Understanding the transport and processing of proteins in their journey from the cytosol to the extracellular milieu has driven significant advances in elucidating the molecular interactions between an organism and its environment. These interactions are particularly important at the host-pathogen interface, where bacterial adhesins, toxins, and other virulence factors interact with host tissues (31). In gram-negative organisms, transit from the cytosol to the extracellular environment occurs by several mechanisms that either bypass the periplasm or use it as an organelle to process and fold proteins destined for secretion (46). Gram-positive organisms lack a membrane-bound periplasm but nevertheless secrete many virulence factors that require posttranslational modification (21). It has been proposed that the space between the cell membrane and cell wall provides a protected environment for folding and processing of secreted proteins in gram-positive bacteria (23, 24, 36, 52). Once translocated across the membrane, many virulence factors, such as the *Streptococcus pyogenes* SpeB protease, are secreted into the extracellular milieu (4), while adhesins are retained at the bacterial surface, where they mediate attachment to host tissues. A large subset of adhesins characterized as virulence factors in gram-positive organisms, such as *S. pyogenes* M protein and *Staphylococcus aureus* protein A, are covalently linked to the cell wall by the presence of a cell wall sorting (CWS) signal (1, 8, 41). The CWS signal is comprised of a C-terminal LPXTG motif, a transmembrane domain, and a positively charged tail (41). Proteins containing this CWS signal are recognized by a sortase enzyme, which cleaves the CWS motif between the threonine-glycine bond. Subsequent transpeptidation links the protein to a lipid II intermediate prior to its incorporation into the cell wall (26, 47). The protein-lipid II complex is processed by penicillin binding proteins, which results in the incorporation of the CWS protein into the mature cell wall (48). Despite the great deal known about the biochemical and mechanistic aspects of cell wall synthesis, sorting, and secretion are unclear. Nevertheless, a close association linking these separate processes appears to be critical, because CWS proteins become properly exposed on the surface of the bacteria only after their sortase-mediated incorporation into the cell wall (25).

*Enterococcus faecalis* commonly causes urinary tract infections, endocarditis, intra-abdominal infections, and bacteremia, and it relies on CWS proteins, including Esp, aggregation substance (AS), and pilin, to cause disease (18, 27, 39, 42). While these studies demonstrate the importance of cell wall proteins in *E. faecalis* pathogenesis, the basic mechanisms by
genomic loci are based on this annotation (GenBank accession number E. faecalis streptomycin, 500 mg/liter; tetracycline, 15 mg/liter. added as follows: chloramphenicol, 20 mg/liter; erythromycin, 25 mg/liter; fusidic and used to propagate plasmids.

Escherichia coli grown statically in brain heart infusion (BHI) broth or agar at 37°C for 15 to 18 h and erythromycin (Erm), 750 mg/liter. For E. faecalis regions approximately 800 bp upstream and downstream of the genes were amplified from OG1RF using primer pairs EF3056e-f3/EF3056f3/EF3056f2 and cloned into pAL1 using EcoRI and PstI restriction sites. All constructs were confirmed by sequencing and transformed into MC1061 (50) was grown in Luria-Bertani broth or agar at 37°C and region of localization was done from electron micrographs of E. faecalis and used to propagate plasmids.

which these proteins are localized to the cell surface or secreted remains unclear. We show here that secretion, protein trafficking, and cell wall processing are colocalized at single foci in E. faecalis through the presence of a positively charged retention domain within the localized protein itself, indicating that these processes are compartmentalized into an organelle.

TABLE 1. Strains and plasmids used in this study

<table>
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<th>Species and strain or plasmid (antibiotic)*</th>
<th>Description</th>
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<td>E. faecalis OG1X ΔStrT (Str)</td>
<td>rtaa rtaC</td>
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* Str, streptomycin; Tet, tetracycline; Kan, kanamycin; Erm, erythromycin; Rif, rifampin; Fus, fusidic acid. See Materials and Methods for antibiotic concentrations.

