**Functional Implementation of the Posttranslational SecB-SecA Protein-Targeting Pathway in Bacillus subtilis**

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*Bacillus subtilis* and its close relatives are widely used in industry for the Sec-dependent secretory production of proteins. Like other Gram-positive bacteria, *B. subtilis* does not possess SecB, a dedicated targeting chaperone that posttranslationally delivers exported proteins to the SecA component of the translocase. In the present study, we have implemented a functional SecB-dependent protein-targeting pathway into *B. subtilis* by coexpressing SecB from *Escherichia coli* together with a SecA hybrid protein in which the carboxyl-terminal 32 amino acids of the *B. subtilis* SecA were replaced by the corresponding part of SecA from *E. coli*. In *vitro* pulldown experiments showed that, in contrast to *B. subtilis* SecA, the hybrid SecA protein gained the ability to efficiently bind to *E. coli* SecB, suggesting that the structural details of the extreme C-terminal region of SecA constitute a crucial SecB binding specificity determinant. Using a poorly exported mutant maltose binding protein (MalE11) and alkaline phosphatase (PhoA) as model proteins, we could demonstrate that the secretion of both proteins by *B. subtilis* was significantly enhanced in the presence of the artificial protein targeting pathway. Mutations in SecB that do not influence its chaperone activity but prevent its interaction with SecA abolished the secretion stimulation of both proteins, demonstrating that the implemented pathway in fact critically depends on the SecB targeting function. From a biotechnological view, our results open up a new strategy for the improvement of Gram-positive bacterial host systems for the secretory production of heterologous proteins.

In most bacteria, the major route of protein transport across the cytoplasmic membrane is the general secretion (Sec) pathway (24, 40). Powered by the translocation motor SecA (32), Sec-dependent proteins are translocated across the membrane through a protein-conducting channel (SecYEG) (19). Sec substrates usually are synthesized as precursor proteins possessing an amino-terminal signal peptide. When nascent Sec substrates emerge from the ribosome, they are recognized and targeted to the Sec translocase either by the signal recognition particle (SRP)-mediated cotranslational pathway (6, 12) or, alternatively, by a posttranslational targeting mechanism that involves the SecB chaperone (1, 29, 44). SecB is found in alpha-, beta-, and gamma-proteobacteria, including the Gram-negative model bacterium *Escherichia coli*, while it is absent from Gram-positive bacteria (36). SecB is a secretion-dedicated chaperone that interacts with newly synthesized precursor proteins and maintains them in a translocation-competent state. Besides this function as an anti-folding factor, SecB also acts as a targeting factor that directly binds to the membrane-bound SecA motor protein and thereby delivers the substrate to the export sites. For the high-affinity SecB-SecA interaction, a binding contact between the negatively charged flat β-sheet surface of SecB and the zinc-containing C-terminal 22 amino acids of SecA is of crucial importance (3, 11, 43). The C-terminal region of SecA is highly conserved in bacteria, even in organisms that do not possess a SecB homologue, such as the Gram-positive model organism *Bacillus subtilis* (35). In fact, it has been shown previously that the C-terminal 22 amino acids of *B. subtilis* SecA were able to bind the *E. coli* SecB (37).

Due to their enormous secretion capacity, *B. subtilis* and some of its close relatives are widely used in industry for the secretory production of various endogenous enzymes (33). However, attempts to use these organisms for secreting heterologous proteins often have led to disappointing results (27). For example, it has been found that the SecB-dependent maltose-binding protein (MalE) from *E. coli* was inefficiently translocated across the *B. subtilis* plasma membrane (4, 5). It has been speculated that the lack of SecB contributes to the relatively poor translocation efficiency of MalE in *B. subtilis*. In fact, the coexpression of *E. coli* SecB in *B. subtilis* resulted in an approximately 2-fold enhancement of MalE precursor processing, an effect that has been attributed to the anti-folding activity of SecB (5).

In the present study, we have investigated the requirements for the installation of a functional SecB-SecA protein-targeting pathway in *B. subtilis*. In *vitro* interaction analysis revealed that the full-length *B. subtilis* SecA protein did not efficiently bind to either the *E. coli* SecB or the *Haemophilus influenzae* SecB. In contrast, hybrid SecA proteins beSecA (for *B. subtilis-E. coli* SecA) and bhSecA (for *B. subtilis-H. influenzae* SecA), in which the C-terminal 32 amino acids of the *B. subtilis* SecA have been replaced by the counterpart of the SecA proteins from *E. coli* or *H. influenzae*, respectively, efficiently bound SecB from *E. coli* or from *H. influenzae*, indicating that the structural details of the C
TABLE 1 Strains and plasmids used in this study

