

Dynamic Distribution of the SecA and SecY Translocase Subunits and Septal Localization of the HtrA Surface Chaperone/Protease during *Streptococcus pneumoniae* D39 Cell Division

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ABSTRACT The Sec translocase pathway is the major route for protein transport across and into the cytoplasmic membrane of bacteria. Previous studies reported that the SecA translocase ATP-binding subunit and the cell surface HtrA protease/chaperone formed a single microdomain, termed “ExPortal,” in some species of ellipsoidal (ovococcus) Gram-positive bacteria, including *Streptococcus pyogenes*. To investigate the generality of microdomain formation, we determined the distribution of SecA and SecY by immunofluorescent microscopy in *Streptococcus pneumoniae* (pneumococcus), which is an ovococcus species evolutionarily distant from *S. pyogenes*. In the majority ($\geq 75\%$) of exponentially growing cells, *S. pneumoniae* SecA (SecA_{Spn}) and SecY_{Spn} located dynamically in cells at different stages of division. In early divisional cells, both Sec subunits concentrated at equators, which are future sites of constriction. Further along in division, SecA_{Spn} and SecY_{Spn} remained localized at mid-cell septa. In late divisional cells, both Sec subunits were hemispherically distributed in the regions between septa and the future equators of dividing cells. In contrast, the HtrA_{Spn} homologue localized to the equators and septa of most (>90%) dividing cells, whereas the SrtA_{Spn} sortase located over the surface of cells in no discernable pattern. This dynamic pattern of Sec distribution was not perturbed by the absence of flotillin family proteins, but was largely absent in most cells in early stationary phase and in Δ cls mutants lacking cardiolipin synthase. These results do not support the existence of an ExPortal microdomain in *S. pneumoniae*. Instead, the localization of the pneumococcal Sec translocase depends on the stage of cell division and anionic phospholipid content.

IMPORTANCE Two patterns of Sec translocase distribution, an ExPortal microdomain in certain ovococcus-shaped species like *Streptococcus pyogenes* and a spiral pattern in rod-shaped species like *Bacillus subtilis*, have been reported for Gram-positive bacteria. This study provides evidence for a third pattern of Sec localization in the ovococcus human pathogen *Streptococcus pneumoniae*. The SecA motor and SecY channel subunits of the Sec translocase localize dynamically to different places in the mid-cell region during the division cycle of exponentially growing, but not stationary-phase, *S. pneumoniae*. Unexpectedly, the *S. pneumoniae* HtrA (HtrA_{Spn}) protease/chaperone principally localizes to cell equators and division septa. The coincident localization of SecA_{Spn}, SecY_{Spn}, and HtrA_{Spn} to regions of peptidoglycan (PG) biosynthesis in unstressed, growing cells suggests that the pneumococcal Sec translocase directs assembly of the PG biosynthesis apparatus to regions where it is needed during division and that HtrA_{Spn} may play a general role in quality control of proteins exported by the Sec translocase.

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The Sec translocase is the most important molecular machine for the secretion of proteins and insertion of membrane proteins in bacteria (1, 2). The Sec translocase apparatus consists of the highly conserved SecYEG conducting channel imbedded in the membrane, the peripherally bound SecA ATP-dependent motor protein that mediates posttranslational export, the SRP-FtsY system that mediates cotranslational export, and a variety of ancillary protein factors (reviewed in references 1 and 2). To understand whether protein delivery is coupled to cellular functions, the subcellular location of the Sec translocase has been examined in several bacteria. In the Gram-negative bacterium *Escherichia coli*,

the SecY and SecE subunits showed spiral-like, polar, or no localization, possibly depending on experimental expression levels (3). In the rod-shaped Gram-positive bacterium *Bacillus subtilis*, subcellular localization studies of SecA and SecY indicated that the Sec translocase is present in a spiral-like structure along the cell length (4).

In the ovococcus (American football-shaped) Gram-positive bacterium *Streptococcus pyogenes*, immunogold electron microscopy (IG-EM) was used to locate *S. pyogenes* SecA (SecA_{Spy}) and HtrA_{Spy}, which is thought to function as a dual-function protease and protein chaperone (5, 6). SecA_{Spy} and HtrA_{Spy} were found to

colocalize at only one specific site in each cell, termed the “ExPortal,” which was defined as a microdomain specialized for secretion. It was proposed that concentrated secretion at a specific site might enable some bacteria to coordinate protein translocation and subsequent folding, especially in bacterial pathogens like *S. pyogenes* that secrete many adhesins, toxins, and virulence factors to their cell surfaces (6). More recent IG-EM studies of the ovococcus species *Enterococcus faecalis* (7) and *Streptococcus mutans* (8) also indicate that their homologues of SecA and the general “housekeeping” sortase SrtA localize to a single locus, similar to the ExPortal reported for SecA and HtrA in *S. pyogenes* (6). In contrast, the SrtA sortase of *S. pyogenes* is not located in a single microdomain and seems to distribute around cell peripheries with some localization at the septa of dividing cells (9).

However, not all studies support the existence of a Sec translocase ExPortal. In one IG-EM study, SecA of *S. pyogenes* was distributed throughout the cell periphery and was not confined to a microdomain (10). In other studies of *S. pyogenes* (10) and the spherical coccus bacterium *Staphylococcus aureus* (11), sortase-attached surface proteins were directed to mid-cell or polar locations by a mechanism that involves specific motifs in signal peptides. Secretion at two disparate locations in cells is not consistent with a single ExPortal per cell. In addition, the localization of the Sec apparatus seems to be influenced by the growth phase of some bacteria. The Sec apparatus was localized in spirals in *B. subtilis* cells growing exponentially, but this pattern disappeared in stationary-phase cells (4). In other bacteria, like *E. faecalis*, SecA location in an ExPortal is independent of growth stage (7). To date, no clear picture has emerged about the localization of the Sec translocase during different stages of cell division and culture growth.

