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Subcellular sites for bacterial protein export

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Summary

Most bacterial proteins destined to leave the cytoplasm are exported to extracellular compartments or imported into the cytoplasmic membrane via the highly conserved SecA-YEG pathway. In the present studies, the subcellular distributions of core components of this pathway, SecA and SecY, and of the secretory protein pre-AmyQ, were analysed using green fluorescent protein fusions, immunostaining and/or immunogold labelling techniques. It is shown that SecA, SecY and (pre-)AmyQ are located at specific sites near and/or in the cytoplasmic membrane of *Bacillus subtilis*. The localization patterns of these proteins suggest that the Sec machinery is organized in spiral-like structures along the cell, with most of the translocases organized in specific clusters along these structures. However, this localization appears to be independent of the helicoidal structures formed by the actin-like cytoskeletal proteins, MreB or Mbl. Interestingly, the specific localization of SecA is dynamic, and depends on active translation. Moreover, reducing the phosphatidylglycerol phospho-

lipids content in the bacterial membrane results in delocalization of SecA, suggesting the involvement of membrane phospholipids in the localization process. These data show for the first time that, in contrast to the recently reported uni-ExPortal site in the coccoid *Streptococcus pyogenes*, multiple sites dedicated to protein export are present in the cytoplasmic membrane of rod-shaped *B. subtilis*.

Introduction

Approximately 25% of Gram-positive bacterial proteins are predicted to be transported from the cytoplasm to the cytoplasmic membrane, the cell wall or the growth medium. Translocation of these proteins involves different transport systems such as the general secretory (Sec) pathway, the twin-arginine translocation (Tat) pathway, a pseudopilin-specific secretion and assembly pathway, or ATP-binding cassette (ABC) transporters (Tjalsma *et al.*, 2000). The highly conserved Sec pathway, which represents the main pathway for protein transport in bacteria, has been studied extensively in *Escherichia coli* and *Bacillus subtilis* (Tjalsma *et al.*, 2000; Driessen *et al.*, 2001). This pathway mediates the translocation of secretory and membrane proteins in a (partially) unfolded conformation through a channel formed by the membrane-embedded SecYEG protein complex. These proteins are synthesized with amino-terminal export signals, which are recognized by cytoplasmic export factors/chaperones involved in targeting of the proteins to the translocation machinery. The translocation of these proteins across the channel is driven by the translocation motor SecA, a peripherally bound ATPase, which interacts with its substrate proteins and has affinity for SecYEG. During translocation, the dimeric SecA protein undergoes repeated cycles of binding and hydrolysis of ATP, coupled to conformational changes driving the translocation of membrane and pre-proteins through the SecYEG complex (den Blaauwen *et al.*, 1996; Driessen *et al.*, 2001).

Although the mechanism(s) of protein translocation has been studied in great detail, knowledge about subcellular distributions of protein transport machineries is limited. The Eps type II secretion system of *Vibrio cholerae*, which mediates the secretion of virulence factors, appears to be preferentially located at the old pole of the cell (Scott *et al.*, 2001). Similarly, the IcsA protein, a member of the autotransporter family found in Gram-negative bacteria, is localized to the old cellular pole (Charles *et al.*, 2001).

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Proteins of this family have been shown to use the Sec-dependent translocation pathway to cross the cytoplasmic membrane and then autocatalyse their own insertion into or transport across the outer membrane. Furthermore, the YidC protein of *E. coli*, which interacts with the Sec machinery and mediates membrane protein insertion into the inner membrane, was also shown to be predominantly located at cell poles (Urbanus *et al.*, 2002). While these observations suggest that components of the Sec-translocon or specialized Sec-machineries are assembled at the cell poles in Gram-negative bacteria, SecY and SecE were recently shown to be uniformly distributed within the cytoplasmic membrane of *E. coli* (Brandon *et al.*, 2003). Moreover, the *B. subtilis* YidC homologues, SpoIIIJ and YqjG, were shown to be randomly distributed throughout the cytoplasmic membrane of vegetatively growing cells (Murakami *et al.*, 2002; H. Tjalsma and J.D.H. Jongbloed, unpubl. obs.). In contrast, Rosch and Caparon (2004) very recently showed that secretory proteins and the secretion motor SecA specifically localize to a microdomain distal to the cell poles of the Gram-positive coccoid *Streptococcus pyogenes*.

In the present work, we describe our studies on the subcellular localization of Sec complexes in the cytoplasmic membrane of the rod-shaped Gram-positive bacterium *B. subtilis*. For this purpose, we analysed the subcellular distribution of the machinery components SecA and SecY and the secretory protein AmyQ in this organism by fluorescence microscopy, using green fluorescent protein (GFP) fusions, immunostaining and/or immunogold labelling techniques. Our results show that *B. subtilis* Sec translocases are organized in multiple clusters located at specific sites in the cytoplasmic membrane. This is in contrast to the situations in *E. coli*, in which no subcellular localization of Sec components was observed (Brandon *et al.*, 2003), and *S. pyogenes*, which contains only a single site dedicated to protein secretion (Rosch and Caparon, 2004). Moreover, we show that the specific localization of the translocation motor SecA is not maintained throughout growth; in fact it shows a dynamic nature.

Results

Localization of SecA-GFP in vegetatively growing *B. subtilis* cells

To investigate the subcellular location of SecA, the SecA protein was fused to GFP, and the SecA-GFP-encoding gene was placed under the control of the xylose-inducible P_{xyI} promoter of plasmid pSG1154 (Lewis and Marston, 1999), resulting in plasmid pSAG1 (see also *Experimental procedures*). As *B. subtilis* cells producing a truncated form of SecA, lacking the last 22 amino acids, were shown to be viable and secretion proficient (van Wely *et al.*,

