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Expression of *Escherichia coli* SecB in *Bacillus subtilis* Facilitates Secretion of the SecB-Dependent Maltose-Binding Protein of *E. coli*

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Less than 20% of the *Escherichia coli* maltose-binding protein (MBP) synthesized in *Bacillus subtilis* is exported. However, a portion of the secreted MBP was processed cotranslationally. Coexpression of SecB, a secretion-related chaperone of *E. coli*, stimulated posttranslational export of MBP in *B. subtilis* but inhibited its cotranslational processing. Export of a SecB-independent MBP–ribose-binding protein hybrid precursor was not enhanced by SecB. A slowly folding MBP derivative (MBP-Y283D) was more efficiently secreted than wild-type MBP, suggesting that the antifolding activity of SecB promotes posttranslational secretion of MBP in *B. subtilis*.

A majority of *Escherichia coli* envelope proteins are exported via the Sec pathway, which comprises SecA (PrlD), SecD, SecE (PrlG), SecF, SecY (PrlA), Lep (SPase I), and LspA (SPase II) (reviewed in reference 27). Proteins destined for egress through this pathway are synthesized as precursors with a cleavable amino-terminal extension, known as the signal peptide, which facilitates formation of export-competent conformers of the precursor (24) and targets it to the export apparatus (13).

Homologues of SecA (26), SecE (14), SecY (28), and Lep (31) have been identified in *Bacillus subtilis*, suggesting that signal peptide-dependent protein secretion in *B. subtilis* utilizes a Sec pathway that is similar to that of *E. coli*. However, several secretory proteins of *E. coli*, including the maltose-binding protein (MBP) and OmpA, were shown to be only inefficiently secreted in *B. subtilis* (6). In *E. coli*, efficient secretion of these proteins requires the molecular chaperone SecB (7, 18, 19). Since a SecB homologue has not yet been identified in *B. subtilis*, it is possible that a lack of SecB contributes to the poor secretion of MBP and OmpA in this bacterium. In this report, the capacity of SecB to facilitate secretion of SecB-dependent proteins in *B. subtilis* was evaluated. A slowly folding MBP species was used to probe the relationship between precursor folding and competence for secretion in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and media. Pulse-chase studies employed *B. subtilis* BE2000 (*trpC2 lys-3 ΔaprE66 Δnpr-82*) (6) grown in modified (6) S7 medium (23) containing glycerol (1%), yeast extract (0.05%), and tryptophan and lysine (50 μg/ml). Selection of BE2000 transformants was done on TBAB plates (tryptose blood agar base) (Difco, Detroit, Mich.) containing chloramphenicol (5 μg/ml). Plasmids were prepared in *E. coli* XL1 Blue (Stratagene, La Jolla, Calif.) grown in LB (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl [per liter]) containing ampicillin (50 μg/ml). *secB* and genes encoding envelope proteins were from *E. coli* MC4100 (F⁻ *ΔlacU169 araD139 rspL150 thi flbB5301 deoC7 ptsF25 relA1*) (2).

Plasmids and plasmid constructions. Genes encoding *E. coli* envelope proteins were placed under transcriptional control of the *Bacillus amyloliquefaciens* alkaline protease promoter (*aprp*) (30) provided by the shuttle vector pBE1020 (6). The pBE1020 derivatives pBE1064, pBE1065, and pBE1146, carrying operon fusions Φ(*aprp-rbsB$_{ss}$-malE*)(Hyb), Φ(*aprp-malE*), and Φ(*aprp-ompA*), respectively, have been described previously (6).

secB was obtained by PCR amplification (DNA Thermal Cycler and Gene Amp Kit; Perkin-Elmer Co., Norwalk, Conn.) of MC4100 chromosomal DNA with PCR primers that introduced an *Nde*I restriction site overlapping the translation initiation site and an *Xba*I site immediately 3′ to the termination codon. The resultant PCR product was cleaved with *Nde*I and *Xba*I and ligated with similarly cut pBE1020, generating pBE1042. DNA sequence analysis (3) determined that the *secB* gene carried in pBE1042 had the expected sequence (21).

An artificial intercistronic region (ICR) (5′ TCTAGAG GAGGCATGCAT 3′) was created by annealing a plus-sense oligonucleotide (5′ CTAGAGGAGGCATGCATATGTCG ACCTGCA) and its truncated complement (5′ GGTCGA CATATGCATGCCTCCT). The resulting partially double-stranded oligonucleotide (carrying 5′ *Xba*I and 3′ *Pst*I sticky ends) was ligated with pBE1013 (pBE1020 derivative lacking the *Nde*I site) which had been cut with *Xba*I and *Pst*I, generating pBE1014. The *Nde*I-*Pst*I fragment carrying *secB* (from pBE1042) was placed 3′ of the ICR in pBE1014, yielding pBE1051. Finally, an *Xba*I fragment from pBE1051, carrying the ICR-*secB* fusion, was ligated into the *Xba*I site immediately 3′ of the precursor-encoding genes carried on pBE1064, pBE1065, and pBE1146. Constructs carrying the insert in the forward orientation (pBE1085, pBE1068, and pBE1146) were identified by restriction analysis. Mutations resulting in the MBP-Y283D (MBP with a Tyr-to-Asp change at position 283) (10) and ribose-binding protein (RBP)-V50E (29) substitutions were introduced by oligonucleotide-directed mutagenesis (Muta-Gene Kit; Bio-Rad, Hercules, Calif.) into *malE* and

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rbsB carried on pBE1065 and pBE1064, yielding pBE1075 and pBE1157, respectively.