MATERIALS AND METHODS

Bacterial strains and culture. Strains used in this study are listed in Table 1. Escherichia coli strain MC1061 (50) was grown in Luria-Bertani broth or agar at 37°C and used to propagate plasmids. E. faecalis strains were inoculated 1:1,000 and grown statically in brain heart infusion (BHI) broth or agar at 37°C for 15 to 18 h for all assays unless otherwise noted. Antibiotics were added at the following concentrations for E. coli: chloramphenicol, 20 mg/liter; kanamycin, 50 mg/liter; and erythromycin (Erm), 750 mg/liter. For E. faecalis strains, the antibiotics were added as follows: chloramphenicol, 20 mg/liter; erythromycin, 25 mg/liter; fusidic acid, 25 mg/liter, kanamycin, 500 mg/liter; rifampin (rifampicin), 25 mg/liter; streptomycin, 500 mg/liter; tetracycline, 15 mg/liter.

Genetic manipulations. Genes targeted for mutation were identified based on the annotated complete genome of E. faecalis V583 (32); all references to genomic loci are based on this annotation (GenBank accession number AE016830). In-frame deletions of rta (EF3056) and rta (EF1094) were created according to previously described methods (38). StrA, a third sortase present in a subset of strains (18, 32), was not investigated. To construct in-frame deletions of rta (EF3056) and rta (EF1094), regions approximately 800 bp upstream and downstream of the genes were amplified from OG1RF using primer pairs EF3056e-f3/EF3056f3/EF3056f2 and cloned into pAL1 using EcoRI and PstI restriction sites. All constructs were confirmed by sequencing and transformed into MC1061 (50) and region of localization was done from electron micrographs of E. faecalis and used to propagate plasmids.

Plasmids

- pABG5 (Kan, Cm) Shuttle vector
- pRS233 (Erm) Temperature-sensitive plasmid for generation of deletions
- pAL1 (Kan) Derivative of pABG5 lacking Cm
- pAL1::StrA (Kan) cassette
- pAL1::StrA-HA (Kan) cassette
- pAL1::StrC-HA (Kan) cassette
- prgB promoter (Kan)
- pAL1::SrtA-HA (Kan)
- pAL1::SrtC-HA (+/-) tail
- pAL1::SrtC-HA (-) tail
- pAL1::SrfA-HA tail

Expression and localization of AS. Overnight starter cultures were diluted to an optical density (OD) at 600 nm of ~0.01 in 5 ml BHI supplemented with 0.25 ml sterile supernatant from OG1X. These cultures were then grown with shaking for 2 to 2.5 h at 37°C before resing and localization electron microscope (JEOL USA Inc., Peabody, MA). Parallel controls using species-matched purified rabbit anti-EbpA or anti-EbpC primary antibody overnight at 4°C. Sections were then washed in blocking buffer and probed with 18-22 nm gold conjugated anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) for 1 h at room temperature. Sections were washed in PBS buffer followed by an extensive water rinse and stained with 1% uranyl acetate-1.6% methylcellulose. Samples were viewed with a JEOL 1200EX transmission electron microscope (JEOL USA Inc., Peabody, MA). Parallel controls with the primary antibody omitted were consistently negative at the concentration of colloidal gold-conjugated secondary antibodies used in these studies.

Negative-stain immunogold electron microscopy experiments for labeling of pili were carried out as described previously (27) with the following modifications. Bacterial strains were grown statically overnight in BHI, diluted 1:1,000 to tryptic soy broth containing 0.25% glucose (TSBG), and then grown overnight (~16 h) statically at 37°C. All mutants exhibited growth curves in TSBG that were similar to those of their wild-type controls (data not shown). The bacteria were then pelleted, washed in phosphate-buffered saline (PBS), and resuspended in PBS containing 5% calf serum. The cells were adsorbed to grids and incubated with affinity-purified rabbit anti-EbpA or anti-EbpC for 1 h. The grids were then washed with PBS and incubated with goat anti-rabbit IgG conjugated to 10-nm-diameter colloidal gold particles for 30 min. The grids were again washed with PBS, fixed with 1% glutaraldehyde for 20 min, and stained with 0.1% uranyl acetate for 30 min. After three subsequent washes with PBS, the grids were examined in a JEOL 1200EX transmission electron microscope as described above. Parallel controls using species-matched preimmune serum were consistently negative.