2000), we anticipated that fusing the GFP to the C-terminus of SecA would circumvent potential steric hindrance and reduce the chance of interfering with SecA function. Moreover, the disordered organization of the final 39 residues in the crystal structure of SecA from *B. subtilis* suggests that the C-terminal part of this protein is flexible (Hunt *et al.*, 2002), supporting the aforementioned hypothesis. Plasmid pSAG1 was used for integration of the *secA-gfp* gene into the *B. subtilis* 168 chromosome either via a single cross-over event at the *secA* locus, or via a double cross-over event at the *amyE* locus (the construction of strains is schematically represented in Fig. 1). Integration at the *secA* locus resulted in strain *secA::pSAG1*, which contains a wild-type copy of *secA* under the control of the xylose-inducible P_{xyI} promoter, and multiple copies of *secA-gfp*. Of these *secA-gfp* copies, one is under the control of the P_{secA} promoter, while the other(s) is/are inducible with xylose (Fig. 1A). Western blot analyses using anti-SecA antibodies revealed that the SecA-GFP protein was produced at approximately the same levels as SecA is produced in cells expressing wild-type SecA (strain *amyE::pSG1154*), when cells were grown in the absence of xylose (Fig. 2A, compare lanes 1 and 3). Integration of plasmid pSAG1 at the *amyE* locus resulted in strain *amyE::pSAG1*, which contains an unmodified copy of *secA* and the *secA-gfp* gene under the control of the P_{xyI} promoter (Fig. 1B). To determine whether the SecA-GFP fusion protein is biologically active, a mutant copy of *secA-gfp*, lacking a large 5' part of the *secA* gene, was cloned into plasmid pSG1151. By integration of the resulting plasmid pSAG2 via a single cross-over event at the *secA* locus, *B. subtilis* strain *secA::pSAG2* was constructed. As a result, the only functional copy of the essential *secA* gene is the one fused to *gfp* and expressed from the *secA* promoter (Fig. 1C). This strain showed no growth defects at 37°C or 20°C and the secretion of α -amylase and proteases in this strain was not affected, indicating that SecA-GFP is functional (data not shown). Moreover, the fact that only the full-length fusion protein could be observed in cell samples of strain *secA::pSAG2* (Fig. 2) excludes the possibility that SecA activity resulted from SecA-like degradation products lacking (major parts of) GFP.

To visualize SecA-GFP in living cells, *B. subtilis* strains *secA::pSAG1* and *amyE::pSAG1* were grown in TY medium in the absence or presence of increasing amounts of xylose. Cells from the mid-exponential growth phase were collected and examined by fluorescence microscopy. The intensity of the fluorescence signal resulting from the presence of SecA-GFP increased in both strains when xylose was added. However, when compared with strain *secA::pSAG1*, higher induction levels and higher exposure times were required to detect the fluorescence signal of SecA-GFP in cells of *B. subtilis*

Fig. 1. Construction of strains containing *secA-gfp* fusions.

A. Schematic representation of the construction of *B. subtilis* 168 *secA*::pSAG1. Plasmid pSAG1 was integrated into the chromosomal *secA* gene by a single (Campbell-type) cross-over (sco) event. The resulting strain contains one copy of *secA-gfp* under the control of the P_{secA} promoter, one copy of *secA* under the control of the xylose-inducible P_{xyI} promoter, and at least one copy of *secA-gfp* under the control of the P_{xyI} promoter.

B. Schematic representation of the construction of *B. subtilis* 168 *amyE*::pSAG1. Plasmid pSAG1 was integrated at the chromosomal *amyE* gene by a double cross-over (dco) event. The resulting strain contains an unmodified copy of *secA* and one copy of *secA-gfp* under the control of the xylose-inducible P_{xyI} promoter.

C. Schematic representation of the construction of *B. subtilis* 168 *secA*::pSAG2. Plasmid pSAG2 was integrated into the chromosomal *secA* gene by a single (Campbell-type) cross-over event. The resulting strain contains one copy of *secA-gfp* under the control of the P_{secA} promoter, and a truncated *secA* gene, encoding a non-functional form of SecA.

amyE', 3' truncated amyE gene; 'amyE, 5' truncated amyE gene; 'secA-gfp, 5' truncated secA-gfp gene; 'secA, 5' truncated secA gene.

Fig. 2. Western blot analyses of SecA-GFP and SecA production in *B. subtilis* strains *secA*::pSAG1 and *amyE*::pSAG1. Cells were grown at 37°C in TY medium until stationary growth phase in the presence of different concentrations of xylose and treated as described in *Experimental procedures*. Equal amounts of protein from total cell extracts were analysed by Western blotting using anti-SecA (top) or anti-GFP (bottom) antibodies. (A and C) *B. subtilis* strain *secA*::pSAG1. (B) *B. subtilis* strain *amyE*::pSAG1. As controls, cell extracts from *B. subtilis* *amyE*::pSG1154, which contains the *gfp* gene under the control of the P_{xy} promoter integrated into the chromosomal *amyE* locus, and total cell extracts from *B. subtilis* strain *secA*::pSAG2 were used. *B. subtilis* strain *amyE*::pSG1154 was grown in TY medium supplemented with 0.5% xylose.

amyE::pSAG1 (Fig. 3). Notably, although SecA-GFP protein levels were similar in *B. subtilis* *secA*::pSAG1 grown in the absence of xylose and *B. subtilis* *amyE*::pSAG1 grown in the presence of 0.5% xylose (Fig. 2A, lanes 1 and 3, and Fig. 2B, lanes 1, 3 and 8), the intensity of the fluorescence signal appeared to be higher in the latter strain (Fig. 3, compare B and F). Taken together, these observations suggest that the intensity of the fluorescence signal from a SecA-GFP homodimer is lower than that of a SecA/SecA-GFP heterodimer and therefore that the amounts of both SecA and SecA-GFP have to be increased to enable detection of the fluorescence emerging from the fusion protein. Indeed, it has been shown that physical interaction between two GFP molecules can result in shifts in the relative intensities of GFP emission (De Angelis *et al.*, 1998). In agreement with this hypothesis, the intensity of the fluorescence signal from SecA-GFP in *B. subtilis* strain *secA*::pSAG2, in which no wild-type SecA is present and only SecA-GFP homodimers can be formed, was very low (data not shown). Moreover, the specific localization of SecA-GFP appears less clear in cells of strain *amyE*::pSAG1 grown in the presence of 2% xylose (Fig. 3G). In these cells, SecA is produced at lower levels than SecA-GFP, as verified by Western blot experiments (data not shown), presumably resulting in the production of higher amounts

of SecA-GFP homodimers. Competition of these SecA-GFP homodimers (low fluorescence) with SecA/SecA homodimers (no fluorescence) and SecA/SecA-GFP heterodimers (high fluorescence) for binding to the translocon leads to a loss of fluorescence at the export sites. In contrast, when cells of strain *secA*::pSAG1 were grown in the presence of increasing amounts of xylose, distinct localization of SecA-GFP could be observed under all xylose concentrations tested, but with increasing levels of fluorescence signals in the cytoplasm (Fig. 3A–E). This suggests that the production of higher amounts of both SecA and SecA-GFP results in the formation of more of all three dimer variants, but judging on the relatively intense fluorescent loci still observed, with SecA/SecA-GFP heterodimers still localized at protein export sites. However, because of saturation of these export sites under these overproduction conditions, a significant portion of SecA/SecA-GFP heterodimers cannot be bound by the translocon causing the increased fluorescence in the cytoplasm. Notably, the pattern of SecA localization observed in strain *secA*::pSAG2 was identical to that observed in strain *secA*::pSAG1, when the latter strain was grown in the absence or presence of xylose. As it was easier to detect the fluorescent signal, and thus analyse the latter strain, further studies were performed with *B. subtilis* strain *secA*::pSAG1, grown in the presence of 1% xylose.