Like *S. pyogenes*, *Streptococcus pneumoniae* (pneumococcus) is an important opportunistic, ovococcus-shaped, Gram-positive human pathogen (12, 13). However, *S. pneumoniae* (*mitis* group) is evolutionarily distant from other species of *Streptococcus* in which SecA and HtrA homologues have been localized, including *S. pyogenes* (*pyogenic* group) and *S. mutans* (*mutans* group) (reviewed in reference 14). This evolutionary distance is reflected by the remarkably different sets of virulence factors used and different diseases caused by these different commensal species of *Streptococcus* (14–17). *S. pneumoniae* is an aerotolerant anaerobe that colonizes the nasopharyngeal cavities of children and adults (12, 13, 16). Besides acting as a commensal, *S. pneumoniae* is a human opportunistic pathogen that causes several serious invasive diseases, including pneumonia, otitis media (earache), meningitis, and bacteremia, that result in at least 1.6 million deaths annually worldwide (13). The majority of pneumococcal virulence factors, including choline binding proteins, pneumococcal surface proteins A and C (PspA and PspC, respectively), proteinase maturation protein A (PpmA), autolysin B (LytB), and metal receptor binding proteins (e.g., PiaA, PiuA, and PsaA), contain signal peptides and are transported to the cell surface by the *S. pneumoniae* Sec (Sec_{Spn}) translocase (16, 18). Essential proteins involved in pneumococcal cell division, such as PcsB, are also delivered to the cell surface by the Sec system (19).

We report here the first determination of the distribution of two Sec translocase subunits, SecA and SecY, as well as HtrA and SrtA in *S. pneumoniae* during different stages of cell division and phases of growth. In this study, we constructed strains that express the respective FLAG-tagged proteins from their native chromo-

somal loci in an unencapsulated derivative of the prototypic virulent serotype 2 strain D39 (20). Lack of capsule causes these strains to form mainly diplococci (19) that can be binned according to stage of division before immunofluorescent images are analyzed (21, 22). We show that during exponential growth, SecA_{Spn} and SecY_{Spn} distribute dynamically to mid-cell and division regions active in peripheral and septal peptidoglycan (PG) synthesis in ovococcus-shaped cells (see references 21, 23, and 24). Likewise, the HtrA_{Spn} protease/chaperone localizes to regions of PG biosynthesis and cell division, whereas the SrtA_{Spn} general sortase is present over the entire cell periphery. We also provide evidence that entry into stationary phase and Δ *cls* mutations that block synthesis of the anionic lipid cardiolipin lead to loss of localization of SecA_{Spn} and HtrA_{Spn}. Besides not supporting the existence of an ExPortal microdomain in *S. pneumoniae*, these results suggest that Sec-mediated secretion and membrane protein insertion and HtrA-catalyzed protein quality control localize to regions of active PG biosynthesis and cell division.

RESULTS

SecA_{Spn}-L-FLAG³-, SecY_{Spn}-L-FLAG³-, HtrA_{Spn}-L-FLAG³-, and SrtA_{Spn}-L-FLAG³-expressing strains exhibit wild-type phenotypes. We constructed strains expressing FLAG³-tagged proteins by fusing a 10-amino-acid linker followed by three tandem copies of the FLAG epitope (FLAG³) to the carboxyl terminus of SecA_{Spn}, SecY_{Spn}, HtrA_{Spn}, or SrtA_{Spn}. Each resulting markerless construct was expressed at its native chromosomal locus in place of the native gene (see Tables S1 and S2 and Fig. S1 in the supplemental material). The resulting strains, IU4468 (D39 Δ *cps rpsL1 htrA*-L-FLAG³), IU4470 (D39 Δ *cps rpsL1 srtA*-L-FLAG³), IU4472 (D39 Δ *cps rpsL1 secA*-L-FLAG³), and IU5471 (D39 Δ *cps rpsL1 secY*-L-FLAG³) showed no aberrant growth phenotypes compared to the parent strain IU1824 (D39 Δ *cps* not expressing a FLAG³ fusion protein) at either 37°C or 41.2°C in brain heart infusion (BHI) broth (data not shown). The four strains expressing FLAG³-tagged proteins showed no differences in colony morphology on Trypticase soy agar II (TSAIL) blood agar plates or cell morphology as observed by phase-contrast microscopy compared to the IU1824 parent strain (column 1 in Fig. 1 and 4; see Fig. S2 and S5 in the supplemental material). Since *S. pneumoniae* Δ *htrA* mutants exhibit a temperature-sensitive growth phenotype (25), the normal growth of IU4468 (D39 Δ *cps htrA*-L-FLAG³) at 41.2°C indicated that the HtrA-L-FLAG³ protein was functional. Likewise, the normal growth of IU4472 (D39 Δ *cps secA*-L-FLAG³) and IU5471 (D39 Δ *cps secY*-L-FLAG³) indicated that the FLAG³ tag did not significantly impede function of the essential SecA and SecY proteins. We confirmed that the SrtA-L-FLAG³ fusion sortase was functional in strain IU4470 (D39 Δ *cps srtA*-L-FLAG³) based on its wild-type level of cell-associated β -galactosidase activity compared to a Δ *srtA* mutant, which secretes this activity into the growth medium (see Text S2 in the supplemental material). Finally, Western blotting (see Text S2) showed that the FLAG³-tagged SecA, HtrA, and SrtA proteins were present as single bands with the expected molecular masses and that the relative cellular amount of each protein did not change in cells in exponential or transition phase (see Text S2) sampled for immunofluorescent microscopy (IFM) studies (Fig. 1–4).