Quantification of SecA and sortase foci. Quantitative analysis of the frequency and region of localization was done from electron micrographs of ~600 repre...
sentative bacteria per strain. A focus of localization was defined as a focus containing ≥3 gold particles clustered together. The frequency of focal forma-
determination was described as the total number of bacterial cells containing foci divided by the total number of cells counted. Focal determination was divided by
dividing each focus-containing bacterium into three regions from youngest visible
sections analyzed and multiple spots should never be observed. If the SrtC
sections into 70-nm sections (see above), and that the microdomain where SrtC
localizes is small relative to the section size and cell size. If the SrtC microdomain
exists as only a single spot, it should be present in one-seventh (14%) of the
number of bacterial cells containing ≥3 gold particle anywhere on the cell.
Significance was measured by Fisher’s exact test.

Theoretical mathematical predictions for SrtC foci were based on the condi-
tions that the diameter of an enterococcal cell is ~500 nm, that the bacteria are
sectioned into 70-nm sections (see above), and that the microdomain where SrtC
localizes is small relative to the section size and cell size. If the SrtC microdomain
exists as only a single spot, it should be present in one-seventh (14%) of the
number of bacterial cells containing ≥3 gold particle anywhere on the cell.
Significance was measured by Fisher’s exact test.

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RESULTS

Sortase A localizes in single foci in E. faecalis. To investigate the overlap of secretion with cell wall assembly, we first con-
structed in-frame deletions of both sortases A and C (SrtA and SrtC), which are present in all examined strains of E. faecalis (27), creating strains ΔSrtA and ΔSrtC. To verify the loss of SrtA activity in E. faecalis, we monitored the phenotypic ex-
expression of the SrtA CWS-containing substrate, AS (15). AS expression leads to a marked, SrtA-dependent clumping of bacteria (11, 19). *E. faecalis* ΔSrtA, but not ΔSrtC, lost the ability to aggregate compared to the wild type (Fig. 1A). In complementation analyses, plasmids expressing either wild-type *srtA* (ΔSrtA + SrtA) or *srtA* constructed to express SrtA fused with a dual influenza virus HA epitope tag (SrtA-HA) restored AS-mediated aggregation in ΔSrtA to levels identical on growth phase, we next directly compared SecA localization in mid-logarithmic phase versus stationary phase. Localization of SecA resembled a stationary-phase population (68.1%, 32/47). In the remaining 31.9% (15/47) SecA foci were observed in both log- and stationary-phase cultures were found either in region 1 (41.3% or 32.4%, respectively, for log- or stationary-phase cultures) or in region 3 (38.3% or 47.1%, respectively, for log- or stationary-phase cultures). Only 21% were localized to region 2. Together these data suggest that SrtA localizes to the active division plane but also remains associated with polar regions, the sites of previous cell division. This pattern of localization was not altered when actively dividing cells in mid-logarithmic growth were examined, indicating that our observations of SrtA foci were not growth stage dependent (Fig. 1C).

