A similar loss occurred upon treatment with gallidermin, a nonpermeabilizing lantibiotic that targets the lipid II precursor of peptidoglycan synthesis, and this treatment disrupted the secretion of several ExPortal substrates. Furthermore, several enzymes involved in the membrane-associated steps of lipid II synthesis, including MraY and MurN, were found to localize to a single discrete focus in the membrane that was coincident with the focal location of the secretory translocons and the anionic lipid microdomain. These data suggest that the ExPortal is associated with the site of peptidoglycan precursor synthesis and that peptidoglycan biogenesis influences ExPortal organization. These data add to an emerging literature indicating that cell wall biogenesis, cell division, and protein secretion are spatially coorganized processes.

IMPORTANCE Since Gram-positive bacteria lack a periplasmic space, they lack a protected compartment to spatially coordinate interaction between newly secreted proteins and the factors required to process them. This represents a significant problem for pathogens that depend on the secretion of toxins and cell wall-associated adhesins to cause disease. Streptococci solve this dilemma by restricting secretion and processing factors to a defined region of the membrane. However, the mechanisms that promote restriction are not understood. In this study, we show that restriction of these factors in the pathogen *Streptococcus pyogenes* is intimately linked with the presence of the cell wall and its synthesis. Furthermore, several cell wall synthesis proteins are also restricted to the site of protein secretion. This study contributes to our understanding of how the Gram-positive cell is organized to coordinate protein secretion and biogenesis with cell wall synthesis and to the ongoing development of antibiotics that target these processes.

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Coordination of the biogenesis of secreted proteins can present a significant challenge to Gram-positive bacteria. One problem is that because these bacteria lack a periplasmic space, they lack a specialized compartment that can facilitate the interaction of nascently secreted polypeptides with accessory folding and processing factors. One solution found in many streptococcal species is to cluster translocons and accessory factors together in a focal microdomain in the cytoplasmic membrane known as the ExPortal (1–3). However, the mechanisms underlying the organization of this organelle are still poorly understood.

The ExPortal has been most extensively studied in *Streptococcus pyogenes*, the causative agent of diseases that range from largely superficial infections of the skin and mucous membranes (impetigo, pharyngitis), to highly invasive diseases (necrotizing fasciitis) and serious postinfection sequelae (rheumatic fever, glomerulonephritis; reviewed in reference 4). The ability of *S. pyogenes* to

cause disease depends on the secretion of an extensive network of virulence proteins (4, 5). Since its genome lacks any alternative secretion system (6), it relies on the general secretory pathway for protein export (for a review, see reference 7).

One feature of the *S. pyogenes* ExPortal that may play a key role in organization is its asymmetric lipid content, enriched in anionic phospholipids (8), that contributes to the retention of certain proteins at the ExPortal versus the peripheral membrane (2). Among the proteins that are retained are several that function in the biogenesis of virulence proteins, including sortases and HtrA. The former are involved in the biogenesis of pili and the covalent attachment of proteins to the cell wall (1, 2, 9), while the latter is involved in the maturation of several secreted proteins (10, 11). Mutations that mislocalize HtrA or sortase reduce the efficiency of both pilus (2) and SpeB (11) biogenesis. In the case of sortase, mutations that reduce the density of positively charged residues on the cytoplasmic tail of its single transmembrane domain result

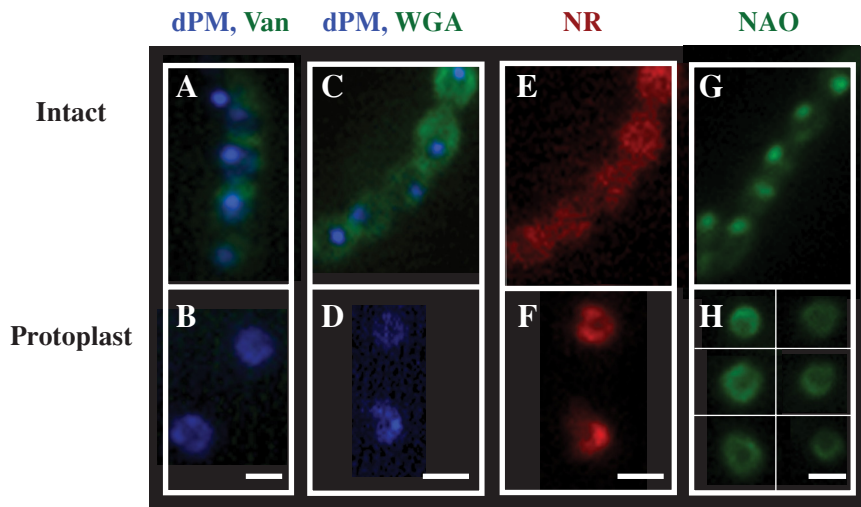


FIG 1 Peptidoglycan is required for maintenance of the ExPortal anionic lipid microdomain. Intact cells (top row) or protoplasts (bottom row) of *S. pyogenes* were examined by fluorescence microscopy following staining with dansyl-polymyxin B (dPM) and FL-vancomycin (Van) (A, B), dansyl-polymyxin B and WGA, (C, D), Nile Red (NR) (E, F), and NAO (G, H). The cells in panels A and B are from the same PlyC-treated culture. The cells in panels C, E, and G and those in panels D, F, and H are from untreated and PlyC-treated cultures, respectively. Panels C and E show different fluorescence channels of the same stained cells, as do panels D and F. The thin lines in panel H separate images assembled from different fields. The fluorescence from each probe is pseudocolored in micrographs as indicated at the top. Probes were used at the following concentrations: dansyl-polymyxin B, 10 μ M; FL-vancomycin, 1.0 μ g/ml; WGA, 5 μ g/ml; Nile Red, 2.5 μ g/ml; NAO, 20 μ M. Scale bars, 1.0 μ m.

in mislocalization (2), indicating charge interactions with the anionic microdomain in ExPortal organization.