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a) ecSecB, E. coli SecB; hiSecA, H. influenzae SecB; bsSecA, B. subtilis SecA; ecSecA, E. coli SecA; hiSecA, H. influenzae SecA; Ap+, ampicillin resistance; Cm+, chloramphenicol resistance; Em+, erythromycin resistance; Km+, kanamycin resistance; T7p, T7 promoter; tacp, tac promoter; xylAp, xylose-inducible promoter; HpaIlp, HpaI promoter; GST, glutathione S-transferase.

b) BGSC, Bacillus Genetics Stock Center.

tail of the SecA proteins dictate whether it can tightly bind to the SecB chaperone or not. Using a poorly exported MalE variant (MalE11) as a reporter protein, we have shown that the expression of beSecA and E. coli SecB in B. subtilis resulted in a significant improvement of MalE11 secretion, and that this improvement was dependent on the SecB targeting function. Interestingly, similar secretion stimulation also was observed for the SecB-independent alkaline phosphatase (PhoA) from E. coli. Taken together, our results indicate that we successfully introduced a functional SecB-SecA protein targeting pathway into B. subtilis, and that this pathway can improve the secretory production of heterologous proteins.

MATERIALS AND METHODS

Plasmids, bacterial strains, media, and growth conditions. The plasmids and bacterial strains used in this study are listed in Table 1. E. coli and B. subtilis strains were grown in Luria-Bertani (LB) medium consisting of 1% tryptone, 0.5% yeast extract, and 1% NaCl. If appropriate, media were supplemented with 100 μg ml⁻¹ ampicillin, 100 μg ml⁻¹ kanamycin, 5 μg ml⁻¹ chloramphenicol, or 5 μg ml⁻¹ erythromycin. secB gene expression in B. subtilis was induced by the addition of 0.5% (wt/vol) xylose unless indicated otherwise.

Bacterial strain constructions. B. subtilis LD1 containing the E. coli secB gene under the regulatory control of the xylose-inducible promoter xylAp integrated into the lacA locus of the chromosome was constructed by transforming B. subtilis 168 with integration plasmid pAX01-ecSecB, followed by the selection of erythromycin-resistant colonies. B. subtilis LD2, LD3, and LD4 were constructed by the same procedure using the integration plasmids pAX01-ecSecBL75Q, pAX01-ecSecBE77K, and pAX01-ecSecBL75Q&E77K, respectively.

Plasmid constructions. The primers used in this study are listed in Table S1 in the supplemental material. For protein purification purposes in E. coli, the various SecA and SecB proteins were expressed in E. coli as...
fusion proteins containing an N-terminal His8 tag that is cleavable by tobacco etch virus (TEV) protease. To construct pSJ3-ecSecA, the E. coli secA gene was amplified using primers ecSecA-N5/ecSecA-B3 and E. coli W3110 genomic DNA as the template. The resulting PCR fragment was digested with NdeI/BamHI and ligated into pSJ3 digested with NdeI/HindIII and ligated into psJ3 digested with NdeI/BamHI. pSJ3-bsSecA was constructed by amplifying the E. coli secA gene using primers bsSecA-B5/bsSecA-X3 and pTiDiv (20) as the template. The resulting PCR fragment was cleaved with BamHI/Xhol and ligated into psJ3. To construct pSJ3-hiSecA, the H. influenzae secA gene was amplified using primers hiSecA-N5/hiSecA-H3 and H. influenzae genomic DNA as the template. The resulting PCR fragment was digested with NdeI/HindIII and ligated into psJ3.