SecA localizes to a distinct membrane domain of *Enterococcus faecalis*. Frequent localization of SrtA to the equatorial region, the site of the nascent cell division septum, suggested a link between secretion, cell division, and cell wall synthesis. To address whether localized sites of SecA-mediated membrane translocation occur in *E. faecalis*, we first examined the distribution of SecA translocons in the membrane. The SecA antibody used in these studies was raised against *Bacillus subtilis* SecA and was shown to cross-react in immunoblots with a band of the size expected for SecA in both *S. pyogenes* (35) and *E. faecalis* (data not shown) whole-cell lysates. When examined by immunogold electron microscopy of thin-sectioned bacteria, SecA-specific staining of *E. faecalis* grown to early stationary phase was observed in single membrane domains in 3% of sections; the remaining 97% of cells were unlabeled. As thin sections reflect only a fraction of the cell, this frequency does not reflect the actual percentage of cells in which foci occur. To examine the site of SecA localization in each bacterium, we collected an additional 50 bacterial cells from the population examined, indicating whether localized sites of SecA were observed in single membrane domains in 3% of sections; the remaining 97% of cells were unlabeled. As thin sections reflect only a fraction of the cell, this frequency does not reflect the actual percentage of cells in which foci occur. To examine the site of SecA localization in each bacterium, we collected an additional 50 bacterial cells from the population labeled with three or more gold particles corresponding to SecA. Fifty-four percent (30/56) of SecA foci were located in the equatorial region 1 of the bacteria, compared to 30.4% (17/56) and 16.1% (9/56) in regions 2 and 3, respectively (Fig. 2B). Multiple foci were never observed (0/56) under the early-stationary-phase growth conditions examined here.

To address the possibility that SecA localization was dependent on growth phase, we next directly compared SecA localization in mid-logarithmic phase versus stationary phase. Among bacteria positively labeled with three or more gold particles, single SecA foci were observed at approximately the same frequency in a log-phase population (72.2%, 39/54) as in a stationary-phase population (68.1%, 32/47). In the remaining 15/54 and 15/47 SecA-labeled bacteria from each growth phase, respectively, the gold particles were not clustered. These observations indicate that SecA focus formation is not dependent on growth phase. Localization of SecA resembled the pattern observed for SrtA. Thus, we tested the hypothesis that both SrtA and SecA are localized at the same membrane...
We observed consistent colocalization of both SrtA-HA and SecA to a single membrane site (Fig. 2C).

**Disruption of sorting leads to focal substrate accumulation.** Following translation, sortase substrates such as AS are targeted to the membrane for Sec-mediated translocation across the membrane in a process facilitated by the Sec signal sequence. Current models predict that after translocation, sortase substrates are retained in the membrane by their transmembrane helix and flanking positively charged tail until sortase cleavage removes the helix and tail and covalently couples the substrate to the cell wall (41). Once secreted through the Sec pathway and processed by sortase, the distribution of sortase substrates around the cell is driven by incorporation into nascent cell wall components used in peptidoglycan biogenesis. Colocalization of SecA with SrtA at a single domain suggested that the secretion and processing machineries for CWS proteins are spatially coupled. This hypothesis was investigated by studying the localization pattern of AS using immunogold electron microscopy. In wild-type (30) or in \(E. faecalis\) SrtA, AS was no longer assembled around the periphery of the cell wall but instead localized to a single site (Fig. 3B). These results suggested that AS is retained at membrane microdomains when it is not properly incorporated into the cell wall by SrtA.

**Pilus-associated sortase C and substrates localize to single sites.** While many SrtA enzymes are considered “housekeeping” sortases responsible for the surface attachment of most cell wall proteins, a subset of sortases function in pilus biogenesis (6, 9). \(E. faecalis\) SrtC is required for the polymerization of pili that consist of three proteins: EbpA, EbpB, and EbpC (27). We hypothesized that efficient pilus biogenesis is facilitated by focal localization of SrtC. To assess SrtC localization, we constructed a strain in which \(srtC\) is deleted and complemented with a plasmid expressing an HA-tagged SrtC (\(\Delta srtC\) SrtC-HA). The HA epitope tag on SrtC did not alter its function in pilus production, since immunolabeling of negatively stained bacteria for the major pilus subunit EbpC showed that approximately 35% of the wild-type cells expressed pili, similar to levels for \(\Delta srtC\) + SrtC-HA (Fig. 4A) and similar to previously reported levels for the same growth conditions (27).