The importance of the anionic character of the ExPortal is further illustrated by its interaction with several cationic antimicrobial proteins (CAPs). These proteins preferentially bind to anionic lipids and have been found to target the *S. pyogenes* ExPortal at nonpermeabilizing, sublethal concentrations. Binding then acts to disorganize the localization of anionic lipids, HtrA, and the SecA ATPase in the ExPortal microdomain (12). Furthermore, disruption is associated with inhibition of the secretion of several toxins (12), supporting a role for the ExPortal in the coordination of secretion-related events.

Interestingly, one of the CAPs revealed to interfere with ExPortal organization, human neutrophil peptide 1, has also been shown to target lipid II in bacterial membranes (13). This molecule plays an essential role in cell wall synthesis and is responsible for linking the cytoplasmic synthesis of the peptidoglycan monomer unit to its extracytoplasmic polymerization reactions (reviewed in references 14 and 15). Many CAPs target lipid II to form pores that efficiently permeabilize the bacterial cell membrane (for a review, see reference 16). However, a subset including the lantibiotic gallidermin are not efficient at forming pores and promote lethality by disrupting cell wall synthesis (17–19). These act through an alternative mechanism, sequestering lipid II into patches of the membrane away from the regions where peptidoglycan synthesis occurs (17–19).

Lipid II is also the substrate for cell wall sorting, in which a protein secreted by the Sec pathway containing the appropriate sorting signal is covalently attached to the peptide moieties of lipid II by sortase. Subsequent transglycosylation and transpeptidation reactions then incorporate this product into the maturing cell wall (reviewed in reference 20). Emerging evidence indicates that secretion and sorting of virulence factors are parts of a highly organized process coordinated with the site of *de novo* peptidoglycan

synthesis (1, 2, 9, 21). These data suggest that peptidoglycan biogenesis and ExPortal-mediated secretion may be spatially linked processes.

In the present study, we examined the relationships among the streptococcal cell wall, peptidoglycan synthesis, and the ExPortal. We report that the cell wall is required for maintenance of the proper localization of both lipid and protein components of the ExPortal. Furthermore, proteins responsible for the membrane-associated steps of lipid II synthesis localize to foci that coincide with the anionic lipid microdomain. Together, these data reveal an intimate association between cell wall synthesis and ExPortal organization.

RESULTS

Peptidoglycan is required for organization of the ExPortal anionic lipid microdomain. 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 lysin of logarithmic and stationary-phase streptococcal cultures resuspended in either raffinose- or glycerol-supplemented medium removed detectable surface peptidoglycan from the majority of streptococci, as monitored by the breakdown of streptococcal chains into individual protoplasts and the absence of cell wall staining by fluorescent vancomycin (FL-vancomycin) or wheat germ agglutinin (WGA) (Fig. 1). In treated cultures, the minority of cells that retained their cell wall, as evidenced by staining with FL-vancomycin, also displayed the focal staining pattern of labeled polymyxin B (Fig. 1A) characteristic of cells from untreated cultures (Fig. 1C) (12). However, protoplasting coincided with the redistribution of labeled polymyxin B from unique foci to a more uniform circumferential pattern, giving the protoplasts a “halo” appearance (Fig. 1B and

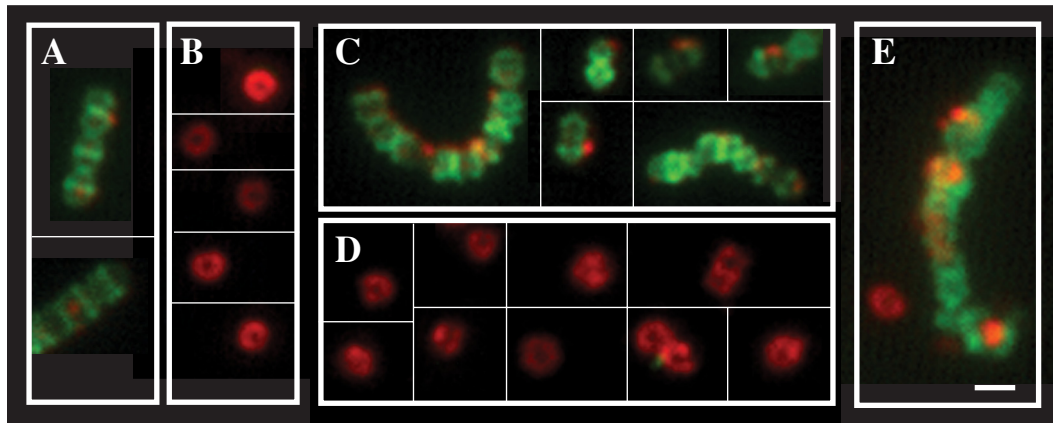


FIG 2 Redistribution of ExPortal proteins following removal of peptidoglycan. The distribution of several ExPortal-localized proteins on intact cells and protoplasts of *S. pyogenes* was examined by immunofluorescence microscopy as follows: HtrA, intact cells (A), protoplasts (B); SecA, intact cells (C), protoplasts (D). Also shown are an intact chain of cells and a protoplast from a PlyC-treated culture stained with anti-HA antibody to detect HtrA-HA (E). All cells were costained with WGA (5 $\mu\text{g}/\text{ml}$) to detect the cell wall. The thin lines in panels A to D separate images assembled from different fields. Immunofluorescence is red, and WGA staining is green. Scale bar, 1 μm .