The plasmids for the expression of chimeric SecA proteins in E. coli were constructed by megaprimer PCR (31). To construct pSJ3-heSecA, two rounds of PCR were performed. First, a DNA fragment encoding amino acid residues 870 to 901 from the C terminus of E. coli SecA was amplified using primers heSecA-mp5p/ecSecA-B3 and pSJ3-ecSecA as the template. The PCR product fragment was used as the megaprimer in the second PCR together with primer hiSecA-N5 and pSJ3-hiSecA as the template. The new PCR fragment was digested with NdeI/BamHI and ligated into psJ3 digested with the same enzymes to generate pSJ3-heSecA. To construct pSJ3-hiSecA, the megaprimer encoding amino acid residues 867 to 901 from the C terminus of H. influenzae SecA was amplified using primers ehSecA-mp5p/ecSecA-H3 and pSJ3-hiSecA as the template. Next, the megaprimer and ecSecA-N5 were used to amplify the fragment encoding the E. coli-H. influenzae hybrid protein (ehSecA) using pSJ3-ecSecA as the template. The PCR product was restricted with NdeI/HindIII and ligated into NdeI/HindIII-digested psJ3. To construct pSJ3-beSecA, the megaprimer encoding amino acid residues 870 to 901 from the C terminus of E. coli SecA was amplified using primers beSecA-mp5p/ecSecA-B3 and pSJ3-ecSecA as the template. Next, the megaprimer and bsSecA-B5 were used to amplify the fragment encoding beSecA using pSJ3-bsSecA as the template. The PCR product was restricted with BamHI and ligated into BamHI-digested psJ3. To construct pSJ3-hiSecA, the megaprimer encoding amino acid residues 867 to 901 from the C terminus of H. influenzae SecA was amplified using primers bhSecA-mp5p/hiSecA-H3 and pSJ3-hiSecA as the template. Next, the megaprimer and bsSecA-B5 were used to amplify the fragment encoding bhSecA using pSJ3-bsSecA as the template. The PCR product was restricted with BamHI/HindIII and ligated into BamHI/HindIII-digested psJ3. pSJ3-hiScB, encoding H. influenzae SecB (hiSecB), was constructed as described previously (39). pSJ2-ecSecB was constructed to express SecB in E. coli (ecSecB). First, the E. coli secB gene was amplified by PCR using primers ecSecB-B5/ecSecB-H3 and E. coli W3110 genomic DNA as the template. The resulting PCR fragment was digested with BamHI/HindIII and ligated into psJ2 digested with the same enzymes.

The construction of pGEX-2T-hiSecAc, which encodes glutathione S-transferase (GST) fused to residues 878 to 901 of hiSecAc, was described previously (43). To construct pGEX-2T-hiSecAc, which encodes GST fused to residues 878 to 901 of E. coli SecA (ecSecA), the DNA fragment encoding residues 878 to 901 of ecSecA was amplified using primers ecSecA-A5/ebSecAc-A3 and pSJ3-ecSecA as the template. The resulting PCR fragment was digested with BamHI/EcoRI and ligated into pGEX-2T digested with the same enzymes. The construction of pGEX-2T-bsSecA, which encodes glutathione S-transferase fused to residues 878 to 901 of bsSecA, was described previously (43). To construct pGEX-2T-bsSecA, which encodes GST fused to residues 878 to 901 of E. coli SecA (ecSecA), the DNA fragment encoding residues 878 to 901 of ecSecA was amplified using primers ecSecA-A5/ebSecAc-A3 and pSJ3-ecSecA as the template. The resulting PCR fragment was digested with BamHI/EcoRI and ligated into psJ2 digested with the same enzymes.

To construct SecA proteins in B. subtilis, the corresponding secA genes were cloned into pOE, a PHMC05 derivative (the original spc expression cassette was replaced by the constitutive promoter HpaIIp) that is compatible with pMA5 to coexist in B. subtilis. pOE-bsSecAc was constructed by amplifying the B. subtilis secA gene using primers bsSecAcA1/bsSecAcA2 and pSJ3-bsSecAcA2 as the template. The resulting PCR fragment was digested with KpnI and ligated into KpnI-digested pOE. pOE-bsSecAc was constructed identically using primers bsSecAcA1/bsSecAcA2 and B. subtilis genomic DNA as the template.

To express wild-type and mutant ecSecB proteins in B. subtilis, the corresponding genes were cloned into the integration vector pAX01 (13) under the control of the xylose-inducible xyIA promoter. First, the wild-type secB gene was amplified using primers ecSecB0/ecSecB1 and E. coli W3110 genomic DNA as the template. The obtained PCR fragment was cleaved with BamHI and ligated into BamHI-digested pAX01, resulting in pAX01-ecSecB. To introduce the mutation L75Q into ecSecB, overlap extension PCR was used. First, two DNA fragments were amplified using E. coli W3110 genomic DNA as the template and primer pairs ecSecB6/ecSecB7 and ecSecBL75Qf/ecSecBr, respectively. Subsequently, the full-length gene for ecSecBL75Q was assembled in the second round of PCR using primers ecSecB7f/ecSecB7r and a mixture of the two PCR fragments described above as the template. The obtained PCR fragment was cleaved with BamHI and ligated into BamHI-digested pAX01, resulting in pAX01-ecSecB7Q5. pAX01-ecSecB77K and pAX01-ecSecBL75QfE77K were constructed identically, except that the primer ecSecBL75Qf was replaced by ecSecB77Kf and ecSecBL75QfE77Kf, respectively.