Radiolabeling, immunoprecipitation, SDS-PAGE, and fluorography. Cultures of BE2000 harboring the appropriate plasmids were grown at 30°C to late logarithmic phase in modified S7 media and pulse-chase radiolabeled with L-[^{35}S]methionine (DuPont Co. NEN Research Products, Boston, Mass.) as previously described (6). At various times after initiation of chase, samples were processed for immunoprecipitation (23) with polyclonal rabbit antisera specific for MBP, RBP, OmpA, or SecB (kind gift of P. J. Bassford, Jr.). Immunoprecipitates were resolved by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography as described elsewhere (6). Precursor and mature levels were quantitated by a "cut-and-count" method (6).

The two-dimensional electrophoresis technique developed by Josefsson and Randall (15, 16) was employed to monitor the temporal mode of MBP processing. Cultures of BE2000 harboring the appropriate plasmid were grown as described above and pulse-radiolabeled with [^{35}S]methionine (30 μCi/ml) for 30 s. Labeling was terminated by the addition of ice-cold trichloroacetic acid to 5%, and the immunoprecipitable MBP species were resolved by SDS–10% PAGE. The portion of the dried gel containing the precursor, mature, and incomplete MBP chains was rehydrated, and the polypeptides contained in the gel slice were subjected to limited proteolysis by V8 protease (1 μg of V8 protease per gel slice). The resultant proteolytic fragments were resolved in a second dimension by SDS–15% PAGE. Gels were soaked in an autoradiography enhancer (Entensify; DuPont Co. NEN Research Products) before being dried.

RESULTS

Effect of SecB synthesis on precursor processing. Cells of *B. subtilis* BE2000 carrying Φ(*aprp-malE-secB*) (encoding the MBP precursor [pMBP] and SecB) were pulse-chase radiolabeled and processed for immunoprecipitation (see Materials and Methods) with SecB antiserum. This analysis indicated that the ICR promoted translation of the *secB* cistron and that SecB was stable throughout the 20-min chase period (Fig. 1A). Similar results (data not shown) were obtained with BE2000 cells harboring Φ(*aprp-malE$_{ss}$-rbsB-secB*) (encoding SecB and the hybrid precursor MBP$_{SP}$-RBP [MBP signal peptide fused to mature RBP {mRBP}]) and Φ(*aprp-ompA-secB*) (encoding SecB and precursor OmpA [pOmpA]). Consistent with the previously reported failure to detect SecB antigen in another *Bacillus* species (*Bacillus cereus* [11]), SecB antiserum did not specifically precipitate radiolabeled bands from BE2000 cells harboring Φ(*aprp-malE*) (Fig. 1A) or other constructs encoding precursors only (data not shown).

As reported previously (6), only about 17% of the total MBP synthesized in BE2000 was recovered as the mature form after 20 min of chase. In contrast, 38% of MBP was matured in cells expressing SecB (Fig. 1). Processing of MBP$_{SP}$-RBP was not influenced by coexpression of SecB (Fig. 1). Although the alkaline protease signal peptide (Apr$_{SP}$) directs relatively efficient secretion of MBP in *B. subtilis*, up to 10% of the Apr$_{SP}$-MBP hybrid precursor is not processed (6). Coexpression of SecB had no influence on either the kinetics or extent of Apr$_{SP}$-MBP processing (data not shown). Similarly, pOmpA processing in BE2000 (6) was not enhanced by coexpression of SecB (data not shown).

Temporal mode of MBP processing in *B. subtilis*. As previously reported (6), little or no additional processing of pMBP occurred after the first 0.5 min of chase (Fig. 1B). Coexpression of SecB not only stimulated processing of MBP during the last 19.5 min of chase but also increased the level of mature MBP (mMBP) present at the earliest chase points (Fig. 1B). To determine if SecB sponsors cotranslational export of MBP in *B. subtilis*, the two-dimensional SDS-PAGE technique of Josefsson and Randall (15, 16) was employed. The faint horizontal smear, beginning at "m′" in the left panel of Fig. 2, results from heterogeneity in the length of pMBP chains that were processed, indicating that at least a fraction of pMBP was processed cotranslationally. In cells coexpressing pMBP and SecB (Fig. 2, right panel), no horizontal smear originating at m′ was detected, indicating that the cotranslational element of pMBP processing was abolished by coexpression of SecB.