D). The circumferential labeling pattern of polymyxin B in protoplasts also resembled that of the neutral lipid-binding dye Nile Red (Fig. 1F), showing that in the absence of cell wall peptidoglycan, the integrity of the anionic lipid microdomain of the ExPortal is lost and anionic lipids are uniformly distributed in the membrane, in a manner similar to that of neutral lipids in untreated cells (Fig. 1E). The redistribution of anionic lipids was confirmed with the fluorescent dye 10-*N*-nonyl-acridine orange (NAO). In intact streptococci, NAO staining revealed the unique anionic lipid microdomains described previously (Fig. 1G) (8, 12), whereas protoplasts displayed the same “halo” staining pattern observed with fluorescent polymyxin B (Fig. 1H). It is unlikely that redistribution of polymyxin B and NAO in protoplasts is due to increased accessibility of the labeling agents to the cytoplasmic membrane, since cultures were washed to remove excess fluorescent label prior to protoplasting. Furthermore, redistribution of anionic lipids was not due to a perturbation of membrane integrity, as quantitation of polymyxin B-treated protoplasts with a fluorescent vital dye indicated that their membranes were not permeabilized (see Fig. S1 in the supplemental material).

Peptidoglycan is required for focal localization of ExPortal proteins. Previous work has shown that localization of ExPortal-associated proteins such as SecA and HtrA coincided with the anionic lipid microdomain of the ExPortal (8, 11) and that disruption of the microdomain by CAPs resulted in redistribution of these proteins (12). Examination of protoplasts by immunofluorescence microscopy revealed that removal of peptidoglycan led to a similar redistribution of HtrA from a focal (Fig. 2A) to a circumferential (Fig. 2B) pattern. The translocon ATPase SecA also redistributed from foci in intact cells (Fig. 2C) to either multiple foci or a more uniform circumferential pattern in protoplasts (Fig. 2D). Redistribution was not observed in the minor population of treated cells, where peptidoglycan removal was incomplete, as WGA-staining cells retained unique foci of both HtrA (Fig. 2E) and SecA (data not shown).

Gallidermin disrupts the ExPortal. The data above suggest that an intact cell wall is required for ExPortal organization. To examine the relationship between the ExPortal and the site of peptidoglycan synthesis, we first examined whether compounds

that disrupt cell wall synthesis also affect ExPortal organization and function. The lantibiotic gallidermin binds lipid II and promotes lethality primarily by arresting cell wall synthesis by sequestering lipid II (17, 22, 23). Consistent with prior reports (17, 23), gallidermin’s inefficient pore-forming activity was reflected by the higher concentration required to compromise *S. pyogenes* membrane integrity compared to that of nisin, an efficient pore-forming lantibiotic. Cell viability was lost at nisin concentrations of $>0.5 \mu\text{M}$ but was unaffected at $2.0 \mu\text{M}$ gallidermin (see Fig. S2 in the supplemental material). Cultures were then treated with a nonlytic concentration of gallidermin ($1.0 \mu\text{M}$), and several ExPortal-related phenotypes were monitored. Examination by fluorescence microscopy revealed that the typical focal staining pattern of a sublethal concentration of boron-dipyrromethene (BODIPY)–polymyxin B (Fig. 3A) was disrupted by treatment with a nonlytic concentration of gallidermin (Fig. 3B) and appeared similar to the pattern observed following exposure to ExPortal-disrupting amounts of polymyxin B (Fig. 3C) (12). Treatment with gallidermin also resulted in the redistribution of HtrA from single foci (Fig. 4A) to cells with multiple foci or a diffuse pattern (Fig. 4D). Costaining with dansyl-polymyxin B (Fig. 4B and E) revealed an overlap with HtrA foci in untreated cells (Fig. 4C) and diffuse HtrA staining in gallidermin-treated cells (Fig. 4F). These data suggest that interaction between gallidermin and lipid II promotes the disruption of ExPortal organization.

Gallidermin disrupts ExPortal-dependent secretion. Cationic antimicrobial peptides that disrupt the ExPortal can also inhibit the translocation of a subset of the toxins secreted by *S. pyogenes* (12). Treatment with a nonlytic concentration of gallidermin also reduced the levels of the SpeB cysteine protease and the streptolysin O (SLO) cytolysin in culture supernatants, as revealed by Western blot analysis (Fig. 5), similar to what has been observed with polymyxin B (12). This inhibition was not the result of a generalized defect in protein secretion and synthesis because (i) cells remained viable (see Fig. S1 in the supplemental material), (ii) levels of SecA in gallidermin-exposed streptococci were unaltered relative to those in untreated bacteria (Fig. 5), and (iii) *de novo* synthesis of a plasmid-encoded green fluorescent protein

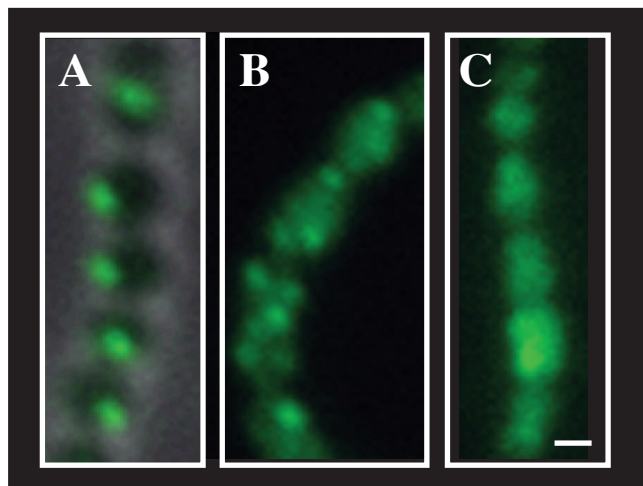


FIG 3 Gallidermin disrupts localization of anionic lipids at nonlytic concentrations. Cultures of *S. pyogenes* were challenged with a nonlytic concentration of gallidermin ($1.0 \mu\text{M}$), and the distribution of anionic lipids was assessed by staining with a nonanionic microdomain-disrupting level of polymyxin B-BODIPY conjugate ($10 \mu\text{M}$) and fluorescence microscopy. (A) Overlay of BODIPY-polymyxin B-stained and phase-contrast images of untreated cells. (B) BODIPY-polymyxin B-stained, gallidermin-treated cells. (C) Cells treated with a sublethal but anionic lipid microdomain-disrupting concentration of BODIPY-polymyxin B ($60 \mu\text{M}$). Scale bar, $1 \mu\text{m}$.