To express E. coli wild-type MalE in B. subtilis, pMA5-ecMalE was constructed as follows. Primers ecMalE1f/ecMalE1r were used to amplify the malE gene using E. coli genomic DNA as the template. The PCR fragment was digested with NdeI/HindIII and cloned into pMA5 (8) treated with the same enzymes. To express MalE11 (harboring the mutations K2T and K4T in the signal peptide and E29G in the early mature region) in B. subtilis, pMA5-ecMalE11 was constructed by overlap extension PCR. First, two separate PCR products were used, E. coli W3110 genomic DNA as the template to perform the amplification of the DNA fragment encoding the mutated signal peptide using primers ecMalE11spf/ecMalE11spr and the mutated mature MalE using primers ecMalE11spf/ecMalE11mr, respectively. In the following step, the full-length malE11 gene was assembled using a mixture of the two overlapping PCR fragments described above as the template and primers ecMalE11spf/ecMalE11mr. The resulting PCR fragment was cleaved with NdeI/HindIII and ligated into pMA5 digested with the same enzymes.

To express E. coli alkaline phosphatase (PhoA) in B. subtilis, the phoA gene was amplified using primers ecPhoAf/ecPhoAr and E. coli W3110 genomic DNA as the template. The resulting PCR fragment was cleaved with NdeI/HindIII and ligated into pMA5 digested with the same enzymes, resulting in pMA5-ecPhoA.

**Protein expression and purification.** The different His-tagged SecA proteins were expressed in E. coli as described previously (43), with slight modifications. E. coli BL21(DE3) strains transformed with pSJ3-derived plasmids expressing the different SecA proteins were grown at 37°C in LB medium supplemented with 100 mM ZnCl2. Protein production was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 20°C when the cells reached an optical density of 0.6 at 600 nm (OD600). The cells were harvested 20 h after IPTG induction.

The recombinant SecA proteins for isotopic titration calorimetry (ITC) were purified by two rounds of nickel affinity chromatography followed by gel filtration chromatography. In brief, the His-tagged SecA proteins purified from the first nickel affinity chromatography were digested with TEV protease overnight at 4°C to remove the His tags. The cleaved His tags, further protein contaminants, and the His-tagged TEV protease were removed by the second nickel affinity chromatography. Proteins from the flowthrough were pooled, concentrated, and run on a Superdex 200 16/60 column (GE Healthcare) with 25 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM MgCl2, 10 μM ZnCl2, and 1 mM diithiothreitol (DTT). Protein concentrations were determined by measuring the OD280 of SecB proteins and GST fused to the C-terminal 22 amino acids of SecA proteins (SecAc) were expressed and purified as described previously (39, 43).

**In vitro SecB-SecA binding assay.** The interaction between His-tagged SecA and SecB proteins was analyzed by a pulldown assay as described previously (43). To analyze the interaction between GST-SecAc and SecB proteins, purified GST-hiSecAc (2.5 mg ml⁻¹) or GST-ecSecAc (2.5 mg ml⁻¹) and hiSecB (14 mg ml⁻¹) or ecSecB (12.5 mg ml⁻¹) were used at the indicated concentrations. Thirty μl GST-hiSecAc or GST-
ecSecA, 9 μl hiSecB or ecSecB, and 61 μl phosphate-buffered saline (PBS) buffer (150 mM NaCl, 16 mM Na₂HPO₄, pH 7.3, 1 mM DTT) were mixed and incubated at room temperature for 10 min. Thirty μl of glutathione agarose beads (equilibrated with PBS already) was added, and the mixture was left on ice for another 10 min. The beads were sedimented by centrifugation for 5 min at 5,000 × g at 4°C, followed by three washing steps with 200 μl PBS buffer. The elution of the bound proteins from the beads was done by resuspending the beads in 50 μl 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione. The beads were centrifuged down, and the resulting supernatant was analyzed subsequently by 20% SDS-PAGE.

ITC. ITC was performed at 20°C with a Microcal (Piscataway, NJ) ITC200 calorimeter calibrated according to the manufacturer’s instructions. All of the protein samples were dialyzed against the ITC buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl) before use. The sample cell (200 μl) was loaded with B. subtilis SecA (bsSecA; 32 μM), ecSecA (13.3 μM), or beSecA (35 μM). ecSecB (376 μM for bsSecA, 251 μM for ecSecA, and beSecA) in the syringe was added in a sequence of 20 to 30 injections of 1.2 μl (for bsSecA), 2 μl (for ecSecA), or 1.33 μl (for beSecA) at 3-min intervals. The data were analyzed with the Origin 7.0 software supplied with the instrument.