To determine if SrtC, like SrtA, is found at sites of secretion, we examined localization of SrtC-HA in early stationary phase. We found that SrtC-HA colocalized with SecA in discrete foci at the membrane (Fig. 4B). Examination of immunolabeled thin sections revealed focal SrtC localization on 18.4% (109/593) of these cells, while the rest were unstained. Of the 109 stained cells, 95.4% (104 bacteria) had a single focus, 3.7% (4 bacteria) had two foci, and 0.9% (1 bacterium) had three foci (Fig. 4C). This last class represented 0.2% (1/593) of all cells expressing SrtC-HA and was below the background frequency for single foci of 0.7% (4/577) observed in wild-type cells that do not express SrtC-HA (data not shown). Since a ring structure would be expected to produce a majority of cells with two...
foci and since the frequency of single foci is consistent with that predicted for 0.5-μm cells cut in 70-nm sections (17.5% observed versus 14.3% predicted; see Materials and Methods), these data support the conclusion that SrtC is localized to a single membrane domain rather than organized into a circumferential ring; however, we cannot exclude the possibility that more than one focus of localized protein exist on a cell. In addition, quantitative analysis of the locations of foci indicated a tendency toward localization in the vicinity of the septum versus the periphery or poles (Fig. 4D, region 1).

To examine whether other sortase substrates accumulate in foci in the absence of their cognate sortase, we analyzed the fate of the pilin subunit EbpA in the absence of SrtC. In a ΔSrtC strain, EbpA is not incorporated into pilus fibers in wild-type cells (27), and immunoblot analysis showed that EbpA monomers are instead both cell wall associated and also secreted into the culture supernatant in the absence of SrtC (see Fig. S1 in the supplemental material). Similar to the accumulation of AS in the absence of SrtA (Fig. 3B), immunoelectron microscopy showed that the cell wall-associated EbpA monomers formed foci in 53% of the EbpA-labeled cells in the absence of SrtC (Fig. 5A and C, left panel) while no foci were observed in wild-type cells (Fig. 5A and D, left panel), a statistically significantly enrichment ($P < 0.00001$). One explanation for the incomplete focal localization of EbpA in the ΔSrtC strain is that SrtA is able to attach a subset of EbpA subunits to the cell wall as has been described for *Corynebacterium diphtheriae* and *Streptococcus pneumoniae* (22, 28). Thus, to further validate focal pilin subunit accumulation in the absence of SrtC, immunofluorescence microscopy on whole bacterial cells was performed. EbpA and EbpC were shown to be distributed in a uniform pattern on the surface of wild-type cells (Fig. 5A, B, and C, right panel). Similarly, in ΔSrtA cells, pilin subunits exhibited uniformly distributed staining (Fig. 5A and B). In contrast, both the ΔSrtC and ΔSrtA ΔSrtC double mutant strains were significantly enriched for EbpA staining of singular foci ($P < 0.0001$) (Fig. 5A). Similar observations were made for EbpC (Fig. 5B). Thus, we conclude that the surface distribution of pilin subunits is altered in strains lacking the SrtC enzyme necessary for pilus polymerization.

A positively charged sequence is important for focal sortase localization retention. The clustering of SrtA and SrtC in singular foci, as well as sortase substrate clustering in the absence of the cognate sortase, suggested that sortases and CWS proteins may contain a signature domain that retains them within membrane microdomains prior to processing. Sequence comparisons revealed that each of these proteins has a single trans-

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**FIG. 4.** Sortase C localizes to single foci in *E. faecalis*. (A) Piliation levels of wild-type OG1X and sortase mutants. Results are from a representative experiment in which $\geq 100$ cells/strain/experiment were counted. Statistical significance measured by chi-square test: *, $P < 0.001$. ***, $P < 0.0001$. (B) Coimmunolocalization of SecA (large particles) and SrtC (small particles) found together in foci. Inset, close-up of colocalized SecA and SrtC. Scale bar, 0.5 μm. (C) Quantitative analysis of SrtC immunoelectron micrographs. *, $P < 0.0001$ by chi-square test. (D) Location of SrtC foci in bacteria equally divided into three regions from youngest visible septum to pole. Statistical significance measured by Fisher’s exact test: *, $P < 0.05$; ***, $P < 0.001$. 


membrane domain that is flanked by a highly positively charged region. Positive charges flanking a transmembrane helix have been well characterized as a determinant of membrane topology (49). In SrtA, this positively charged domain is in the uncleaved secretion sequence of the protein (which also serves as the membrane-spanning domain), whereas in SrtC this domain is at the C terminus. Sequence comparisons of sortase substrates show that cell wall sorted proteins, including AS and the Ebps, contain a positively charged tail as part of their CWS (Fig. 6A and data not shown) (40, 41). Therefore, we hypothesized that in these proteins, a higher positive charge not only determines topology but also mediates localization to and retention within membrane microdomains.