(GFP) was unaffected in untreated and treated cultures when its expression was induced from a tetracycline-regulated promoter at the time of gallidermin or polymyxin B exposure, as assessed by Western blotting and fluorescence microscopy (Fig. 5). Altogether, these results indicate that the lipid II-targeting activity of a lantibiotic can disrupt ExPortal function in the absence of significant membrane damage.

Several lipid II synthesis enzymes localize to membrane foci.

Given the restriction of lipid II to sites of cell division and the high affinity of lantibiotics for the pyrophosphate moiety of lipid II, lantibiotic-lipid II interaction likely occurs in proximity to the membrane site where lipid II synthesis takes place (16). To further examine whether peptidoglycan synthesis and ExPortal-mediated protein biogenesis are spatially coordinated processes, the localization of two enzymes involved in the membrane-associated steps of lipid II synthesis was monitored. MraY is a membrane-intrinsic protein that catalyzes the transfer of the phospho-*N*-acetylmuramoyl (MurNAc) pentapeptide from the soluble cytoplasmic UDP-MurNAc pentapeptide substrate to the membrane-embedded undecaprenyl-phosphate ($C_{55}\text{-P}$) acceptor to produce undecaprenyl-pyrophosphoryl-MurNAc pentapeptide (lipid I) (15, 24). MurN cycles between the cytoplasm and the inner leaflet of the membrane in order to attach *L*-alanine to the lipid II substrate. This represents the final synthetic step prior to the translocation of lipid II to the outer leaflet of the membrane (15, 24). Strains for this analysis were constructed by modification of the native chromosomal loci in order to produce alleles that expressed proteins with C-terminal fusions to superfolder GFP (sfGFP). Since the genes encoding MraY and MurN are essential (25) and the modified alleles represent the only copy of each gene in the genome, the observation that the resulting strains were viable and had no growth rate defect indicates that the fusion proteins (MraY_{sfGFP}, MurN_{sfGFP}) were fully functional. Consistent with

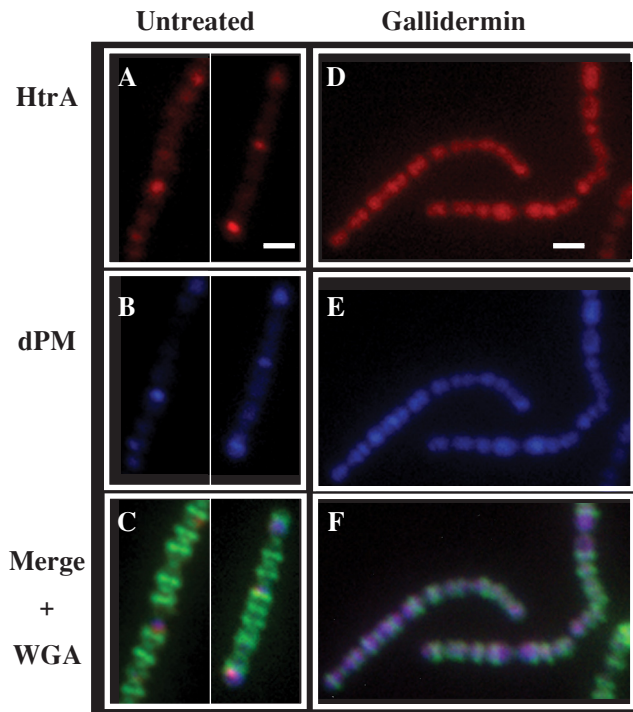


FIG 4 Redistribution of HtrA following a nonlytic gallidermin challenge. The distribution of HtrA in the *S. pyogenes* membrane was assessed by immunofluorescence microscopy in cultures untreated or challenged with gallidermin ($1 \mu\text{M}$) as indicated. Cells were stained with anti-HA antibody to detect HtrA-HA, dansyl-polymyxin B (dPM, $10 \mu\text{M}$), and WGA ($5 \mu\text{g/ml}$) as indicated. The bottom row shows images merged from all three fluorescence channels (Merge + WGA). The thin lines in panels A to C separate images assembled from different fields. Scale bars, $1 \mu\text{m}$.

their role in growth, peak expression of MraY_{sfGFP} and MurN_{sfGFP} occurred during the log phase of culture when examined by Western blotting (data not shown). When examined by fluorescence microscopy, MraY_{sfGFP} localized to unique foci in the membrane, most prominently during log phase (Fig. 6A). This focal pattern was confirmed when cells were examined by immunoelectron microscopy (Fig. 6A). Similarly, MurN_{sfGFP} also localized to unique foci when log-phase cells were examined by both fluorescence and immunoelectron microscopy (Fig. 6B), although some localization throughout the cytoplasm was also observed. The focal localization of MraY and MurN contrasted with that of a fusion protein based on the membrane protein YajC, which demonstrated a uniform circumferential distribution when examined by fluorescence and electron microscopy (Fig. 6C) that was unaltered by treatment with polymyxin B (Fig. 6F). Finally, since it has been reported that fusion to a fluorescent partner can alter the localization patterns of certain proteins (26), the localization of MurN was also examined following fusion to a small epitope tag (LFFF) developed for analysis of protein localization in streptococci (27). Immunofluorescence microscopy demonstrated that MurN_{LFFF} localized to unique focal sites (Fig. 6E) in a pattern indistinguishable from that of an LFFF-tagged version of HtrA (Fig. 6D).