Protein localization and Western blotting. B. subtilis whole-cell extracts and supernatant fractions were prepared as described previously (21), with minor modifications. From 1 ml culture, cells and supernatant were separated by centrifugation. The cell pellet was washed once with lysis buffer (10 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 200 mM NaCl) and resuspended in 100 μl lysis buffer. For the disruption of the cells, lysozyme was added to a final concentration of 5 mg ml⁻¹, and the mixture was incubated for 30 min at 37°C. The proteins in the supernatant were precipitated by adding 10% trichloroacetic acid for 12 h at 4°C. The distribution of MalE- or PhoA-derived polypeptides in the corresponding fractions was analyzed by SDS-PAGE and Western blotting using MalE- or PhoA-specific antibody and the Western blotting detection kit (Amer sham Biosciences) as described previously (2). The chemiluminescent protein bands were recorded using a charge-coupled device (CCD) camera and the image analyzing system Fujifilm LAS-1000 (Fuji Photo Film).

Enzyme assay. PhoA activity was determined as described previously (7).

RESULTS
The extreme carboxyl terminus (C tail) of SecA is an important SecB binding specificity determinant. As the first step for the establishment of a functional SecB-SecA protein targeting pathway in B. subtilis, we analyzed whether the full-length B. subtilis SecA protein (bsSecA) is able to interact with SecB from E. coli (ecSecB) or from H. influenzae (hiSecB) in an in vitro binding assay. In contrast to the C-terminal 22 amino acids of bsSecA (bsSecAc) that could bind ecSecB in the context of a GST-bsSecAc fusion (37), no such interaction between a His-tagged version of full-length bsSecA and ecSecB or hiSecB could be detected (Fig. 1A, lanes 2 and 3). Next, two hybrid SecA proteins were constructed in which the C-terminal 32 amino acids of bsSecA were replaced by the corresponding residues of SecA from E. coli (resulting in beSecA) or H. influenzae (resulting in bhSecA), respectively (Fig. 2). Strikingly, the beSecA and the bhSecA hybrid proteins gained the ability to interact with ecSecB (Fig. 1A, lanes 5 and 7). Furthermore, bhSecA (but not beSecA) also could bind hiSecB (Fig. 1A, lanes 4 and 6). A similar scenario was observed when the C tail was swapped between the SecA proteins of E. coli (ecSecA) and H. influenzae (hiSecA). Also in this case, the C tail derived from ecSecA conferred the binding of H. influenzae-E. coli SecA (heSecA) (Fig. 2) to ecSecB but not hiSecB, whereas the C tail from hiSecA promoted the binding of ehSecA (Fig. 2) to both ecSecB and hiSecB (see Fig. S1 in the supplemental material). Interestingly, the C tails alone (SecAc) did not show any SecB preference, since both ecSecAc and hiSecAc efficiently interacted with ecSecB and hiSecB (see Fig. S2 in the supplemental material). These results suggested that the structural details of the conserved zinc-containing C tail strongly influence the specificity as well as the binding affinity between the respective full-length SecA proteins and SecB proteins from different species.

To further confirm our hypothesis, the binding affinities of ecSecB to three different SecA proteins were determined by isothermal titration calorimetry (ITC). The dissociation constant for the binding of ecSecB to bsSecA was in the submillimolar range (K_d = 0.17 ± 0.03 mM) (Fig. 1B, left), which is 200-fold higher than the dissociation constant observed for the binding between ecSecB and ecSecA (K_d = 0.8 ± 0.3 μM) (Fig. 1B, middle). The binding affinity between ecSecB and beSecA (K_d = 2.2 ± 0.4 μM) (Fig. 1B, right) was comparable to that between ecSecB and ecSecA and significantly higher than the affinity between ecSecB and bsSecA. The determined affinity constants are consistent with the results obtained in the in vitro binding assay described above.

Coexpression of ecSecB and beSecA in B. subtilis improves secretion of an inefficiently exported heterologous model protein. Since the results described above have shown that the beSecA hybrid protein can bind to ecSecB in vitro, we investigated whether the coexpression of these two proteins in B. subtilis results in a functional SecB-SecA protein-targeting pathway in vivo. For the expression of ecSecB in B. subtilis, its gene was cloned under the control of a xylose-inducible promoter and subsequently integrated into the lacA locus in the chromosome. In the corresponding xylose-induced cells, ecSecB can be detected in the cell (C) fraction (Fig. 3B). For the expression of bsSecA or beSecA in B. subtilis, a plasmid-based expression system containing the respective secA genes under the control of a constitutive promoter (HpaIIp) was used. The expression of plasmid-encoded SecA proteins in addition to the chromosomally encoded bsSecA resulted in increased amounts of total SecA protein in the C fraction (Fig. 3B). As a model Sec protein, we chose a variant of the SecB-dependent E. coli maltose-binding protein (MalE11) that possesses three amino acid alterations to convert the protein into a less efficient Sec substrate (Fig. 3A). When MalE11 was expressed in B. subtilis, small amounts of secreted mature MalE protein could be detected in the supernatant (S) fraction (Fig. 3B, panel 1). In the C fraction of the same strain, a mature-sized form of MalE was present which might correspond to the translocated and processed MalE that has not been released from the cell wall or, alternatively, to a nonexported degradation product of the MalE11 precursor in which the signal peptide has been clipped off by cytosolic proteases. Importantly, however, a significant amount of full-length MalE11 precursor was found to accumulate in the C fraction, suggesting that MalE11 export in B. subtilis occurred by an inefficient and most likely posttranslational mode of membrane translocation.