Fig. 3. Specific localization of SecA-GFP in *B. subtilis* strains *secA*::pSAG1 and *amyE*::pSAG1. *B. subtilis* cells expressing SecA-GFP were grown in TY medium until mid-exponential growth phase in the absence or presence of increasing amounts of xylose, and analysed by fluorescence microscopy.

A–E. *B. subtilis secA*::pSAG1 grown without xylose (A and B) or with 0.5% (C), 1% (D), or 2% (E) xylose.

F and G. *B. subtilis amyE*::pSAG1 grown with 0.5% (F) or 2% (G) xylose.

Exposure times applied: 3 s (A, C–E) or 6 s (B, F and G).

SecA-GFP was found to be concentrated into foci at the poles, the septum and at intermediate positions of growing cells (Fig. 4A–B). The foci appeared as pairs of dots usually not perpendicular to the long axis of the cells. This localization pattern was confirmed in comparable studies in which cells of strain *secA*::pSAG1 were grown to mid-exponential growth phase in a minimal medium, in the presence of 1% xylose, and subsequently analysed by microscopy as described for cells grown in TY medium (data not shown). Three-dimensional reconstruction of the fluorescent signals by deconvolution microscopy suggests that SecA-GFP is distributed along a helical-like pattern (Fig. 4C). If so, at least two spiral-like structures seem to be running along the longitudinal axis of the cell, with most of the protein concentrated in clusters along these structures.

Localization of SecY

To examine whether the location pattern observed for SecA-GFP is related to specific localization of the Sec machinery, we studied the cellular distribution of SecY in a similar way. For this purpose, a plasmid containing the *secY*-encoding sequence fused to the 3' end of the *gfp* gene and under the control of the P_{xyI} promoter was constructed. Next, the resulting plasmid pGY1 was used for integration of the resulting P_{xyI} -*gfp*-*secY* cassette into the *B. subtilis* chromosome via a double cross-over event at the *amyE* locus (see *Experimental procedures*). This resulted in strain *amyE*::pGY1, which contains both a wild-type copy of *secY* and a copy of *gfp*-*secY*, integrated at the *amyE* locus and under transcriptional control of the

Fig. 4. Localization of protein transport sites. *B. subtilis* cells grown in TY or minimal medium at 37°C.

A–C. Localization of SecA-GFP. (A) *B. subtilis* strain *secA::pSAG1* was grown until mid-exponential growth phase in the presence of 1% xylose, and cells were analysed by fluorescence microscopy. (B) Magnified image of three typical cells and a schematic interpretation of the subcellular SecA-GFP distribution. For reason of clarity, only one of putatively two helices is represented. (C) Image processed by deconvolution microscopy showing spiral-like structures in two typical cells expressing SecA-GFP.

D–G. Localization of GFP-SecY. *B. subtilis* strain *amyE::pGY1* was grown until the transition point between exponential and post-exponential growth in the presence of 1% xylose. (D and E) Fluorescence microscopy of cells grown in TY medium. (F) Fluorescence microscopy of cells grown in minimal medium. (G) Image processed by deconvolution microscopy showing GFP-SecY distribution in two typical cells of *B. subtilis* strain *amyE::pGY1*.

H. Fluorescence microscopy of *B. subtilis* strain bdr 524 expressing the SpoIVFB-GFP membrane protein. Cells were grown until the early exponential growth phase in the presence of 0.5% xylose.

I–K. Immunogold labelling of SecY and SpoIVFB-GFP in *B. subtilis*. (I and J) Cells of *B. subtilis* 168 were grown to transition point, freeze fractured and labelled with anti-SecY antibodies. Electron micrographs of the exoplasmic face (I) and the protoplasmic face (J) of the cytoplasmic membrane are shown. (K) Cells of *B. subtilis* strain bdr 524 were grown until mid-exponential growth phase in the presence of 0.5% xylose, freeze fractured and labelled with anti-GFP antibodies. An electron micrograph of the protoplasmic face of the cytoplasmic membrane is shown.

L. Immunofluorescence staining of fixed cells of *B. subtilis* 168 using anti-SecY antibodies and secondary Oregon Green anti-rabbit antibodies. M. Localization of (pre-)AmyQ in *B. subtilis* strain 168 (pKTH10). This strain, expressing the *B. amyloliquefaciens* α -amylase precursor pre-AmyQ, was grown until the transition phase, and cells were analysed by fluorescence microscopy. A typical example of cells after immunofluorescence microscopy using anti-AmyQ antibodies is shown.

Scale bars: 5 μ m in (immuno)fluorescence pictures, and 0.5 μ m in pictures of immunogold-labelled membranes.

xylose-inducible promoter (notably, the strain construction is comparable to that of strain *amyE::pSAG1*; Fig. 1B). To visualize GFP-SecY, *B. subtilis* strain *amyE::pGY1* was grown in TY or minimal medium in the presence of 1% xylose to the transition point from exponential to stationary growth. Cells were collected, and samples were prepared for microscopic analysis and examined as described above. A typical dotted pattern, resembling that of SecA-GFP, was visible in microscopic images of *B. subtilis* strain *amyE::pGY1*, when cells were grown either in TY medium (Fig. 4D–E; see Fig. S1 in *Supplementary material*) or in minimal medium (Fig. 4F). Moreover, deconvolution microscopy analyses of cells grown in TY medium confirmed these observations and showed a subcellular distribution of GFP-SecY that is similar to that shown for SecA-GFP. However, in contrast to SecA-GFP, which seems to be organized in continuous helices, GFP-SecY was shown to form fluorescent 'arcs' but never complete helicoidal structures (Fig. 4G; compare with Fig. 4C).