SecA_{Spn} and SecY_{Spn} localize dynamically during cell division. The localization patterns of SecA-L-FLAG³ were determined by IFM in cells at the following three stages of division (Materials

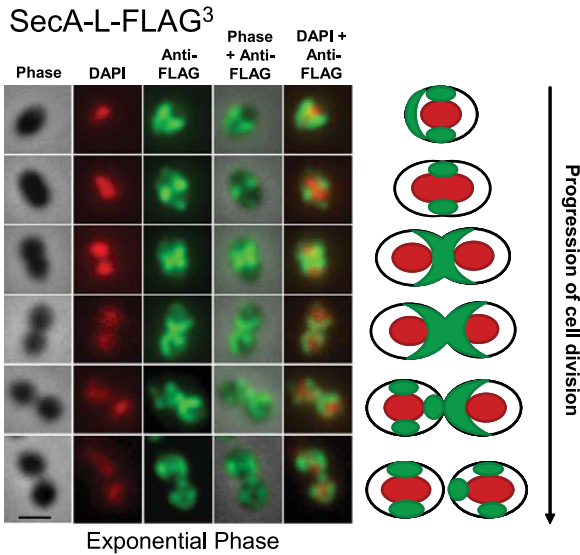


FIG 1 SecA-L-FLAG³ localizes primarily to equators, septa, and hemispheres of exponentially growing *S. pneumoniae* cells. Strain IU4472 (D39 Δ *cps rpsL1 secA-L-FLAG³*) was grown to mid-exponential phase in BHI broth, and DAPI staining and IFM of cells were performed as described in Materials and Methods. Cells at different stages of division were binned based on images from phase-contrast microscopy (column 1), and localization patterns of DAPI (pseudocolored red; column 2) and SecA-L-FLAG³ (pseudocolored green; column 3) were determined from images obtained by epifluorescent microscopy. Columns 4 and 5 show overlays of columns 1 and 3 or columns 1 and 2, respectively. Images of representative cells are shown at different stages of division. A schematic summary at right shows the SecA_{Spn} localization pattern for >75% of the cell population at each stage (Fig. 2). The experiment was performed more than 5 times independently. Multiple fields were observed in each experiment, with similar results. Scale bar = 1 μ m.

and Methods): predivisional single cells (oval cells) (Fig. 1, rows 1 and 2), early divisional cells (slight indentation at mid-cell) (Fig. 1, row 3), and late divisional cells (septum width of less than half the cell width) (Fig. 1, row 4). Visual patterns of localization were confirmed by determining pixel intensities in different regions of cells (Materials and Methods; data not shown). Among predivisional single cells, 78% showed localization of SecA-L-FLAG³ primarily at equators in the region between separating nucleoids (Fig. 1, row 2; Fig. 2A). An additional 15% of predivisional cells had SecA-L-FLAG³ spreading from equators to over half of the cell (Fig. 1; row 1). In cells with this hemispherical distribution, nucleoids appeared condensed and spherical, suggesting that these cells might have been newly divided. Besides this prominent equatorial localization of SecA-L-FLAG³ in 93% of predivisional cells, weaker labeling of SecA-L-FLAG³ was sometimes present at one pole (36%; e.g., see Fig. 1, row 2), both poles (16%), or at other areas (46%), consistent with a dynamic redistribution of SecA_{Spn} and the translocase apparatus during division.

This conclusion was supported by patterns seen in early and late divisional cells. About 75% of cells at these stages (Fig. 2A) had a “ χ ” (Chi)-like distribution of SecA-L-FLAG³ along the division septum between the divided nucleoids, eventually forming two hemispheres surrounding the middle halves of the daughter cells (Fig. 1, rows 3 and 4). Finally, in postdivisional diplococci (Fig. 1, rows 5 and 6), SecA-L-FLAG³ was distributed in each cell similar to the patterns observed in separated single cells (Fig. 1, rows 1 and 2). Daughter cells that contained spherical nucleoids showed a

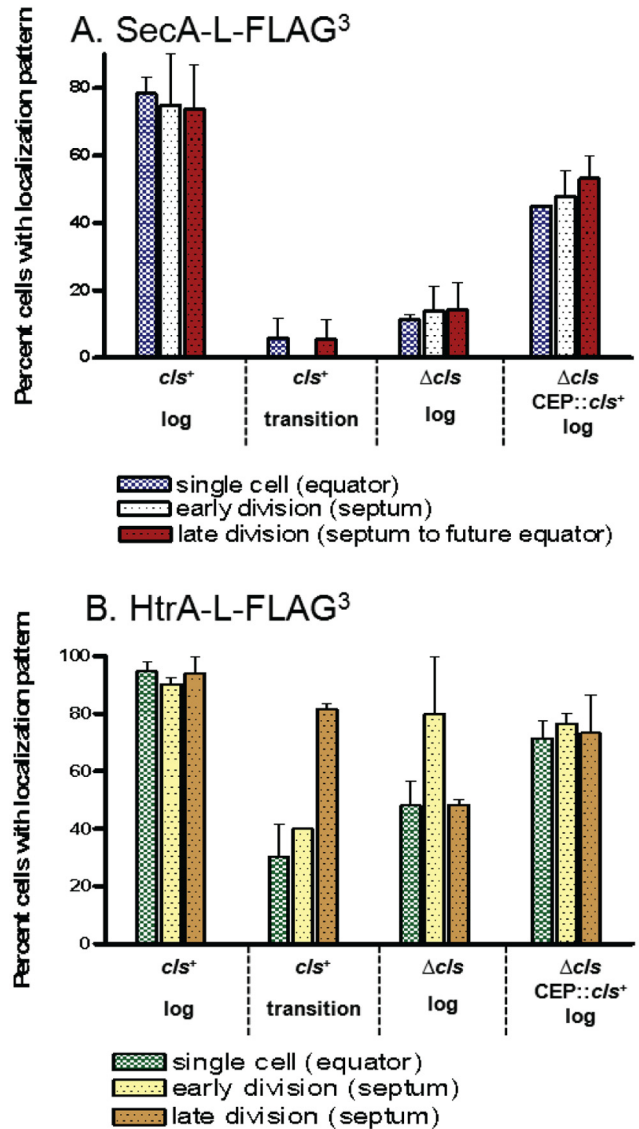


FIG 2 Distribution of predivisional, early divisional, and late-divisional cells with SecA-L-FLAG³ (A) and HtrA-L-FLAG³ (B) localized at equators, division septa, or hemispheres between septa and future equators, as indicated. Cells were grown exponentially (log) or to the transition to stationary phase, and IFM was performed as described in Materials and Methods. (A) *cls*⁺ parent strain IU4472 (D39 Δ *cps rpsL1 secA-L-FLAG³*), Δ *cls*::P_{c-erm} mutant IU5093, and complemented Δ *cls* mutant IU5138 (Δ *cls*::P_{c-erm}/CEP::*cls*⁺, where “*ll*” indicates a separate ectopic location in the chromosome). (B) *cls*⁺ parent strain IU4468 (D39 Δ *cps rpsL1 htrA-L-FLAG³*), Δ *cls*::P_{c-erm} mutant IU5089, and complemented Δ *cls* mutant IU5136 (Δ *cls*::P_{c-erm}/CEP::*cls*⁺). See the text for additional details.