Since the SrtC charged domain is at the C terminus and thus is not part of the signal peptide, we used it as a model protein to investigate whether the positively charged residues play a role in localizing membrane proteins to discrete foci, distinct from the signal peptide. This was examined by mutating the positively charged C-terminal domain in SrtC. Three different mutants were constructed: a mutant with a deletion of all positively charged residues from the C terminus, a mutant with every other positively charged amino acid replaced by a negatively charged residue, and a mutant with every positively charged residue replaced by a negative one. The effect of these mutations on SrtC localization was determined by immunogold electron microscopy. In contrast to the discrete localization observed for wild-type SrtC (Fig. 4B and 6D), all three mutants displayed an altered pattern of localization (Fig. 6E to G) corresponding to a decrease in the frequency of focal localization of gold particles and an increase in random gold labeling. Quantifying these results, we observed 36% fewer foci for SrtCΔtail, 72% fewer for SrtC(+/−)tail, and 70% fewer for SrtC(−)tail compared to wild-type SrtC (Fig. 6B). Mislocalized SrtC typically appeared as multiple smaller SrtC clusters along with several single gold particles. SrtC mislocalization is not due to instability of the mutated proteins, as verified by Western blot analysis (Fig. 6C). The clustering pattern of SrtC is unlikely to be mediated by the HA tag, since the clustering is dispersed in the SrtC mutants (which also have an HA tag). Thus, we conclude that the positively charged domain flanking the transmembrane helix is necessary for sortase localization.

**Focal localization of SrtC is necessary for efficient pilus formation.** If SrtC localization to single foci is important for coupling secretion of pilus subunits and their subsequent processing into pilus fibers, then disruption of SrtC localization should also disrupt pilus biogenesis. We examined the location and extent of pilus expression on negatively stained bacteria with anti-EbpC antibodies. As reported for pili of other gram-positive organisms (14, 27), only a subset of the cells in any culture expressed pili, and for those cells that did express pili, no differences were observed in either the structure of the pili or their localization pattern on the cell surface for the wild-type, ∆SrtC + SrtCWT, and ∆SrtC + SrtCΔtail strains (see Fig. S2 in the supplemental material). In contrast, the overall proportion of piliated cells in each culture differed markedly. Complementation of ∆SrtC with

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**FIG. 5.** Pilus subunits accumulate focally in the absence of SrtC. (A and B) Quantification of EbpA (A) or EbpC (B) immunofluorescent labeling of whole *E. faecalis* OG1RF wild-type or sortase mutant cells grown to stationary phase. *, *P < 0.0000001 by Fisher’s exact test. (C and D) EbpA labeling of wild-type (WT) (C) or ∆SrtC bacteria (D) and localization by electron microscopy (left panels). Representative images of whole-cell immunofluorescence labeling of EbpA (red), DNA (blue), and cell wall (green) are also shown (right panels).
a plasmid-encoded copy of srtC restored piliation to wild-type levels (Fig. 4A). However, mislocalization of SrtC through expression of SrtC\(\Delta\)tail, resulted in a significantly reduced level of piliation (15% piliation for SrtC\(\Delta\)tail versus 55% piliation for SrtCwt, \(P < 0.0001\)) (Fig. 4A), suggesting that focal subcellular localization of SrtC is required for efficient pilus biogenesis. Taken together, these data indicate that while SrtC mislocalization has no effect on the final destination of the sortase

![Figure 6](https://example.com/figure6.png)