To confirm that the specific localization pattern of GFP-SecY corresponds to a biologically relevant distribution of the SecY protein, we examined the subcellular location of wild-type SecY in *B. subtilis* strain 168. For this purpose, *B. subtilis* 168 was grown as described above, and SecY was visualized by both electron microscopy of immunogold-labelled, freeze-fractured cells, and immunofluorescence microscopy of fixed, permeabilized cells using anti-SecY antibodies. As a control in the electron microscopy analyses, the distribution of SpoIVFB-GFP in cells expressing this fusion protein was studied using anti-GFP antibodies. This latter protein was selected as a control within these experiments, because it was previously shown to localize randomly into the cytoplasmic membrane when its production is induced during early exponential growth (Rudner *et al.*, 2002; Fig. 4H). As expected, a random distribution of immunogold-labelled GFP-SpoIVFB protein in freeze-fractured membranes of *B.*

subtilis bdr547 was observed (Fig. 4K). Notably, immunogold labelling of SpoIVFB-GFP could only be detected at the protoplasmic face of freeze-fractured membranes, as the GFP moiety of this fusion protein is exclusively present at that cytoplasmic side of the membrane (Fig. 4K). In contrast to the results with SpoIVFB-GFP, immunogold labels corresponding to the presence of SecY in freeze-fractured membranes of *B. subtilis* 168 show a clustered organization both at the protoplasmic and at the exoplasmic side of the cytoplasmic membrane. (Fig. 4I and J). Moreover, neighbouring SecY clusters seem to be transversely organized. In addition to these observations, immunofluorescence microscopy of *B. subtilis* 168 cells treated with SecY antibodies and Secondary Oregon Green anti-rabbit antibodies showed patterns of fluorescent pairs of dots, also suggesting a clustered cellular distribution of SecY (Fig. 4L; see Fig. S2 in *Supplementary material*). Moreover, to investigate whether SecA-GFP proteins localize near SecY clusters, the localization pattern of both proteins within single cells of *B. subtilis* *secA::pSAG1* were studied by immunofluorescence microscopy. These experiments showed that the majority of SecY and SecA-GFP colocalize in these cells (data not shown). Taken together, our observations indicate that SecY is localized in specific clusters that laterally track along the cytoplasmic membrane, apparently forming a discontinuous helicoidal structure.

Localization of export sites

In a complementary approach to localize active Sec translocase complexes, the sites at which secretory proteins are exported were determined. *B. subtilis* strains containing plasmid pKTH10, which encodes the precursor of *Bacillus amyloliquefaciens* α -amylase, produce this protein at high levels, and as a result accumulate cell-associated precursor and mature forms of AmyQ (Palva,

1982; Kontinen and Sarvas, 1988; Vitikainen *et al.*, 2001; Wahlström *et al.*, 2003). The accumulated (pre-)AmyQ, which was shown to be primarily exposed on the outer surface, is at least partly translocated suggesting that a post-translocational step is rate limiting in the secretion process (Vitikainen *et al.*, 2001). Thus, assuming that (pre-)AmyQ would be retained for a prolonged period of time in the vicinity of the translocation apparatus, *B. subtilis* 168 (pKTH10) was used to examine the subcellular localization of this Sec substrate. For this purpose, the strain was grown to mid-exponential growth phase in TY medium, and cells were collected and analysed by immunofluorescence microscopy using anti-AmyQ antibodies. As shown in Fig. 4M (for additional pictures, see Fig. S3 in *Supplementary material*), fluorescent transverse bands and dots, dispersed over the cell surface in a pattern comparable to those shown for SecA and SecY, could be visualized in *B. subtilis* strain 168 (pKTH10). This result indicates that the cellular distribution of AmyQ export sites is similar to that of the Sec machinery. In conclusion, our observations demonstrate that protein transport via the general Sec pathway occurs clustered at specific locations in cells of *B. subtilis*, and that these clusters might be organized along a helicoidal structure.

SecA-GFP localization depends on active transcription and translation

Bacillus subtilis cells normally secrete proteins at high levels in the stationary growth phase, starting shortly after transition point. To determine whether the subcellular localization of Sec components is maintained during the different growth stages, the subcellular distribution of SecA and SecY was studied at several time points during growth. For this purpose, *B. subtilis* strains *secA*::pSAG1 and *amyE*::pGY1 were grown in TY medium in the presence of 1% xylose, and cells from different growth stages were collected and examined by fluorescence microscopy. The subcellular localization of the GFP-SecY protein remained unchanged during growth (data not shown), localized as pairs of dots in the cytoplasmic membrane. Surprisingly, however, the specific localization of SecA-GFP was less apparent in cells derived from cultures reaching the stationary phase of growth, and its fluorescent signal was shown to be completely delocalized, and dispersed throughout the cytoplasm after continued growth into the stationary growth phase (Fig. 5). The possibility that delocalized fluorescence resulted from SecA-GFP degradation products lacking (large parts of) SecA

Fig. 5. Localization of SecA-GFP is growth phase dependent. *B. subtilis* strain *secA*::pSAG1 was grown in TY medium at 37°C, in the presence of 1% xylose. Samples were withdrawn at mid-exponential (A), late exponential (B), early stationary (C) and late stationary growth phase (D), and examined by fluorescence microscopy.

is highly unlikely because Western blot experiments, using anti-GFP antibodies, showed no degradation products (data not shown). This indicates that the SecA-GFP fusion protein is stable during the different growth stages.

The fact that SecA-GFP was shown to be localized specifically during exponential growth phase suggested that this localization is related to the high levels of RNA and protein synthesis during this growth phase. To test whether the delocalized fluorescence in cells at later growth stages was the result of a reduction in active RNA and protein synthesis, cells of *B. subtilis* strain *secA::pSAG1* were grown in TY medium until mid-exponential growth phase, treated for 30 min with 100 µg ml⁻¹ rifampicin or 200 µg ml⁻¹ chloramphenicol, respectively, and analysed by fluorescence microscopy (Fig. 6). As expected, DNA-DAPI staining showed that chloramphenicol treatment induced nucleoid compaction. Examination of the fluorescent signals of SecA-GFP in these cells revealed that the fusion protein was no longer localized but uniformly distributed through the cytoplasm, except for the area that is occupied by the condensed nucleoid (compare Fig. 6C and D). Moreover, in cells that showed lower levels of nucleoid compaction, dots of localized SecA-GFP can still be observed. Similar results were obtained when cells of strain *secA::pSAG1* were treated with rifampicin, which blocks mRNA synthesis, but not protein synthesis (Fig. 6F). It has to be noted that rifampicin did not result in nucleoid compaction as was previously also observed by Weber and co-workers (Fig. 6E; Weber *et al.*, 2001). However, RNA isolations showed that rifampicin treatment resulted in ≈15 times lower amounts of RNA in rifampicin-treated cells when compared with that isolated from untreated cells, indicating that RNA synthesis was significantly affected (data not shown). In contrast, RNA amounts isolated from chloramphenicol-treated cells was approximately two times lower than that isolated from untreated cells. Notably, the observed delocalization of the fluorescent signal in chloramphenicol- and rifampicin-treated cells corresponds to the intact SecA-GFP protein, as Western blot experiments showed that none of these antibiotics induced degradation of this protein (data not shown). These observations suggest that the localization of SecA at discrete sites close to the membrane is related to active transcription and translation.