half-cell labeling pattern of SecA-L-FLAG³ (Fig. 1, row 5, right cell), similar to that of late divisional cells (Fig. 1, row 4), while daughter cells with duplicating nucleoids showed the strongest SecA-L-FLAG³ labeling at mid-cell (Fig. 1, row 4, left cell; row 6, both cells). Taken together, these images indicate that SecA_{Spn} distributes dynamically during the cell cycle and goes through stages where it is distributed over the hemispherical halves between the separated nucleoids of dividing pneumococcal cells (Fig. 1, right).

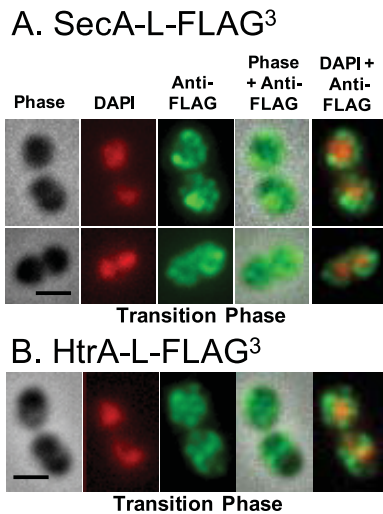


FIG 3 SecA-L-FLAG³ (A) and HtrA-L-FLAG³ (B) do not localize in cells during the transition from exponential to stationary phase in BHI broth. Strains IU4472 (Fig. 1) and IU4468 (Fig. 4) were grown to transition phase, and DAPI staining and IFM of cells were performed as described in Materials and Methods. Representative cells are shown with the same column arrangement as in Fig. 1. The experiment was performed more than 2 times independently. Multiple fields were observed in each experiment, with similar results. Scale bar = 1 μ m.

The localization patterns of SecY-L-FLAG³ determined by IFM were remarkably similar to those of SecA-L-FLAG³ at all three stages of division of exponentially growing cells (see Fig. S2 in the supplemental material). Similar to SecA-L-FLAG³, SecY-L-FLAG³ localized chiefly at equators of single predivisional cells, at septa of early divisional cells, and as hemispheres in late-divisional cells in the majority (>75%) of cells at each stage of division (data not shown). This result indicates that SecA_{Spn} tracks with SecY_{Spn}, which is a member of the translocase channel complex, in exponentially growing pneumococcal cells.

SecA_{Spn} and SecY_{Spn} are not localized in transition-phase cells. We performed similar IFM and DAPI (4',6-diamidino-2-phenylindole) labeling of cells grown to transition phase before reaching stationary phase (optical density at 620 nm [OD₆₂₀] of 0.78, 15 min before the maximal OD₆₂₀ of 0.82). The localization patterns of SecA-L-FLAG³ and SecY-L-FLAG³ observed in exponentially growing cells (Fig. 1 and 2A; see Fig. S2 in the supplemental material) were present in fewer than 6% of transition-phase cells (Fig. 2A and 3A) (data not shown). Instead, SecA-L-FLAG³ and SecY-L-FLAG³ appeared as puncta in no discernible pattern in a large majority of transition-phase cells, including single cells and diplococci at different stages of division (Fig. 3A) (data not shown).

SecA_{Spn} is not localized in Δ cls (cardiolipin synthase) mutants. The anionic lipids phosphatidylglycerol (PGly) and cardiolipin (CL) have been implicated in protein translocation across the bacterial membrane (3, 4, 26), and depletion of anionic lipids results in rapid loss of the spiral localization of the Sec components in *B. subtilis* (4). In addition, the ExPortal microdomain of *S. pyogenes* was postulated to be enriched in anionic phospholipids (27). Consequently, we determined the distribution of SecA-L-FLAG³ in *cls* (*spd_0185*) deletion mutants lacking cardiolipin synthase, which converts PGly to CL. A Δ cls::P_{c-erm} deletion/in-

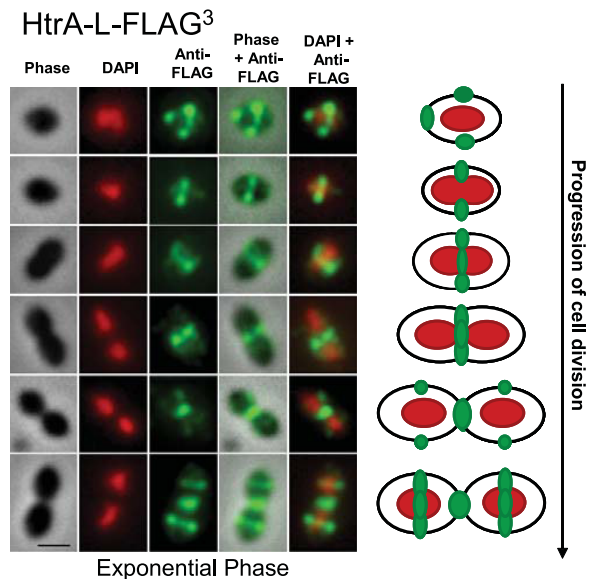


FIG 4 HtrA-L-FLAG³ localizes to equators and septa of exponentially growing *S. pneumoniae* cells. Strain IU4468 (D39 Δ cps *rpsL1* *htrA*-L-FLAG³) was grown to mid-exponential phase in BHI broth, and DAPI staining and IFM of cells were performed as described in Materials and Methods. Representative images are shown with the same row and column arrangements as in Fig. 1. A schematic summary at right shows the HtrA_{Spn} distribution pattern in >90% of the cell population (Fig. 2). The experiment was performed more than 3 times independently. Multiple fields were observed in each experiment, with similar results. Scale bar = 1 μ m.