Expression of ecSecB, bsSecA, or beSecA alone had no effect on the secretion of MalE11 in B. subtilis (Fig. 3B, compare panel 1 to panels 2, 3, and 5). In contrast, the coexpression of ecSecB with bsSecA resulted in a slight increase in MalE11 secretion (Fig. 3B, panel 4), indicating that the weak interaction between bsSecA and ecSecB allowed a slightly improved targeting of the MalE11 precursor to the translocase. Strikingly, however, the coexpression of ecSecB and beSecA with MalE11 resulted in a clear improvement...
of MalE11 secretion, since significantly more mature MalE protein was present in the S fraction and the amount of accumulated precursor in the C fraction was decreased (Fig. 3B, panel 6). Furthermore, the modulation of the amount of ecSecB in the cell by varying the concentration of the inducer xylose clearly showed that the beSecA-dependent stimulation of MalE11 secretion correlated with ecSecB availability (Fig. 3C, panels 4 to 6). Taken together, these findings suggest that a functional SecB-mediated protein-targeting pathway is operational in B. subtilis cells coexpressing ecSecB and the SecB-binding-proficient beSecA hybrid protein.

**Improved secretion of MalE11 in B. subtilis is dependent on the SecB-targeting function.** To further support our view that the stimulation of MalE11 secretion by the coexpression of ecSecB and beSecA was due to a functional SecB-mediated protein-targeting pathway, we analyzed the effect of SecB mutations (L75Q and E77K) that are known to cause a marked reduction in the affinity of SecB for SecA but do not affect its substrate.

![Figure 1](http://aem.asm.org/)

**FIG 1** beSecA hybrid protein efficiently binds ecSecB. (A) Analysis of SecA-SecB interactions by an in vitro pulldown assay. The SecA and SecB proteins analyzed in each experiment are indicated by a plus sign above the respective lanes. SecA, position of the corresponding SecA proteins; hiSecB, SecB from H. influenzae; ecSecB, SecB from E. coli; bsSecA, SecA from B. subtilis; beSecA, B. subtilis-E. coli SecA hybrid protein; bhSecA, B. subtilis-H. influenzae SecA hybrid protein. Lane 1, molecular size markers. (B) Determination of SecA-SecB dissociation constants by isothermal titration calorimetry (ITC). ITC assays of ecSecB interacting with bsSecA, ecSecA, and beSecA were performed at 20°C in triplicate for each interaction. One of the respective experiments is shown for each interaction. Integration of the raw data (top of panel B) yielded the heat in kcal/mol of SecB injected versus the SecB-to-SecA molar ratio (bottom of panel B). The dissociation constant we determined for the complex of ecSecA and ecSecB ($K_d = 0.8 \pm 0.3 \mu M$) is slightly different from the value ($K_d = 1.7 \pm 0.2 \mu M$) described previously (26), which most likely is due to differences in the temperature and the buffers used for ITC between the two experiments and the scatter in our data from the ITC200 titration.
binding ability (10, 23, 38). As shown in the lower part of Fig. 4, the SecB mutant proteins ecSecBL75Q, ecSecBE77K, and ecSecBL75Q&E77K were expressed at a level comparable to that of the wild-type ecSecB protein (compare panel 3 to panels 4 to 6). In contrast to the wild-type ecSecB, these mutants no longer promoted an improved secretion of MalE11 when coexpressed with beSecA (Fig. 4, panels 3 to 6), as indicated by a reduction of the amount of mature MalE in the S fraction and a concomitant increase in the amount of accumulated MalE11 precursor in the cytosol. From these results, we conclude that the ecSecB-beSecA interaction-dependent protein-targeting pathway delivers ecSecB-bound MalE11 precursor to beSecA protein and thereby improves MalE11 secretion in B. subtilis.