Localization of Sec components in *mreB* and *mbi* mutants

Our deconvolution microscopy studies indicate that SecA and SecY of *B. subtilis* are organized in clusters along spiral-like structures. Comparable helicoidal structures have previously been described in *B. subtilis* for the actin-like cytoskeletal elements MreB and Mbl (Jones *et al.*, 2001). These proteins, visualized by immunofluorescence microscopy as bands/pairs of dots, were

shown to assemble close to the membrane into helical fibres that are required for rod-shaped growth. To investigate whether the subcellular distribution of SecA and SecY is related to the presence of this cytoskeletal structure, the localization of Sec components was examined in *B. subtilis* strains mutated in either the *mbi* or the *mreB* gene (Jones *et al.*, 2001). To study the localization of SecY, strain 2060 (Jones *et al.*, 2001), which contains a xylose-inducible *mreB* gene, was grown in TY medium and depleted for MreB by growth in the absence of xylose (for details, see *Experimental procedures*), and analysed by immunofluorescence microscopy using anti-SecY antibodies. To enable SecA-GFP localization studies in the *mbi* mutant strain 2505 (Jones *et al.*, 2001), the SecA-GFP encoding fragment of plasmid pSAG1 was cloned into plasmid pSG1164. The resulting plasmid pSAG_{cam}, containing *secA-gfp* under the control of the P_{xyi} promoter, was integrated via a single cross-over event into the chromosomal *secA* locus of *B. subtilis* strain 2505 (see also *Experimental procedures*). This resulted in *B. subtilis* strain 2505 *secA::pSAG_{cam}*, which carries a wild-type copy of *secA* under the control of the xylose-inducible promoter and a copy of *secA-gfp* under the control of the P_{secA} promoter. Finally, strain 2505 *secA::pSAG_{cam}* was grown in TY medium, and cells were collected and analysed by fluorescence microscopy. As shown in Fig. 7, the depletion of MreB or the absence of Mbl resulted in the typical modifications in cell morphology observed previously (Jones *et al.*, 2001). However, although less evident than in parental strains, both SecY in MreB-depleted cells (Fig. 7A) and SecA-GFP in *mbi* mutant cells (Fig. 7B) still localized in a dotted pattern along the entire length of the cell. Together, these observations indicate that the subcellular localization of Sec components does not depend on the presence of MreB or Mbl.

Specific localization of SecA depends on membrane phospholipids composition

SecA favours the presence of acidic phosphatidylglycerol (PG) phospholipids in the cytoplasmic membrane for optimal activity (de Vrije *et al.*, 1988). The *B. subtilis* *pgsA* gene encodes the essential enzyme phosphatidylglycerolphosphate synthase, which is involved in the biosynthesis of the acidic phospholipids PG and cardiolipin (de Mendoza *et al.*, 2002). In order to test whether PG phospholipids are determinants in the specific localization of SecA, the subcellular distribution of SecA-GFP was analysed in a *B. subtilis* strain in which the expression of the essential *pgsA* gene is under control of the IPTG-inducible P_{spac} promoter. To this end, chromosomal DNA from *B. subtilis* strain BFA2809 (Kobayashi *et al.*, 2003) was used to transform *B. subtilis* *secA::pSAG1*.

Fig. 6. Specific localization of SecA-GFP is lost upon chloramphenicol or rifampicin treatment. *B. subtilis* strain *secA::pSAG1* was grown until mid-exponential growth phase in TY medium at 37°C, in the presence of 1% xylose, cells stained with DAPI, and analysed by fluorescence microscopy.

A and B. Exponentially growing cells.

C and D. Exponentially growing cells, grown in the presence of chloramphenicol for 30 min.

E and F. Exponentially growing cells, grown in the presence of rifampicin for 30 min..

A, C and E. Blue fluorescence signal from DAPI-stained DNA.

B, D and F. Green fluorescence signal from SecA-GFP.

Fig. 7. Localization of Sec components in *B. subtilis* *mbl* or *mreB* mutants.

A and B. Immunostaining of SecY in the *mreB* mutant strain 2060, in which *mreB* expression is dependent on the presence of xylose. Cells were grown in TY medium until transition phase in the presence of 1% xylose (A), or without xylose (B).

C and D. Localization of SecA-GFP in *B. subtilis* strain 2505 (Δmbl). Cells were grown until mid-exponential growth phase in TY medium at 37°C, supplemented with 1% xylose. (C) Phase-contrast image showing the typical cell morphology of *mbl* mutants. (D) Subcellular distribution of SecA-GFP, as visualized by fluorescence microscopy.

Scale bar: 5 μ m.

Transformants were selected on TY agar plates supplemented with IPTG. When the resulting strain, *B. subtilis* *secA::pSAG1 pgsA::pMutin4*, was grown in TY medium in the presence of 100 μ M IPTG, cell size and morphology were normal. Microscopic examination of the distribution of SecA-GFP in these cells (Fig. 8A) showed that fluorescent signals were concentrated into pairs of dots regularly spread along the cytoplasmic membrane in a pattern similar to that observed for the parental strain *secA::pSAG1*. However, depletion of PgsA in the absence of IPTG resulted in curved cells, with the SecA-GFP signal dispersed throughout the cytoplasm (Fig. 8B). These observations indicate that depletion of acidic phospholipids results in the loss of specific localization of the SecA protein.

Discussion

Most bacterial extracytoplasmic proteins use the general Sec pathway for export across or integration into the cytoplasmic membrane. To date, the mechanistic properties of this transport system are known in considerable detail. Interestingly, although the general Sec pathway is highly conserved, striking functional and mechanistic differences exist, which might reveal the various strategies that bacteria have developed to export proteins. At the spatial level, the *E. coli* YidC protein, which is involved in the transfer of membrane proteins from the Sec apparatus into the membrane, locates predominantly at cell poles (Urbanus *et al.*, 2002), whereas the *B. subtilis* homologues SpoIIJ and YqjG are randomly dispersed through-

Fig. 8. The presence of acidic phospholipids is required for specific localization of SecA-GFP. Fluorescence microscopy was performed with cells grown until mid-exponential growth phase in TY medium at 37°C, supplemented with 1% xylose. *B. subtilis* strain *secA::pSAG1 psgA::pMutin4* was grown in the presence (A) or absence (B) of 100 μ M IPTG (B). Scale bar: 1 μ m.