sertion mutation and a nonpolar markerless Δ cls mutation were constructed (see Tables S1 and S2 in the supplemental material), and both types of mutations caused similar phenotypes. Growth of unencapsulated D39 Δ cps Δ cls::P_{c-erm}, D39 Δ cps Δ cls::P_{c-erm} *secA*-L-FLAG³, and D39 Δ cps Δ cls *secA*-L-FLAG³ mutants was impaired in BHI broth compared to that of their *cls*⁺ parents, with longer lags and lower (63 to 86%) growth yields (see Fig. S3 in the supplemental material) (data not shown). At low (OD₆₂₀ of <0.1) densities, Δ cls mutant cells often formed long chains (>30 cells), which occasionally contained lysed cells (block arrows, column 1 in Fig. S4B in the supplemental material) (data not shown) and typically were wider and more spherical than the *cls*⁺ parent cells (column 1 in Fig. S4A and S4B in the supplemental material) (data not shown). SecA_{Spn}-L-FLAG³ was mislocalized in most cells containing either type of Δ cls mutation (see Fig. S4B) (data not shown), and only 11 to 27% of Δ cls mutant cells showed the patterns of SecA_{Spn}-L-FLAG³ localization observed in the *cls*⁺ parent (Fig. 1 and 2A; see Fig. S4A and S4B) (data not shown). At higher culture densities, the number of lysed Δ cls cells within chains increased, leading to shorter chains among many lysed cells (data not shown).

These defects in growth yield, chain length, cell lysis, and SecA_{Spn}-L-FLAG³ localization caused by both Δ cls mutations were largely complemented by expression of Cls⁺ protein from the P_{fcsK} promoter in the ectopic CEP site (Fig. 2A; see Fig. S3 and S4C in the supplemental material) (data not shown) (28, 29). Complementation levels for both Δ cls mutations were similar in the presence or absence of 0.8% (wt/vol) fucose, which is an inducer of the P_{fcsK} promoter (19, 21, 30). This result suggests that sufficient Cls⁺ enzymatic activity is expressed by leakiness of the P_{fcsK} promoter

in the absence of inducer to complement the phenotypes. Since the complementation levels were similar for the $\Delta cls::P_c-erm$ and the nonpolar Δcls mutations, the lack of full complementation is not likely due to polarity of the Δcls mutations on expression of the downstream *spd_0186* and *spd_0187* genes of unknown function. The nearly complete complementation of SecA-L-FLAG³ mislocalization (Fig. 2A; see Fig. S4A and S4C) (data not shown) supports the notion that CL (or less likely, the Cls⁺ protein itself) plays a role in proper localization of the protein translocase in *S. pneumoniae*.

HtrA localizes at equatorial rings and division septa in exponentially growing pneumococcus. Despite its importance to *S. pneumoniae* physiology and virulence (see Discussion), the cellular localization of HtrA_{Spn} on cell surfaces has never been reported. We determined the localization of HtrA-L-FLAG³ at different stages of the cell cycle in exponentially growing cells (Fig. 4). Strikingly, HtrA-L-FLAG³ localized to the equators and division septa of >90% of cells (Fig. 2B and 4). Among single cells (Fig. 4, rows 1 and 2), HtrA-L-FLAG³ was located at equators, and 20% of these cells showed additional strong labeling at one pole, possibly from the septum of the previous division (e.g., Fig. 4, row 1). As equators became division septa, HtrA-L-FLAG³ retained its mid-cell localization (Fig. 4, rows 3 and 4). HtrA-L-FLAG³ remained at closing septa, after daughter cells appeared to be separated, and then started to appear at the equators of newly divided cells (Fig. 4, rows 5 and 6).

Like SecA-L-FLAG³, HtrA-L-FLAG³ delocalized in cultures transitioning to stationary phase (Fig. 2B and 3B). Although some transition-phase cells still contained HtrA-L-FLAG³ at equators and septa, HtrA-L-FLAG³ was also located randomly over the surfaces of many cells (Fig. 3B). The localization of HtrA-L-FLAG³ to equators and septa observed in the exponentially growing parent strains (Fig. 4) was diminished in the $\Delta cls::P_c-erm$ mutant, but to a lesser extent than for SecA-L-FLAG³ (Fig. 2A and B). The mislocalization of HtrA-L-FLAG³ in the $\Delta cls::P_c-erm$ mutant was again complemented by ectopic expression of Cls⁺ (Fig. 2B). Because HtrA_{Spn} localizes to the sites of peptidoglycan (PG) biosynthesis and cell division (see references 21 and 24), we examined the cell morphologies of isogenic $\Delta htrA$ and *htrA*⁺ strains during growth at 37°C and 41.2°C in BHI broth at culture densities with OD_{620s} of 0.1 (mid-log) and 0.4 (late-log phase). We did not observe any obvious differences in cell shape, cell size, or chaining under these conditions between the two strains (data not shown).

The SrtA_{Spn} sortase locates randomly on cell surfaces. Sortase was reported to be in the ExPortal structure of some bacteria (7, 8). Consequently, we determined the distribution of SrtA-L-FLAG³ during the cell cycle. In contrast to SecA-L-FLAG³, SecY-L-FLAG³, and HtrA-L-FLAG³, SrtA-L-FLAG³ did not localize and was distributed in an apparently random punctate pattern over the surface of cells at all stages of division (see Fig. S5 in the supplemental material). This conclusion was supported by preliminary three-dimensional (3D)-reconstructed images showing SrtA-L-FLAG³ located randomly on the pneumococcal cell surface (data not shown).