MraY and MurN colocalize with the ExPortal. Since the pattern of localization of MraY and MurN resembled that of the ExPortal protein HtrA_{LFFF}, it was of interest to determine if these lipid II enzymes also localize to the ExPortal. Fluorescence mi-

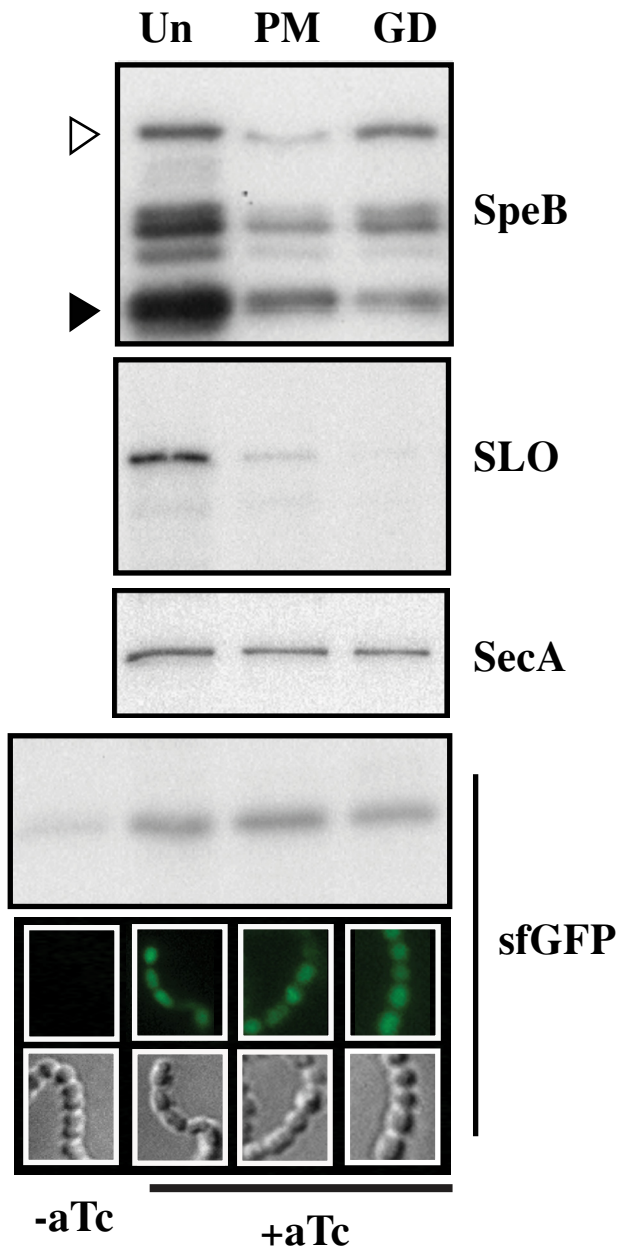


FIG 5 Nonlytic challenge with gallidermin inhibits secretion of SpeB and SLO. Secretion of the cysteine protease SpeB and SLO following a challenge with gallidermin (GD) or polymyxin B (PM) was assessed by Western blotting of culture supernatants and compared to that of an untreated (Un) culture as shown. Open and filled triangles indicate the migration of the zymogen and the mature form of SpeB, respectively. Also shown are Western blot assays to monitor SecA and expression of sfGFP from a plasmid-borne gene under the control of a tetracycline-regulated promoter in the absence ($-aTc$) or presence ($+aTc$) of induction by anhydrous tetracycline. Fluorescence and phase-contrast micrographs of GFP-expressing cells are shown. Cultures were challenged with gallidermin at $1 \mu M$ (SpeB, SecA) or $0.5 \mu M$ (SLO, GFP) and polymyxin B at $30 \mu M$ (SecA) or $45 \mu M$ (SpeB, SLO, GFP).

scopy revealed that the localization of MraY (Fig. 7A to C) and MurN (Fig. 7D to F) coincided with the focal site bound by dansyl-polymyxin B (12). Approximately 25 to 30% of the cells were observed to be colabeled, and $>90\%$ of these demonstrated coin-

cident labeling (Fig. 7I). Finally, the location of the lipid II synthetic enzymes was compared with that of SecA. Immunoelectron microscopy after costaining with antibodies conjugated to gold beads of different sizes revealed that the anti-SecA and anti-MurN_{sfGFP} conjugates recognized the same focal site on the streptococcal surface (Fig. 7G and H). These data indicate that synthesis of the peptidoglycan precursor and the site of polypeptide secretion are spatially coordinated.

DISCUSSION

The mechanism by which the cell wall supports ExPortal organization is unknown. However, the influence of the cell wall on lipid microdomains may be a feature shared with rod-shaped Gram-positive bacteria. For example, in *Bacillus subtilis*, anionic phospholipids are organized into discrete microdomains that adopt a spiral configuration relative to the longitudinal cell axis (28, 29). Removal of peptidoglycan or inhibition of lipid II synthesis results in the disruption of these spiral microdomains (30). This suggests that either (i) peptidoglycan synthesis is necessary for the establishment of specific lipid microdomains in *B. subtilis* or (ii) disruption of lipid II production causes the disassembly of existing microdomains (30). This also implies a link between the cell wall and protein secretion in *B. subtilis*, since it has also been shown that the anionic lipids are required for the organization of the Sec translocons into a longitudinal spiral pattern (31).