out the cytoplasmic membrane of vegetatively growing cells (Murakami *et al.*, 2002). Moreover, studies concerning the subcellular localization of the SecY and SecE proteins of *E. coli* suggested that SecYEG translocons are uniformly distributed within the cytoplasmic membrane of this bacterium (Brandon *et al.*, 2003). In contrast, the specific localization of a single export site for secretory proteins was very recently shown in the Gram-positive coccoid *S. pyogenes* (Rosch and Caparon, 2004). Here, we show that two essential components of the Sec transport machinery, SecA and SecY, are located at specific sites in *B. subtilis*, in particular during exponential growth. These proteins, which are shown to colocalize, appear concentrated into foci at the poles, at the septum and at intermediate positions along the longitudinal axis of cells. The specific localization of SecA and SecY seems to be an intrinsic property of these proteins and not a general phenomenon of GFP-fused cytoplasmic and/or transmembrane proteins, as such fusions with the cytoplasmic CspB, CspC, CspD and HBSu proteins (Weber *et al.*, 2001), or the membrane proteins SpoIVFB (Rudner *et al.*, 2002), and SpoIIJ and YqjG (Murakami *et al.*, 2002)

showed divergent localization patterns. Moreover, in the case of SecY, the subcellular localization observed for the GFP-SecY fusion protein was further substantiated by immunofluorescence microscopy and immunogold labelling and subsequent electron microscopy analyses of the cellular distribution of wild-type SecY. In addition, immunofluorescence microscopy of vegetatively growing *B. subtilis* cells overproducing pre-AmyQ indicates that protein export sites are organized in clusters, located at discrete sites in the bacterial cytoplasmic membrane. Taken together, the studies presented here demonstrate that Sec-mediated protein transport is spatially organized and takes place at specific cellular sites in *B. subtilis* cells.

Interestingly, while comparable studies in *S. pyogenes* showed the clustered organization of the Sec machinery at a single site in the membrane (Rosch and Caparon, 2004), Sec translocons in *B. subtilis* cluster at multiple sites in the cytoplasmic membrane. Moreover, confocal fluorescence microscopy analyses combined with deconvolution of stacked images suggest that SecA and SecY of *B. subtilis* are organized in spiral-like structures running between the two opposite cellular poles in and/or in close

proximity to the cytoplasmic membrane. However, if so, SecA and SecY are not continuously distributed along these structures, but accumulate in clusters. Such helical distribution is reminiscent of the pattern observed for the *B. subtilis* proteins MreB and Mbl. MreB and Mbl assemble into helical fibres forming cytoskeletal-like structures that are required for growth into rod-shaped cells. These homologues, which are only found in rod-shaped bacteria, have been proposed to determine the elongation mode of peptidoglycan synthesis (Jones *et al.*, 2001; Carballido-Lopez and Errington, 2003). Our data indicate that the specific distribution of the Sec components does not rely on the presence of MreB or Mbl. Similarly, Shih *et al.* (2003) suggested that MinCDE and MreB are not part of the same helical structure in *E. coli*. Although the list of proteins shown to be organized in spiral-shaped structures is increasing, there is no evidence for the presence of a unique scaffold responsible for the assembly of these proteins. It should be noted, however, that the structure formed by either MreB or Mbl in the *mbl* or *mreB* mutants, respectively, could partially maintain the shape of the *B. subtilis* cell. Thus, it cannot be excluded that one of the two cytoskeletal proteins is involved in the specific location of the Sec components. Interestingly, recent studies suggest that penicillin-binding proteins, PBP3 and PBP4a, are located to the lateral cell wall, and individually or together distributed along helical-like, although not continuous, cables (Scheffers *et al.*, 2004). The specific localization of these PBPs was also shown to be independent of the internal MreB/Mbl cytoskeleton. One attractive hypothesis is that the localization of proteins involved in cell wall synthesis is related to the specific subcellular localization of Sec translocases. This, together with the observation that the secretion-dedicated microdomain of *S. pyogenes* is often associated with a nascent division septum (Rosch and Caparon, 2004), suggests that protein secretion in Gram-positive bacteria occurs at positions in the cell envelope with a less rigid composition of the cell wall, thus facilitating the diffusion of secretory proteins through this barrier.

Interestingly, the localization of SecA is dynamic. First, reducing the phosphatidylglycerol phospholipids content of the cytoplasmic membrane resulted in the loss of localization of SecA, suggesting that the membrane phospholipids composition is a determinant for the localization process. This shows that the subcellular distribution of SecA is related to its function, as SecA activity was shown to depend on acidic phospholipids (de Vrije *et al.*, 1988; Lill *et al.*, 1990). Second, the specific localization of SecA seems to be growth phase dependent, as it is only observed during exponential growth. This suggests that it depends on the high levels of synthesis of exported and membrane proteins in rapid growing cells (Vos-Scheperkeuter and Witholt, 1982; Norris and Madsen,

1995). This idea was supported by the observation that inhibition of protein synthesis by chloramphenicol and inhibition of transcription by rifampicin impaired the specific localization of the SecA protein. Interestingly, treatment of cells with chloramphenicol, rifampicin and other inhibitors of macromolecular synthesis destroys the structure created by transertion, the process of coupled transcription, translation and insertion of nascent membrane and exported proteins (Norris and Madsen, 1995; Woldringh *et al.*, 1995; Binenbaum *et al.*, 1999; Fishov and Woldringh, 1999; Woldringh, 2002). Our observations indicate that the Sec machinery, which is responsible for the insertion of membrane proteins into the cytoplasmic membrane and secretion of exported proteins, could contribute to such transertion structure. However, at this stage it is unclear whether this putative transertion structure is constrained by the specific localization of Sec machinery components, or whether the opposite occurs. Alternatively, other components and/or mechanisms might define the localization sites for protein transport in *B. subtilis*. We are currently aiming at the identification and analysis of the mechanisms and components involved in the specific subcellular localization of the Sec machinery.

Experimental procedures

Plasmids, bacterial strains and media

Table 1 lists the strains and plasmids used in this study. Strains were grown at 37°C in TY (Tryptone/Yeast extract) broth containing Bacto-Tryptone (1%), Bacto-Yeast extract (0.5%) and NaCl (1%). Minimal medium for *B. subtilis* was prepared as described previously (Tjalsma *et al.*, 1998). To visualize α -amylase and protease activity, TY agar plates were supplemented with 1% starch or 1% skim milk respectively. When required, media for *E. coli* were supplemented with ampicillin or spectinomycin (100 μ g ml⁻¹); media for *B. subtilis* were supplemented with spectinomycin (100 μ g ml⁻¹) or chloramphenicol (5 μ g ml⁻¹). To deplete for PgsA in *B. subtilis* strain *secA::pSAG1 pgsA::pMutin4* or to deplete for MreB in *B. subtilis* strain 2060, these strains were grown overnight on nutrient agar plates with inducer (100 μ M IPTG for strain *secA::pSAG1 pgsA::pMutin4*, and 1% xylose for strain 2060 respectively). Next, a colony from each plate was used to inoculate 10 ml of TY medium with or without inducer. Subsequently, cultures were diluted 100-fold after reaching an OD₆₀₀ of 0.5, and cells were grown for an additional 2 h until the respective cultures reached an OD₆₀₀ of 0.5 for *B. subtilis* strain *secA::pSAG1 pgsA::pMutin4* or an OD₆₀₀ of 0.8 for *B. subtilis* strain 2060. Finally, cells were prepared for fluorescence or immunofluorescence microscopy as described below.