SecA_{Spn}, HtrA_{Spn}, and SrtA_{Spn} locations are not affected by the *rpsL1* allele or absence of flotillin family proteins or each other. Markerless unencapsulated strains expressing SecA-L-FLAG³, HtrA-L-FLAG³, and SrtA-L-FLAG³ from their native loci (IU4468, IU4470, and IU4472; see Table S1 in the supplemental

material) contained the *rpsL1* allele imparting streptomycin (Sm) resistance used in allele exchange. We complemented the *rpsL1* mutation by a dominant P_c-[Kan^r-*rpsL*⁺] insertion into the ectopic CEP site (strains IU4648, IU4650 and IU4652) (see Table S1), restoring Sm sensitivity. The cellular locations of SecA-L-FLAG³, HtrA-L-FLAG³, and SrtA-L-FLAG³ were the same in the *rpsL*⁺/*rpsL1* complemented strains as in the *rpsL1* background (data not shown). We conclude that the *rpsL1* allele did not influence the location patterns of these proteins.

Flotillin family (also designated SPFH or PHB) proteins copurify with SecA in a detergent-resistant membrane (DRM) fraction of *S. aureus* (31). Therefore, we investigated whether the locations of SecA_{Spn}, HtrA_{Spn}, and SrtA_{Spn} were altered in mutants lacking the pneumococcal homologues of the flotillin family proteins. Two genes in *S. pneumoniae* strain D39, *spd_1962* and *spd_1984*, encode proteins containing PHB domains, identified by the SMART program (<http://smart.embl-heidelberg.de/>). Knockout of *spd_1962*, *spd_1984*, or both *spd_1962* and *spd_1984* (see Table S1 in the supplemental material) did not result in any overt growth or cell morphology defects under the conditions tested so far (data not shown). In addition, SecA-L-FLAG³, HtrA-L-FLAG³, and SrtA-L-FLAG³ showed wild-type localization patterns in the single *spd_1962* and *spd_1984* mutants and the double *spd_1962 spd_1984* mutant (data not shown). We conclude that the putative pneumococcal flotillin proteins do not influence cell division or cellular location of SecA_{Spn}, HtrA_{Spn}, and SrtA_{Spn}.

Last, we tested whether SecA_{Spn} or HtrA_{Spn} localization depended on SrtA_{Spn} and whether SecA_{Spn} and SrtA_{Spn} location depended on HtrA_{Spn}. SecA-L-FLAG³ and HtrA-L-FLAG³ showed wild-type localization patterns in an *srtA*_{Spn} mutant (data not shown) (Fig. 1 and 4; see Table S1 in the supplemental material). Likewise, the localization pattern of SecA-L-FLAG³ and the random distribution of SrtA-L-FLAG³ were the same in an *htrA*_{Spn} mutant as in the isogenic *htrA*⁺ parent (data not shown) (Fig. 1; see Fig. S5 and Table S1 in the supplemental material). The SecA_{Spn} gene is essential and could not be knocked out in these experiments. We conclude that SecA_{Spn} and HtrA_{Spn} localization does not depend on the presence of SrtA_{Spn} and SecA_{Spn} and SrtA_{Spn} localization does not depend on HtrA_{Spn}.

DISCUSSION

We show herein that the SecA and SecY translocase subunits of *S. pneumoniae* localize to mid-cell regions of actively dividing cells of *S. pneumoniae* (Fig. 1). In predivisional cells, SecA_{Spn} and SecY_{Spn} are primarily located at equators, which will develop into divisional septa. Some predivisional cells also have SecA_{Spn} and SecY_{Spn} at one pole, possibly as remnants of the previous division. As division progresses and septation begins, both Sec subunits cover the new hemispheres of cells in a distinctive “ χ ” (Chi)-like distribution between the septa and future equators of the dividing cell. This “ χ ” distribution is maintained in newly divided cells, until SecA_{Spn} and SecY_{Spn} relocate to equators. This localization of the Sec translocase subunits overlaps the regions of peptidoglycan (PG) biosynthesis in dividing pneumococcal cells (see references 21 and 24). Localization of SecA_{Spn} and SecY_{Spn} to equators and septa is lost as cells stop growing and transition to stationary phase (Fig. 3) (data not shown), which eventually induces autolysis of pneumococcal cultures.

Notably, the localization of SecA in *S. pneumoniae* reported here (Fig. 1 and 3) is markedly different from the concentration of

SecA at a single ExPortal microdomain reported previously for *S. pyogenes* (5, 6) and *E. faecalis* (7). There are two possible explanations for this difference. Although all are ovococcus species, *S. pneumoniae* is evolutionarily distant from both *S. pyogenes* and *E. faecalis* (14), and it is possible that different mechanisms of protein export are used, especially by species like *S. pyogenes* that secrete numerous toxins and superantigens (17). On the other hand, the difference may reflect the methods of detection. This study used IFM to locate Sec subunits in a *Streptococcus* species for the first time, whereas in previous studies, SecA_{Spn} and *E. faecalis* SecA (SecA_{Efa}) were detected using IG-EM techniques (5, 7, 8). It is possible that the thin sectioning or high levels of fixation used in IG-EM methods did not allow the entire distribution pattern of SecA_{Spn} and SecA_{Efa} to be detected (see reference 32). Consistent with this interpretation, other studies using the IG-EM method found SecA_{Spn} distributed over the entire surface of cells (10), similar to the pattern reported here for SecA_{Spn} in transition-phase cells (Fig. 3). Moreover, the IFM approach used here has now located several pneumococcal cytoplasmic and surface proteins in different patterns, including FtsZ, whose localization was already established (Fig. 1, 3, and 4; see Fig. S2 and S4 to S6 in the supplemental material) (21, 22, 33–35). These diverse localization patterns of different proteins argue that the IFM method is allowing sufficient access of antibody to target proteins fused to the FLAG³ epitope. Taken together, our results do not support the presence of an ExPortal in *S. pneumoniae* and indicate a dynamic distribution of the Sec translocation apparatus.