Segregation of anionic lipids may be a more general mechanism for organizing macromolecular complexes at membrane sites where their physiological activity is required. In *Caulobacter crescentus*, the organization of the cytoskeleton protein MreB to helical cables is dependent on lipid II (32). Furthermore, the organization of the MinD cell division protein into a helical spiral pattern is dependent on phosphatidylglycerol (33). Examination of cell wall synthesis in growing *B. subtilis* cells found that MreB and its isoforms assembled with cell wall synthesis holoenzyme components into discrete patches in the membrane that moved processively along peripheral tracks perpendicular to the cellular axis (34, 35). The motility of these complexes was driven by cell wall synthesis, and the MreB polymers both restricted the localization of patch components and directed their movement (34, 35). Together, these data point to a link between membrane lipid organization and the molecular machineries of peptidoglycan synthesis and cell division.

One mechanism by which peptidoglycan synthesis might influence membrane segregation is association of cytoskeletal components inside the cell with complexes for cell wall synthesis exterior to the membrane. This arrangement would place constraints on the distribution of anionic phospholipids in the intervening membrane in order to create and stabilize lipid microdomains. However, *S. pyogenes* lacks homologs to the cytoskeletal MreB proteins (6) and does not possess the synthetic machinery required for cell elongation, including RodA, MreC, and MreD (reviewed in reference 36). Rather, the macromolecular complex directing cell division and growth in *S. pyogenes* consists of the tubulin homologue FtsZ, several accessory divisome proteins (FtsA, FtsW, EzrA, DivIB, and DivIVA), and a set of high- and low-molecular-weight penicillin-binding proteins (36). Thus, while both *S. pyogenes* and *B. subtilis* segregate anionic lipids into microdomains, the mechanisms underpinning how peptidoglycan synthesis and cell division are integrated with membrane structure are likely to be fundamentally different.

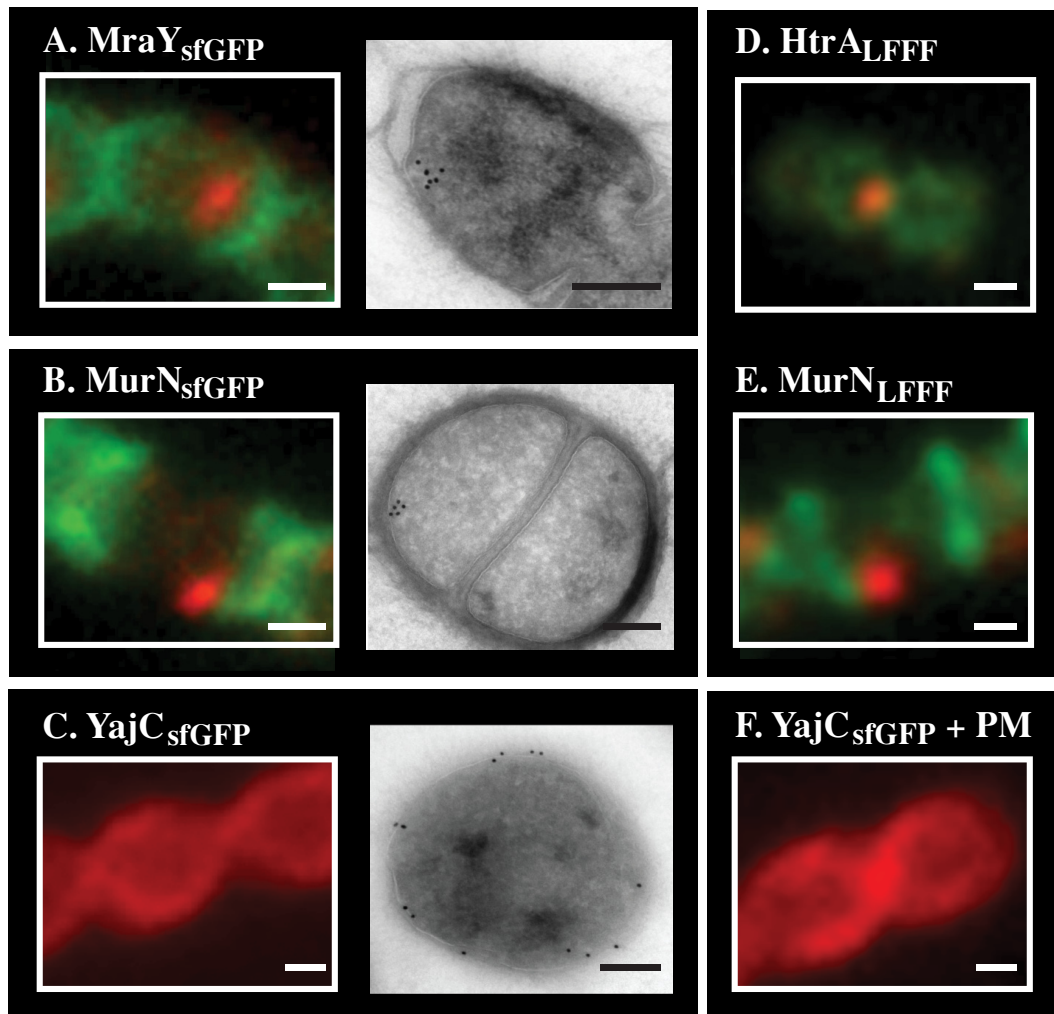


FIG 6 Focal localization of MraY and MurN. Localization of sfGFP-tagged MraY (A), MurN (B), and YajC (C) in the *S. pyogenes* membrane was assessed by fluorescence microscopy (left) and immunogold electron microscopy (right). The location of LFFF epitope-tagged versions of HtrA (D) and MurN (E) was monitored by immunofluorescence microscopy. The distribution of YajC in membranes following a challenge with an ExPortal-disrupting concentration of polymyxin B (PM; 45 μ M) is also shown (F). For all fluorescence images, the cells were counterstained with WGA. Micrographs are pseudocolored as follows: GFP and anti-LFFF, red; WGA, green. Scale bars, 0.25 μ m.