DNA and RNA techniques

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and transformation of *E. coli* were carried

Table 1. Strains and plasmids.

	Genotype/relevant features	References
Plasmids		
pKTH10	Encodes α -amylase (AmyQ) of <i>B. amyloliquefaciens</i> ; 6.8 kb; Km ^r	Palva (1982)
pMutin4	Integrative vector; 8.6 kb; Ap ^r , Em ^r	Vagner <i>et al.</i> (1998)
pSG1151	<i>bla cat gfp_{mut1}</i>	Lewis and Marston (1999)
pSG1154	<i>bla amyE3' spc P_{xyI}-gfp_{mut1} amyE5'</i>	Lewis and Marston (1999)
pSG1164	<i>bla cat P_{xyI}-gfp_{mut1}</i>	Lewis and Marston (1999)
pSG1729	<i>bla amyE3' spc P_{xyI}-gfp_{mut1} amyE5'</i>	Lewis and Marston (1999)
pSAG1	Derivative of pSG1154; <i>bla amyE3' spc P_{xyI}-secA-gfp_{mut1} amyE5'</i>	This work
pSAG2	Derivative of pSAG1; <i>bla cat 'secA-gfp_{mut1}</i>	This work
pSAG _{cam}	Derivative of pSG1164; <i>bla cat P_{xyI}-secA-gfp_{mut1}</i>	This work
pGY1	Derivative of pSG1729; <i>bla amyE3' spc P_{xyI}-gfp_{mut1}-secY amyE5'</i>	This work
Strains		
<i>E. coli</i>		
MC1061	F ⁻ ; <i>araD139</i> ; Δ (<i>ara-leu</i>)7696; Δ (<i>lac</i>)X74; <i>galU</i> ; <i>galk</i> ; <i>hsdR2</i> ; <i>mcrA</i> ; <i>mcrB1</i> ; <i>rspL</i>	Wertman <i>et al.</i> (1986)
<i>B. subtilis</i>		
168	<i>trpC2</i>	Kunst <i>et al.</i> (1997)
2060	<i>trpC2 amyE::</i> (P _{xyI} -c-myc-mreBCD <i>spc</i>) <i>mreB::neo</i>	Jones <i>et al.</i> (2001)
2505	<i>trpC2 mbl::spc</i>	Jones <i>et al.</i> (2001)
BFA2809	<i>trpC2 pgsA::pMutin4</i>	Kobayashi <i>et al.</i> (2003)
bdr524	<i>amyE::</i> (P _{xyI} -spoIVFB-gfp-cam)	Rudner <i>et al.</i> (2002)
<i>secA::pSAG1</i>	<i>trpC2 secA::pSAG1 (secA-gfp_{mut1} spc P_{xyI}-secA)</i>	This work
<i>amyE::pSAG1</i>	<i>trpC2 amyE::pSAG1 (spc P_{xyI}-secA-gfp_{mut1})</i>	This work
<i>secA::pSAG2</i>	<i>trpC2 secA::pSAG2 (secA-gfp_{mut1} cat 'secA)</i>	This work
<i>amyE::pGY1</i>	<i>trpC2 amyE::pGY1 (spc P_{xyI}-gfp_{mut1}-secY)</i>	This work
2505 <i>secA::pSAG_{cam}</i>	<i>trpC2 mbl::spc secA::pSAG_{cam} (secA-gfp_{mut1} cat P_{xyI}-secA)</i>	This work
<i>secA::pSAG1</i>	<i>trpC2, secA::pSAG1 (secA-gfp_{mut1} spc P_{xyI}-secA) pgsA::pMutin4</i>	This work
<i>pgsA::pMutin4</i>		

out essentially as described (Sambrook *et al.*, 1989). Enzymes were from Roche Molecular Biochemicals. *B. subtilis* was transformed as described (Tjalsma *et al.*, 1997) and transformants were selected on solid TY medium with appropriate antibiotics. Polymerase chain reaction (PCR) was carried out with Expand DNA polymerase (Roche Molecular Biochemicals). PCR fragments were purified using the PCR purification kit of Roche Molecular Biochemicals. Southern hybridization analyses were performed using the non-radioactive ECL labelling and detection system (Amersham Biosciences). For RNA isolations, cells were collected by centrifugation and RNA was extracted using the High Pure RNA isolation kit (Roche Molecular Biochemicals). RNA concentrations and quality was checked using a 2100 Bioanalyzer (Agilent Technologies) and a nanodrop ND-1000 (Nanodrop Technologies).

Primers 5'-TTA TAA GGG CCC GGA ATT TTA AAT AAA ATG TTT-3' (forward) and 5'-ACT ACT CGA GAC GGC CGC AGC AAT T-3' (reverse) were used to amplify the *secA* gene from chromosomal DNA of *B. subtilis* 168. The resulting 2538 bp PCR fragment was purified from a 1% agarose gel, cleaved with *Apal* and *XhoI* and cloned into the corresponding sites of pSG1154 (Lewis and Marston, 1999), resulting in pSAG1. *B. subtilis* 168 *secA::pSAG1* was obtained by a Campbell-type (single cross-over) integration of plasmid pSAG1 into the chromosomal *secA* gene. Southern hybridization analysis revealed that more than one copy of the plasmid was integrated into the *secA* locus. Integration via homologous recombination of pSAG1 into the chromosomal *amyE* gene resulted in *B. subtilis* strain *amyE::pSAG1*.

To construct *B. subtilis* strain *secA::pSAG2*, plasmid pSAG1 was digested with *Clal*, the resulting 1710 bp fragment containing the 3'-part of *secA* purified from a 1% aga-

rose gel, and ligated into the *Clal* site of pSG1151 (Lewis and Marston, 1999). The resulting plasmid, pSAG2, was integrated into the genome of *B. subtilis* 168 by a single (Campbell-type) cross-over event. Finally, transformants were examined by Southern hybridization to verify that integration had occurred at the chromosomal *secA* locus. The resulting strain, *B. subtilis secA::pSAG2*, contains a truncated *secA* gene lacking the first 272 codons and expresses a SecA-GFP fusion protein.