To attain their ellipsoid shape, pneumococcal cells use two modes of PG biosynthesis that extend the side walls from mid-cells and close septa between daughter cells, respectively (see references 21 and 24). Consequently, cell division proteins, including FtsZ_{Spn} (see Fig. S6 in the supplemental material), MreC_{Spn} (21), penicillin binding proteins (e.g., PBP2b_{Spn} and PBP2x_{Spn}) (24), and PcsB_{Spn} (L.-T. Sham, unpublished data), and regions of active PG biosynthesis revealed by fluorescent vancomycin staining localize to equators, septa, and mid-cell regions (see references 19 and 21), similar to SecA_{Spn} and SecY_{Spn}. The SecA motor protein plays roles in exporting proteins from cells and in secreting large hydrophilic domains of membrane-anchored proteins into the extracellular space (reviewed in reference 1). Many proteins that carry out or mediate PG biosynthesis, such as the penicillin binding proteins (PBPs), MreC, DivIB, and carboxypeptidase (see references 24, 33, and 36), contain single transmembrane domains or membrane anchors linked to large extracellular domains, which are likely exported in a SecA-dependent manner. Therefore, an attractive model that emerges is that the Sec translocase of *S. pneumoniae* directs secretion of the PG biosynthesis apparatus to regions where PG biosynthesis occurs. The protein interactions that direct SecA_{Spn} and SecY_{Spn} to equators, septa, and mid-cell regions are currently under investigation.

S. pneumoniae serotype 2 strain D39 used in this study contains only the SecA general Sec translocase pathway for protein export (20). Strain D39 lacks the accessory SecA2 system found in some serotypes of *S. pneumoniae* (37). In addition, the twin-arginine transporter (38) is absent from *S. pneumoniae*. Besides PG biosynthetic proteins, 5% of the 1,914 polypeptides encoded by strain D39 are predicted to contain signal peptides according to the LocateP program (<http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py>) (18). These proteins include secreted proteins, lipid-anchored glycoproteins, including 28 substrate binding pro-

teins of ABC transporters, and 12 of the 16 LPXTG motif-containing proteins that are anchored to the PG by sortase. Previous work with *S. aureus* (11) and *S. pyogenes* (10) suggests that signal peptides containing a YSIRK/GS amino acid motif direct the proteins to division septa. In contrast, sortase-attached proteins lacking this motif end up at cell poles (10, 11).

Six of the sortase-attached proteins of strain D39 contain sequences related to the YSIRK/GS motif. The localization patterns of SecA_{Spn}, SecY_{Spn}, and SrtA_{Spn} reported here could accommodate two modalities of localization of sortase-attached proteins. In exponentially growing cells, SecA_{Spn} and SecY_{Spn} are located over hemispheres fanning out from division septa (Fig. 1; see Fig. S2 in the supplemental material), whereas SrtA_{Spn} is distributed over the entire surface of cells (see Fig. S5 in the supplemental material), similar to a fraction of the SrtA in *S. pyogenes* (9). However, even in preliminary experiments that localize SrtA-L-FLAG³ by 3D imaging (data not shown), we did not detect any patterns of SrtA_{Spn} localization or preferential association with septa, as reported for SrtA_{Spn} (9). Nevertheless, the overlap between SecA_{Spn}, SecY_{Spn}, and SrtA_{Spn} in hemispheres of dividing cells (Fig. 1; see Fig. S2 and S5 in the supplemental material) would allow sortase-attached proteins to be directed to septa. In newly divided cells, SecA_{Spn} and SecY_{Spn} remain momentarily at cell poles (Fig. 1; see Fig. S2), and SecA_{Spn} and SecY_{Spn} spread over surfaces as cells stop dividing and enter stationary phase (Fig. 3). Either of these configurations would allow sortase-attached proteins lacking the YSIRK/GS motif to be localized to poles. Experiments are in progress to determine the localization of sortase-attached proteins in *S. pneumoniae*.

Unexpectedly, we found that the HtrA_{Spn} protein is predominantly located at the equators and septa of dividing pneumococcal cells (Fig. 4), largely coincident with SecA_{Spn} and SecY_{Spn} (Fig. 1; see Fig. S2 in the supplemental material). HtrA_{Spn} is a member of a widely conserved family of serine proteases that are crucial for protein homeostasis in extracytoplasmic compartments (39). DegP of *E. coli* is the most studied member of this family and exhibits antagonistic functions as a chaperone and protease within a single polypeptide (40, 41). The substrates of HtrA_{Spn} are unknown, but *htrA* mutants of *S. pneumoniae* show increased sensitivity to thermal and oxidative stress (25), decreased nasopharyngeal colonization (42) and virulence (25), and increased bacteriocin activity (43). Additionally, *htrA*_{Spn} appears to influence expression of pneumococcal competence (see reference 44) and is a member of the regulon controlled by the CiaRH two-component system, which responds to antibiotics that inhibit PG biosynthesis (45). The localization of HtrA_{Spn} to regions of PG biosynthesis in unstressed, exponentially growing cells, coincident with the localization of SecA_{Spn} and SecY_{Spn}, suggests that HtrA_{Spn} may play a general role in the quality control of proteins exported by the Sec translocase in *S. pneumoniae*. The basis for HtrA_{Spn} localization to division sites is unknown.

Finally, normal anionic lipid content was required for localization of SecA_{Spn} and, to a lesser extent, HtrA_{Spn} to cell equators and division septa (Fig. 2; see Fig. S4 in the supplemental material). Previously, the anionic lipids phosphatidylglycerol (PGly) and cardiolipin (CL) were shown to play important roles in protein translocation across the membranes of other bacterial species (26). SecA has a high affinity for anionic lipids (46), and its ATPase activity is enhanced by the presence of acidic phospholipids (47). CL and, to a lesser extent, PGly promote the association of SecA