**FIG. 6.** Sortase C localization is dependent on a positively charged cytoplasmic tail. (A) Alignment of proteins observed in localized foci in *E. faecalis*. The cartoon depicts amino acids adjacent to the transmembrane helices (TMH) of *E. faecalis* SrtA, SrtC, AS, and Ebp pilus subunits. NH\(_3\) and CO\(_2\) indicate the N and C termini of the proteins, respectively. Boldface amino acids are positively charged. (B) Quantitative analysis of SrtC immunoelectron micrographs. Bacteria labeled with three or more gold particles were assessed for the presence or absence of focal localization. The percentage of cells displaying SrtC foci is expressed relative to wild-type (WT) value. Statistical significance measured by Fisher’s exact test: *\(P < 0.05\); **\(P < 0.001\). (C) Anti-HA immunoblot of whole OG1RF, demonstrating stability of SrtC tail mutants. (D) Expression of SrtC-HA under control of the RofA promoter results in localization to single domains on the surface of the bacterium in wild-type *E. faecalis* strain OG1RF. (E to G) Immunolocalization of SrtC tail mutants (the amino acid sequence of the mutagenized tail is indicated). Scale bar, 0.5 \(\mu\)m.
substrate after attachment to the cell wall, proper SrtC placement in the cell membrane facilitates its efficient function.

**DISCUSSION**

Bacterial cells display exquisite subcellular organization in the processing of virulence factors for display on the cell surface. Localization of presecretory proteins facilitates efficient secretion, processing, and assembly of macromolecular structures necessary for pathogenic interactions. This process has been enigmatic in gram-positive organisms due to incomplete understanding of basic molecular secretion mechanisms. Recently, two models have been proposed for how proteins are secreted in gram-positive cocci. In the first model, components of the general secretory pathway (Sec machinery) have been shown to localize to distinct domains in *S. pyogenes* and *Streptococcus mutans* (17, 35), leading to the proposal that protein secretion and processing may be spatially coupled. Supporting this model, two secreted proteins from *S. pyogenes*, SpeB and HtrA, colocalize with this secretion domain, termed the ExPortal. In the second model, domains within the secreted proteins themselves, and not the location of the Sec machinery, are proposed to direct the localization of secreted proteins in gram-positive cocci. A domain of the N-terminal secretion signal was shown to differentially influence the sites of protein F and M protein appearance on the cell surface of *S. pyogenes*, as well as that of a number of LPXTG-containing proteins in *S. aureus* (5, 7). Consistent with the model for localized secretion machinery, Sec components were found to localize in a helical pattern along the lengths of both gram-positive *B. subtilis* and gram-negative *E. coli* rods (3, 43), suggesting that secretion localization may be a conserved phenomenon in similarly shaped bacteria. Nevertheless, the molecular details governing the spatial subcellular distribution of the Sec apparatus in gram-positive cocci is not yet understood. Reconciliation of these models awaits careful examination of the entire secretion apparatus locale, including the SecYEG translocation channel, throughout the cell cycle.

Here we show that both secretion and sortase processing are spatially coupled in *E. faecalis*. The observation that SecA can localize to single domains in both log and stationary phases is consistent with the ExPortal model of localized protein secretion and indicates that sortase proteins are also found at this subcellular location. The significant enrichment of SecA and sortase enzyme domains in the vicinity of the cell division plane is consistent with cell wall synthesis in enterococci and streptococci occurring at the midcell (reviewed in reference 51). Localization of SrtC was facilitated by a positively charged sequence within the C terminus of the protein, distinct from the N-terminal secretion signal. Efficient assembly of sortase-dependent pilus required proper subcellular localization of sortase. Our results suggest a model of coordinated localized secretion and sorting of cell wall proteins and are consistent with a model that in *E. faecalis*, proteins do not always traverse the cell membrane in a random manner but instead have at least one pathway that coordinates protein secretion and subsequent processing in localized regions across the cell membrane. It is likely that gram-positive cocci have evolved multiple mechanisms for subcellular localization. It will be interesting to determine whether signal sequence domains, analogous to YSIRK motifs identified in *S. pyogenes* and *S. aureus* (5, 7), also play a role in cell wall protein deposition in *E. faecalis*. Notably, none of the enterococcal sortases examined in this study and only one of 57 predicted CWS proteins in the sequenced *E. faecalis* V583 genome possesses a signal sequence bearing a canonical YSIRK motif (reference 32 and data not shown).