Our examination of the localization of YajC also highlighted other fundamental differences from *B. subtilis* in the composition of the Sec protein export machinery itself. In *B. subtilis*, YajC is maintained at the membrane by its single transmembrane segment and is considered an auxiliary subunit of the Sec protein translocase by virtue of its coexpression and interaction with SecD and SecF, although it is nonessential for both viability and secretion (37). However, *S. pyogenes* does not possess either SecD or SecF (6). Furthermore, exposure to polymyxin B at a concentration that alters anionic lipid and ExPortal structure did not alter the uniform distribution of YajC in the streptococcal membrane, suggesting that it is independent of the factors that influence ExPortal organization. Thus, the function of YajC in streptococci remains unknown.

In contrast to YajC, the localization of MraY and MurN may suggest a mechanism by which peptidoglycan can influence the formation of lipid microdomains. 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 (38). Depending on composition, structure, and environment, different phases can coexist within a single membrane plane, resulting in lateral phase segregation and the formation of microdomains. The properties of these lipid phases can determine the orientation, mobility, and interaction of proteins and lipids and thus directly influence the biological functionality of the domains (39). This suggests a role for the C₅₅-P anchor of lipid II in organizing lipid microdomains. Spatial constraint of C₅₅-P would create a microdomain of unique composition that may then direct the segregation of anionic lipids into microdomains. This mechanism would resemble how the spatial constraint of squalene/polyisoprenoid synthesis enzymes in *B. subtilis* acts to promote the formation of membrane microdomains with defined functionality (40). Significantly, the C₅₅-P membrane anchor of lipid II is a polyisoprenoid synthesized by the enzyme undecaprenyl pyrophosphate synthase (UppS) (41). Thus, the localization of UppS,

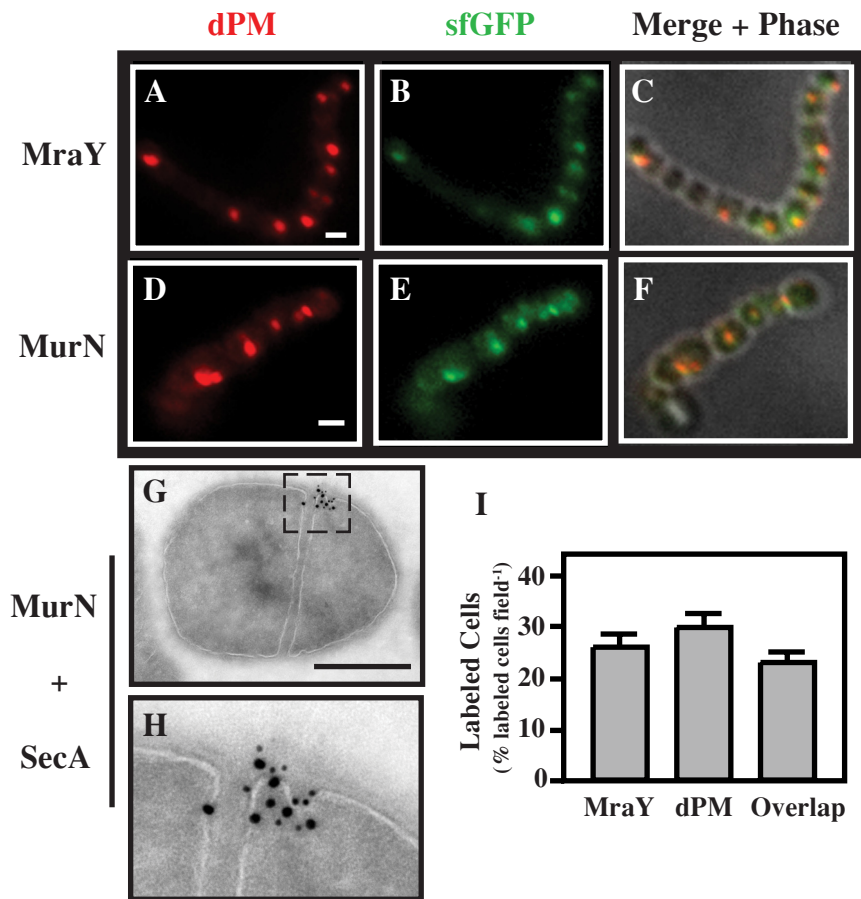


FIG 7 MraY and MurN localization is coincident with the ExPortal. Strains of *S. pyogenes* expressing MraY_{sfGFP} (A to C) and MurN_{sfGFP} (D to F) were stained with a concentration of dansyl-polymyxin B (dPM) that preferentially recognizes the ExPortal microdomain (10 μ M). Images are pseudocolored and merged with a phase-contrast image as indicated at the top. Scale bars, 1.0 μ m. (G) A strain expressing MurN_{sfGFP} was subjected to immunogold electron microscopy and stained to detect GFP (18-nm beads) and SecA (12-nm beads). Scale bar, 0.5 μ m. The boxed area is presented at 3-fold higher magnification in panel H. (I) The percentage of streptococci that exhibited labeling by sfGFP or dansyl-polymyxin B was quantitated, as was the percentage of labeled cells where focal labeling was coincident (Overlap). The quantitative data presented are means and standard errors of the means derived from at least three independent experiments by the examination of a minimum of 1,000 stained cells.

which in turn may involve the action of divisome proteins involved in determining the site of cell wall growth and septation, may be a key element promoting microdomain formation in *S. pyogenes*.