and SecYEG and prime translocation (3). Consistent with these biochemical results in other bacteria, *S. pneumoniae* Δ *cls* (CL synthase) mutants, which lack CL and possibly accumulate PGly, showed mislocalization of SecA_{Spn} and HtrA_{Spn} in growing cells (Fig. 2; see Fig. S4). This mislocalization is reminiscent of the rapid disruption of *B. subtilis* SecA (SecA_{Bsu}) spirals in *B. subtilis* cells depleted of anionic lipids (4). Δ *cls* mutants of *B. subtilis* and several other bacterial species do not show growth defects under non-stressed conditions and may contain biosynthetic routes that compensate for loss of CL synthesis (3, 27, 48, 49). In contrast, *S. pneumoniae* D39 Δ *cls* mutants are impaired for growth and form chains of misshapen cells that readily lyse (see Fig. S3 and S4 in the supplemental material). The defective growth and SecA_{Spn} localization phenotypes of Δ *cls* mutants were largely complemented by ectopic expression of the *cls*⁺ gene (see Fig. S3 and S4). It remains to be determined whether the impaired growth and fragility of pneumococcal Δ *cls* mutants are caused by decreased function and localization of the Sec translocase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Unencapsulated derivatives of *Streptococcus pneumoniae* serotype 2 strain D39 were used in this study (20). Strains containing antibiotic resistance markers were constructed by transforming linear DNA amplicons synthesized by overlapping fusion PCR into competent pneumococcal cells, as described previously (29). All intermediate and final strains synthesized are listed in Table S1 in the supplemental material, and primers used for the generation of the amplicons are listed in Table S2 in the supplemental material. Strains containing markerless *secA*-L-FLAG³, *secY*-L-FLAG³, *htrA*-L-FLAG³, *srtA*-L-FLAG³, or Δ *cls* alleles in native gene loci (see Table S1) were generated using the P_c-[Kan^r-*rpsL*⁺] (Janus cassette [50]) allele replacement method as described in reference 35. Intermediate strains IU4370 (Δ *htrA*::P_c-[Kan^r-*rpsL*⁺]) and IU4372 (Δ *srtA*::P_c-[Kan^r-*rpsL*⁺]) were constructed with the deletion of *htrA* and *srtA* reading frames, except for the 60 bp at the 5' and 3' ends, and replaced with the P_c-[Kan^r-*rpsL*⁺] cassette. Since *secA* and *secY* are essential genes, the intermediate strains IU4404 (*secA*⁺ intergenic::P_c-[Kan^r-*rpsL*⁺]) and IU5445 (*secY*⁺ intergenic::P_c-[Kan^r-*rpsL*⁺]) were constructed by the insertion of the P_c-[Kan^r-*rpsL*⁺] cassette after the *secA* and *secY* coding sequences. Strains were grown on plates containing Trypticase soy agar II (TSII; modified) (Becton-Dickinson; BD) and 5% (vol/vol) defibrinated sheep blood (TSII BA) and incubated at 37°C in an atmosphere of 5% CO₂ (mixed with 95% of the normal atmospheric composition). For antibiotic selections, TSII BA plates were supplemented with 250 μg kanamycin per ml, 250 μg streptomycin per ml, or 0.3 μg erythromycin per ml. For liquid cultures, strains were cultured statically in BD brain heart infusion (BHI) broth at 37°C or 41.2°C (to test for temperature sensitivity) in an atmosphere of 5% CO₂.

Localization of SecA-L-FLAG³, SecY-L-FLAG³, HtrA-L-FLAG³, SrtA-L-FLAG³, and FtsZ-FLAG³ by immunofluorescence microscopy (IFM). IFM localization of FLAG³-tagged proteins using anti-FLAG polyclonal antibody (Sigma, F7425) and nucleoid staining with 4',6-diamidino-2-phenylindole (DAPI) were performed as described previously (35). Control experiments repeatedly showed no detectable labeling of cells that did not express a protein fused to the FLAG epitope tag (data not shown). Strains were cultured in BHI broth to mid-exponential phase (OD₆₂₀ of 0.13) or to the transition to stationary phase (OD₆₂₀ of 0.78 15 min before reaching the maximal culture OD₆₂₀ of 0.82). Stained cells were examined using a Nikon E-400 epifluorescence phase-contrast microscope. Images were captured using a CoolSNAP HQ² charge-coupled device (CCD) camera (Photometrics) and processed with NIS-Elements AR imaging software (Nikon). For the 300-ms exposure time used, micrographic images of control strain IU1824 (D39 Δ *cps* not expressing a FLAG³-tagged protein) did not show detectable labeling. The background pixel intensity corresponded to 1,000 arbitrary units (AU) (dynamic

range, 0 to 16,383), and there was no difference in background pixel intensities between areas within cells or outside cells. Micrographs of strains expressing SecA-L-FLAG³, SecY-L-FLAG³, HtrA-L-FLAG³, or SrtA-L-FLAG³ showed regions of intense labeling (Fig. 1, 3, and 4; see Fig. S2 and S4 to S6 in the supplemental material), corresponding to maximal pixel intensities ranging from 4,000 to 15,000 AU. In comparison, the pixel intensities in regions of cells not showing labeling in micrographs was close to (i.e., 1,200 AU) the background level. Validation experiments by this IFM approach using a strain expressing FtsZ-FLAG³ showed patterns of FtsZ_{Spn} localization during cell division (see Fig. S6) similar to those reported previously using anti-FtsZ_{Spn} antibody (22, 34).

Determination of distribution pattern frequencies. Unencapsulated derivatives of strain D39 grown in BHI broth, which form mainly single cells and diplococci and some short chains of 4 cells (19), were sorted into different stages of division. For each experiment, 50 to 100 cells from multiple phase-contrast microscopic fields of each strain were binned as single cells (oval cells), early divisional cells (slight indentation at mid-cell), or late-divisional cells (width of septum less than half of cell width). Images of immunofluorescently labeled cells using anti-FLAG antibody were then superimposed on phase-contrast images. Cells in each stage of division were then scored for protein localization at equators, septa, poles, hemispheres, or elsewhere. Similar localization patterns were observed in at least five independent experiments, and quantitative data were obtained from two separate experiments.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00202-11/-/DCSupplemental>.

Text S1, DOC file, 0.1 MB.
Text S2, DOC file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.2 MB.
Figure S5, PDF file, 0.1 MB.
Figure S6, PDF file, 0.1 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0.2 MB.

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