The B. subtilis ecSecB-beSecA targeting pathway improves the secretion of a SecB-independent heterologous model protein. Unlike MalE, the E. coli alkaline phosphatase PhoA is considered to be exported in a SecB-independent manner, at least under normal growth conditions (16, 18). To analyze whether the artificially introduced SecB-mediated targeting pathway can be beneficial for the secretion of normally SecB-independent proteins, the PhoA precursor was coexpressed with different combinations of ecSecB, bsSecA, and beSecA in B. subtilis, and the amount of secreted PhoA in the culture supernatant was determined by measuring alkaline phosphatase activity and by Western blot analysis. As shown in Fig. 5A, without the coexpression of Sec components, the basal level of PhoA activity of 220 U ml−1 OD600−1 was found in the culture supernatant of the respective cells (lane 1). Although the expression of ecSecB alone did not result in an improvement in PhoA secretion (lane 2), the overexpression of either bsSecA (lane 3) or beSecA (lane 5) alone increased the secretion of PhoA by approximately 30%. When ecSecB and bsSecA were coexpressed, no further improvement was observed (lane 4). In contrast, the presence of ecSecB together with beSecA increased the PhoA secretion amount up to 60%, indicating that the targeting of ecSecB-bound PhoA precursor to beSecA also contributes significantly to the total increase in PhoA activity. This notion was further supported by the finding that the

FIG 2 SecA hybrid proteins. (A) Overall domain structure of SecA. The functional SecA domains and subdomains are indicated as defined previously (15, 25). NBD, nucleotide binding domain; PBD, preprotein binding domain; IRA2, intramolecular regulator of ATPase 2; SD, scaffold domain, WD, wing domain; IRA1, intramolecular regulator of ATPase 1; CTL, C-terminal linker; CTD, C-terminal domain. (B) Schematic representation of SecA hybrid proteins used in this study. The numbers in parentheses correspond to the amino acids that are derived from the indicated parental SecA proteins. The respective fusion sites are indicated by an arrow.

FIG 3 Improvement of MalE11 secretion in B. subtilis by coexpression of ecSecB and beSecA. (A) Amino acid alterations present in the inefficiently secreted MalE11 protein. MalE, primary sequence of wild-type MalE corresponding to the signal peptide and the early mature region. MalE11, MalE mutant protein possessing mutations K2T and K4T in the signal peptide and mutation E29G in the early mature protein. The corresponding mutations result in a reduction of the positive net charge of the n-region of the signal peptide and in a more positively charged early mature protein domain, both of which are known to slow down the kinetics and overall efficiency of MalE membrane translocation in E. coli (28). The respective amino acid positions are underlined. The arrowhead indicates the signal peptidase cleavage site. (B) MalE11 secretion is enhanced in the presence of ecSecB and beSecA. Cellular (C) and supernatant (S) fractions of B. subtilis expressing the SecA and SecB proteins as indicated above the respective panels were subjected to SDS-PAGE and Western blotting using MalE antibodies (upper). In addition, the cellular fractions were immunoblotted using B. subtilis SecA antibodies (middle) or E. coli SecB antibodies (lower). P, MalE11 precursor protein; m, mature MalE11 protein. (C) Secretion stimulation of MalE11 in the presence of beSecA correlates to ecSecB availability. The amount of ecSecB, as symbolized by the solid triangle, in the cell was modulated by varying the concentration of the inducer xylose in the growth medium. The xylose concentrations used were 0.5% (panel 3), 0.1% (panel 4), 0.05% (panel 5), and 0% (panel 6), respectively. MalE11-derived protein products in the C and S fractions, as well as the amount of ecSecB in the C fractions of the respective cells, were analyzed by immunoblotting as described for panel B.
coexpression of beSecA with the ecSecB(L75Q) mutant protein did not confer an additional increase of PhoA activity above the 30% observed when only beSecA was expressed (lane 7).

Western blot analysis of the S and C fractions of B. subtilis cells that expressed PhoA either with (Fig. 5B, panel 2) or without (Fig. 5B, panel 1) the coexpression of the ecSecB-beSecA targeting pathway clearly supported the beneficial effect of the artificial targeting pathway on PhoA secretion. An increased amount of mature PhoA was detected in the S fraction of cells that coexpressed ecSecB and beSecA compared to the control cells. Interestingly, the amounts of accumulated pre-PhoA precursor in the C fractions were almost identical in both strains, suggesting that the additional amount of secreted PhoA that is observed in the presence of ecSecB-beSecA reflects a fraction of the PhoA precursor that has been rescued from proteolytic degradation by interaction with the targeting pathway.

DISCUSSION

In the present study, we have introduced a functional SecB-dependent protein-targeting pathway in the Gram-positive model bacterium B. subtilis. The replacement of the C-terminal 32 amino acids of bsSecA by the corresponding part of ecSecA resulted in a beSecA hybrid protein that, in contrast to the unaltered bsSecA, possessed a high binding affinity for ecSecB. The coexpression of ecSecB together with the beSecA hybrid in B. subtilis improved the secretion of two heterologous model proteins (MalE11 and PhoA) into the culture supernatant in an ecSecB-beSecA interaction-dependent manner. From a biotechnological view, the construction of an artificial protein-targeting pathway in a Gram-positive bacterium can be considered a novel strategy for the improvement of an artificial protein-targeting pathway in a Gram-positive model bacterium.