The cell wall of gram-positive bacteria is responsible for scaffolding its surface-exposed proteins but is also a significant barrier to secretion (44). This likely requires unique mechanisms for efficient transit and processing of virulence factors across the membrane and into the extracellular space. The assembly and attachment of gram-positive pili to the cell wall constitute an excellent model system for study of such complexity. The genes encoding pilus subunits are found genetically clustered with a sortase that is involved in the covalent polymerization of subunits during pilus biogenesis. After pilus polymerization, anchorage of the pilus to the growing cell wall is facilitated either by a “housekeeping” sortase encoded elsewhere on the chromosomes (2, 29, 45) or by the pilus-associated sortase enzyme itself, as has been reported for *S. pneumoniae* (20). We show that the enterococcal pilus subunits EbpA and EbpC are significantly enriched in single foci in the absence of SrtC. These observations suggest a model in which a microdomain for secretion and sortase action may facilitate a high local concentration of subunits that are primed for pilus assembly. Interestingly, recent findings with *S. pneumoniae* show pilus localization at multiple, symmetric foci in wild-type cells (14). Pilus biogenesis in pneumococci appears to be a more complex process than in enterococci, with three pilin-associated sortase enzymes that not only display substrate specificity but also are required for focal presentation of pilus (14). These findings are consistent with a model in which pilus formation in gram-positive bacteria involves the coordination of subunit secretion, processing by multiple sortase enzymes, and cell wall synthesis. For secreted proteins such as pilin subunits, the enrichment of SecA and SrtC foci at or near the division septum where peptidoglycan synthesis is occurring may also reflect the most energetically favorable site for secretion and processing, where the cell wall barrier is thinnest. Organization of multiple cellular processes at a single site not only facilitates spatial and temporal coordination of these processes but also promotes efficiency in their function.

An interesting prediction of any localized secretion model is that the membrane contains an asymmetric distribution of proteins, with one subset retained in foci while another becomes routed into the peripheral membrane. Thus, the membrane proteins themselves should contain specific motifs that are responsible for their trafficking to their appropriate destination following insertion into the membrane. Our data suggest that a high positive charge flanking a membrane-spanning region can function as a retention sequence for SrtC localization. Interestingly, the CWS sequences of sortase substrates in *S. aureus* and *E. faecalis* also possess a positively charged tail that is necessary for efficient cell wall sorting (19, 41). We hypothesize that this positively charged region may act to retain a sortase substrate within membrane microdomains following its translocation by the Sec pathway in order to promote interaction with the similarly localized sortase enzyme. Thus, in the absence of their cognate sortases, the sortase substrates
AS and Ebp pilin subunits were retained in membrane microdomains. Understanding how positive charge mediates localization of sortase C in E. faecalis will lead to important insights into the molecular underpinnings of gram-positive pathogen secretion and protein processing. One explanation is that cytoskeletal proteins or other subcellularly localized proteins form a scaffold for protein localization. Currently, the streptococci and enterococci lack any of the known bacterial cytoskeletal proteins described to date. Alternatively, a lipid-stabilized domain similar to lipid rafts in eukaryotic cells (34) may facilitate the retention of proteins by protein-lipid interactions. The latter possibility is consistent with findings that negatively charged lipids are necessary for SecA localization in B. subtilis and that anionic lipid domains occur at single sites that are consistent with the location of the ExPortal in S. pyogenes (3, 37).

In this study, we show that secretion and sortase processing occur together in E. faecalis. Sortase localization is facilitated by a positive charge that is necessary for efficient pilus biogenesis. These findings present a novel mechanism for coordinate secretion and processing of cell wall proteins in gram-positive cocci. Together, these data increase our understanding of basic molecular processes in this important category of pathogens and could lead to the identification of novel targets for therapeutic agents.

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