MATERIALS AND METHODS

Strains, media, and growth conditions. Experiments with *S. pyogenes* used strains HSC5 (M serotype 14) (42) and SF370 (M serotype 1) (6). For brevity, data presented are from HSC5 unless otherwise indicated, since identical results were obtained with both. For descriptions of these strains, see Table S3 in the supplemental material. Molecular cloning experiments used *Escherichia coli* DH5 α . Routine culture was at 37°C in Todd-Hewitt broth (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium) for streptococci and LB medium for *E. coli*. Functional assays used cultures grown in C medium without agitation in closed containers for liquid medium or under anaerobic conditions with a commercial gas generator as described previously (43). Antibiotics were used at the following concentrations for *E. coli* and *S. pyogenes*, respectively: kanamycin, 50 and 500 μ g/ml; spectinomycin, 100 and 100 μ g/ml; carbenicillin, 50 μ g/ml (*E. coli* only). In selected experiments, media were supplemented with polymyxin B (Sigma catalog number P0972-50MU), nisin (0.5 μ M; Sigma catalog number N5764), or gallidermin (1.0 μ M; a generous gift from James Smith, Texas A&M University) as described previously (12).

DNA and protein techniques. Plasmid DNA was isolated via standard techniques and used to transform *S. pyogenes* or *E. coli* as described previously (44). Restriction endonucleases, ligases, and polymerases were used according to the manufacturer's recommendations. The fidelity of all molecular constructs and mutated chromosomal loci was confirmed by PCR and determination of DNA sequences with oligonucleotide primers with the appropriate sequences. The concentrations of streptolysin O and the SpeB protease in culture supernatants and SecA in cell extracts were determined by Western blotting as described previously (12, 45). Blots were developed with a Chemidoc XRS imager (Bio-Rad), and relative protein concentrations were determined with Quantity One software (version 4.6.7; Bio-Rad).

Fluorescent and epitope-tagged fusion proteins. Strains for expression of modified proteins were constructed by a standard recombination-based method (see Text S1 in the supplemental material). In the resulting strains, native chromosomal loci were modified to express hybrid proteins fused at their C termini to sfGFP (46), a hemagglutinin (HA) epitope tag (11), or the LFFF epitope tag (27). In each strain, the modified gene represents the only copy of that gene in the genome. For the primers and plasmids used in strain construction and for the resulting strains, see Tables S1 to S3 in the supplemental material.

Preparation of protoplasts. Peptidoglycan was digested with PlyC, a lysin enzyme with activity against the cell wall of *S. pyogenes*, prepared as

described previously (47, 48). Protoplasts were generated by treatment with PlyC (5 U/ml, 10 min) in 30% (wt/vol) raffinose solution supplemented with 5 mM EDTA as described previously (47, 48). For visualization of lipid and protein localization, protoplasts were resuspended in either ThyB or C medium supplemented with 5 to 8% glycerol, as indicated in Results.

Fluorescence microscopy. Intact streptococci, protoplasts, or streptococci from cultures challenged with polymyxin B or lantibiotics were stained with sublethal concentrations of dansyl-polymyxin B (10 μ M; Invitrogen catalog number P13238) or polymyxin B-BODIPYFL conjugate (10 or 60 μ M; Invitrogen catalog number P13235) as described previously (12). Samples were examined with a Leica DM IRE2 fluorescence microscope, and images were captured with a QImaging Retiga 1350 EX charged-coupled device camera and Openlab software (Improvision). Where indicated, cell walls were visualized by staining with fluorescent vancomycin (1 μ g/ml; Invitrogen catalog number V34850) or WGA conjugated to Alexa Fluor 488 or 350 (5 μ g/ml; Invitrogen catalog number W11261 or W11263) and neutral membrane lipids were visualized by staining with Nile Red (2.5 μ g/ml; Invitrogen catalog number N1142). Cellular viability was assessed by staining with a fluorescent vital dye (LIVE/DEAD, BacLight; Invitrogen) (12) and is described in detail in Text S1 in the supplemental material.

Immunofluorescence and immunogold microscopy. Streptococcal cells from untreated or various treated cultures were processed with PlyC and fixed as described previously (9). Immunofluorescence microscopy to detect HtrA-HA and SecA was conducted as described previously (3, 11). Other antisera included a polyclonal rabbit anti-FLAG antibody (Sigma F7425) used at a dilution of 1:250 and detected with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) diluted 1:500. Cell walls were stained with WGA-Alexa Fluor 350 (Invitrogen) as described above. Slides were mounted in an antifade reagent (ProLong Gold; Invitrogen), and images were captured as described above. For immunogold electron microscopy, samples were harvested from the early or mid-logarithmic phase of growth and processed as described previously (3, 11). Colocalization studies used a polyclonal rabbit anti-GFP antibody (ab6556; Abcam) at a dilution of 1:100. Sections were stained with streptavidin-gold conjugates with beads of the various sizes indicated in the figure legends (BBI International) and examined by electron microscopy as previously detailed (3, 11).

Images and quantitative and statistical analyses. Images were processed for publication with Adobe Photoshop CS and represent spliced montages representative of multiple stained cells. Quantitation of foci was performed as described in detail elsewhere (2). The data presented are means and standard error of the means derived from at least three independent experiments consisting of the examination of a minimum of 1,000 stained cells for each experimental condition. Differences between mean values were tested for significance with a two-tailed Student *t* test, and the null hypothesis was rejected for *P* values of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00485-13/-/DCSupplemental>.

Text S1, DOCX file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Figure S1, EPS file, 27.7 MB.
Figure S2, TIF file, 90.8 MB.

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