To construct *B. subtilis* strain *amyE::pGY1*, the *secY* gene was amplified by PCR from chromosomal DNA of *B. subtilis* 168, using primers 5'-CGG GAT CCT GTT TAA AAC AAT CTC CAA C-3' (forward) and 5'-CGG AAT TCG TTT TTC ATA AAT CCA CGG-3' (reverse). The resulting 1308 bp PCR fragment was purified from a 1% agarose gel, cleaved with *BamHI* and *EcoRI* and cloned into the corresponding sites of pSG1729 (Lewis and Marston, 1999), resulting in plasmid pGY1. Finally, this plasmid was used to transform *B. subtilis* 168, in which integration via homologous recombination of pGY1 into the chromosomal *amyE* gene resulted in *B. subtilis* strain *amyE::pGY1*.

To construct *B. subtilis* strain 2505 *secA::pSAG_{cam}*, plasmid pSAG1 was cleaved with *XbaI*, and the resulting 3326 bp DNA fragment was purified from 0.8% agarose gel and ligated into the corresponding site of pSG1164 (Lewis and Marston, 1999). Next, for construction of *B. subtilis* strain 2505 *secA::pSAG_{cam}*, the resulting plasmid pSAG_{cam} was introduced in strain 2505 (Jones *et al.*, 2001). Notably, for the above-described construction of plasmids pSAG1, pSAG2, pGY1 and pSAG_{cam}, *E. coli* strain MC1061 was used as cloning host.

To construct *B. subtilis* strain *secA::pSAG1 pgsA::pMutin4*, strain *B. subtilis secA::pSAG1* was transformed with chromo-

somal DNA from strain BFA2809 (Kobayashi *et al.*, 2003) and transformants were selected for resistance to erythromycin and spectinomycin on TY agar medium supplemented with 100 μ M IPTG.

Western blot analysis and immunodetection

To detect SecA and SecA-GFP, *B. subtilis* cells were grown until mid-exponential growth phase and separated from the growth medium by centrifugation (2 min, 13 000 *g*, room temperature). Samples for SDS–10% polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as described previously (van Dijk *et al.*, 1991). SDS-PAGE was carried out as described by Laemmli (1970). After separation, proteins were transferred to PVDF membranes (Roche Molecular Biochemicals). SecA-GFP and SecA were visualized with specific anti-GFP and/or anti-SecA polyclonal antibodies and horseradish peroxidase-anti-rabbit-IgG conjugates (Amersham Biosciences) according to the manufacturer's instructions.

Fluorescence microscopy

Cells were prepared for immunofluorescence microscopy as described by Glaser *et al.* (1997) with the following modifications: all washing and blocking steps were performed in phosphate-buffered saline (PBS) containing 2% BSA (end-concentration). Anti-SecY and Anti-AmyQ antibodies were used at 1 : 850 and 1 : 400 (v/v) dilutions respectively. Secondary Oregon Green anti-rabbit antibodies or tetramethylrhodamine anti-rabbit antibodies (Molecular Probes) were used at a dilution of 1 : 850.

For fluorescence microscopy, 0.5 ml samples of cultures were withdrawn and cells were collected by centrifugation (2 min, 11 000 *g*, room temperature). Next, cells were resuspended in 100 μ l of minimal medium (Tjalsma *et al.*, 1998), and 2 μ l of this cell suspension was applied to a Polysine™ microscope slide (Omnilabo) and covered with a cover glass. For DAPI staining, 4',6'-diamidino-2-phenylindole (DAPI) was used at a final concentration of 10 μ g ml⁻¹. Fluorescence images were examined using a 100 $\times$ oil immersion objective on a Zeiss microscope (Carl Zeiss) and an Axion Vision camera (Axion Technologies), using the appropriate filters (BP546, FT580, LP590). Image overlays were made and assembly of the final figures was done using ADOBE PHOTOSHOP v. 6.0.

Deconvolution microscopy

Cells were prepared as described above and examined using Polysine™ microscope slides. Microscopy was carried out using a Zeiss 510 META-NLO laser scanning microscope (Carl Zeiss), fitted with a Plan-Apochromat 63 $\times$ N.A. 1.4 objective. Stacks of 60 optical section images were collected at spacings of 176 nm, using standard filter sets for GFP (488 nm excitation and bandpass filter 500–530 nm emission). Images were deconvoluted through 15 iterations using the Huygens Pro deconvolution software (Scientific Volume Imaging). Three-dimensional structures were modelled using the Amira-3.0 software (TGS).

Freeze-fracture electron microscopy

Cells were grown in TY medium until mid-exponential growth phase, concentrated by centrifugation, washed twice with growth medium and resuspended in 10% (v/v) of the initial volume of growth medium containing 15% (w/v) glycerol. Aliquots were enclosed between two 0.1 mm copper profiles as used for the sandwich double-replica technique. The sandwiches were rapidly frozen by plunging them into liquid propane, cooled by liquid nitrogen. Freeze-fracturing was performed in a BAF400T (BAL-TEC, Liechtenstein) freeze-fracture unit at –150°C using a double-replica stage. The fractured samples were shadowed without etching with 2.0–2.5 nm platinum/carbon at an angle of 35°. The evaporation of platinum/carbon with electron guns was controlled by a thin-layer quartz crystal monitor.

Freeze-fracture labelling

For freeze-fracture immunogold labelling and subsequent electron microscopy, the freeze-fracture replica were transferred to a digesting solution (2.5% SDS in 10 mM Tris buffer, pH 8.3, and 30 mM sucrose) and incubated overnight (Fujimoto, 1997). The replica were washed four times in PBS buffer and treated with PBS + 1% bovine serum albumin (BSA) for 30 min. Next, replica were incubated for 1 h with PBS containing 0.5% BSA and monospecific antibodies against SecY or GFP (dilution 1 : 20). Subsequently, replica were washed four times with PBS and incubated for 1 h with a 1 : 50 diluted solution of the second gold-conjugated antibody (goat anti-rabbit IgG with 10 nm of gold; British Biocell International) in PBS containing 0.5% BSA. After immunogold labelling, the replica were immediately rinsed several times in PBS, fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature, washed four times in distilled water and finally picked onto Formvar-coated grids for viewing in an EM 902 electron microscope (Zeiss).

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Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4278/mmi4278sm.htm>

Fig. S1. Specific localization of GFP-SecY in *B. subtilis* strains amyE::pGY1. *B. subtilis* cells expressing GFP-SecY were grown in TY medium until the transition point between exponential and post-exponential growth in the presence of 1% xylose, and analysed by fluorescence microscopy.

Fig. S2. Localization of SecY in wild-type *B. subtilis* cells. Immunofluorescence staining of fixed cells of *B. subtilis* 168 using anti-SecY antibodies and secondary Oregon Green anti-rabbit antibodies.

Fig. S3. Localization of (pre-)AmyQ in *B. subtilis* strain 168 (pKTH10). Cells were grown until the transition phase, and analysed by immunofluorescence microscopy using anti-AmyQ antibodies.

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