Multiple interactions involved in SecB-SecA complex formation have been extensively studied in the E. coli system. One of the contact areas identified is between the negatively charged flat \( \beta \)-sheet of the SecB tetramer and the zinc-containing C-terminal 22 residues of SecA (9–11, 17, 43). Another existing contact area is the \( \beta \)-sheet of the SecB tetramer and the zinc-containing C-terminal 22 residues of SecA (9–11, 17, 43). Another existing contact area is between the C-terminal 13 residues of SecB and the N-terminal 11 residues of SecA (30). Interestingly, the latter seems to negatively contribute to the formation of the SecB-SecA complex, which is thought to play a crucial role in transferring SecB-bound precursors via SecA to the SecYEG translocon (30).

Since SecB is absent from B. subtilis and other Gram-positive bacteria, the targeting of Sec substrates to the translocon in these organisms is thought to be mediated mainly, if not exclusively, by the SRP pathway (14, 34, 41, 42). Nevertheless, the region encompassing the C-terminal 22 residues of the bsSecA is highly homologous to the counterpart of ecSecA that has a high affinity to ecSecB (see Fig. S3 in the supplemental material). Although the isolated bsSecA C-terminal tail can bind ecSecB in the context of a GST fusion protein (37), the full-length bsSecA protein did not form a stable complex with ecSecB in our in vitro binding assay. This suggests that the C tail is not freely accessible for SecB binding or that other unfavorable contacts in bsSecA, such as the extreme N-terminal domain (30), prevent the formation of a stable ecSecB-bsSecA complex. However, the coexpression of ecSecB together with bsSecA in B. subtilis resulted in a slight improvement of MalE11 secretion, indicating that a weak interaction between ecSecB and bsSecA can occur in vivo, thereby allowing an inefficient SecB-dependent targeting of MalE11 precursors to bsSecA.

In contrast, the beSecA hybrid protein can form a stable complex with ecSecB in vitro, and the observed binding affinity closely
resembles that between ecSecB and its cognate interacting partner ecSecA. Thus, the few amino acid differences that exist between the C tails of ecSecA and bsSecA (see Fig. S3 in the supplemental material) preclude an efficient ecSecB-dependent targeting to bsSecA, indicating that the structural details of the extreme C tail of SecA are of crucial importance for the overall SecB-SecA binding affinity. Moreover, the coexpression of ecSecB and bsSecA strongly stimulates MalE11 secretion in vivo due to an efficient delivery of the ecSecB-bound substrate to bsSecA, a notion that was confirmed by the observation that the increased secretion stimulation disappeared when the wild-type ecSecB was replaced by substrate-binding-deficient but SecA-binding-deficient ecSecB mutant proteins.

Compared to that of MalE11, the secretion of wild-type MalE in B. subtilis was more efficient. In the cellular fraction of the corresponding strain, only small amounts of MalE precursor accumulated, indicating that MalE was effectively targeted to the translocase by the B. subtilis in-house targeting systems. Therefore, MalE secretion only marginally benefited from the presence of the implemented SecB-dependent targeting pathway in addition to the slight positive effect by coexpressing ecSecB alone (see Fig. S4 in the supplemental material, compare panels 2 and 6) (5). Interestingly, the yield of secreted PhoA, which in its native host E. coli does not rely on the SecB-dependent targeting pathway under normal conditions (16, 18), was significantly increased in B. subtilis containing the artificial SecB-dependent targeting pathway. Since the increase of secreted PhoA was not paralleled by a complementary decrease of accumulated pre-PhoA precursor, the most reasonable explanation is that the artificially introduced targeting pathway rescued a fraction of PhoA from proteolysis by cytoplasmic proteases.

In general, when the secretion of a heterologous protein by B. subtilis is attempted, the nascent precursor protein faces different potential fates in the foreign environment, such as aggregation, degradation, folding into an export-incompetent state, or successful targeting to the Sec translocase. Although the kinetic partitioning between all those possibilities and, as a consequence, a putative beneficial effect of the SecB-dependent targeting pathway is not predictable in advance for any desired target protein, our results nevertheless show that the implementation of a SecB-dependent targeting pathway adds a promising new tool to the repertoire of strategies aimed at the improvement of the secretory production of heterologous proteins by B. subtilis and, most likely, other SecB null bacteria.

REFERENCES