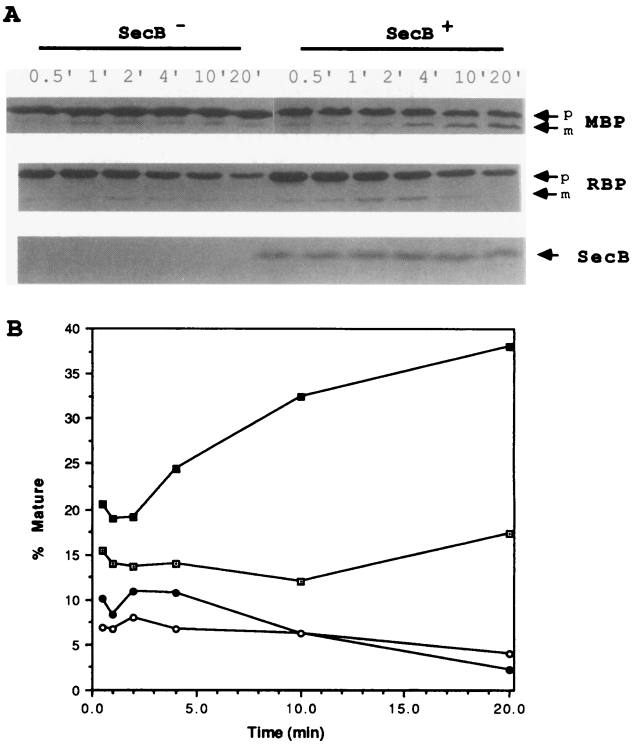

FIG. 1. Export of MBP and MBP$_{SP}$-RBP in *B. subtilis* in the absence and presence of SecB. (A) S7–glycerol-grown cells of strain BE2000 harboring plasmid pBE1065 (encoding MBP) (upper and lower panels, leftmost six lanes), pBE1068 (encoding MBP and SecB) (upper and lower panels, rightmost six lanes), pBE1064 (encoding MBP$_{SP}$-RBP [RBP]) (middle panel, leftmost six lanes), or pBE1085 (encoding MBP$_{SP}$-RBP [RBP] and SecB) (middle panel, rightmost six lanes) were pulse-radiolabeled with [^{35}S]methionine for 30 s and chased with excess unlabeled methionine. At the indicated time points (minutes), samples were removed, the chase was terminated, and the protein products were immunoprecipitated and analyzed by SDS-PAGE and fluorography. The positions of SecB and precursor (p) and mature (m) MBP and MBP$_{SP}$-RBP (RBP) are indicated by arrows. (B) The amounts of radiolabeled MBP and MBP$_{SP}$-RBP recovered as the mature form were determined for each chase point (see Materials and Methods) and are given as percentages of the total (precursor plus mature) recovered at the 1-min chase point. Symbols: ⊡, MBP; ■, MBP plus SecB; ○, MBP$_{SP}$-RBP; ●, MBP$_{SP}$-RBP plus SecB. Values for MBP and MBP plus SecB are the arithmetical means for three different experiments (including the labeling experiment shown in panel A). Values for MBP$_{SP}$-RBP and MBP$_{SP}$-RBP plus SecB are the arithmetical means for two experiments (including that shown in panel A).

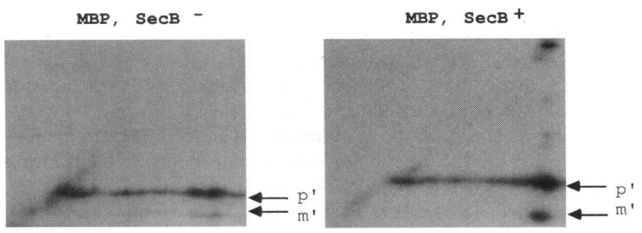

FIG. 2. Two-dimensional analysis of the cotranslational processing of MBP in *B. subtilis* in the absence and presence of SecB. S7–glycerol-grown cells of strain BE2000 harboring pBE1065 (encoding MBP) (left) or pBE1068 (encoding MBP and SecB) (right) were pulse-radiolabeled for 30 s, and the polypeptides related to MBP were immunoprecipitated and resolved by SDS–10% PAGE. These polypeptides, contained in excised portions of the gel, were subjected to partial proteolysis by V8 protease, and the products were resolved in a second dimension by SDS–15% PAGE. The amino-terminal fragments derived from full-length precursors are designated p′, while the amino-terminal fragments derived from mMBP are designated m′. Only the relevant portion of each gel is shown. For a comprehensive discussion of this technique, see the study of Josefsson and Randall (16).

Similar results were obtained with the Apr_{SP}-MBP hybrid (data not shown).

Export of MBP-Y283D. Mutational alterations that reduce the rate of preprotein folding can partially supplant the antifolding activity of SecB (4, 7). To determine if reducing the rate of precursor folding affects secretion in *B. subtilis*, export rates of $pMBP^+$ and the slowly folding derivative pMBP-Y283D (22) were compared. During 60 min of chase, only 19% of the total $pMBP^+$ synthesized was processed, while up to 30% of pMBP-Y283D was matured (Fig. 3). An alteration (RBP-V50E) that slows the folding and facilitates secretion of RBP (29) was introduced into MBP_{SP}-RBP, and export of MBP_{SP}-RBP-V50E was examined. However, MBP_{SP}-RBP-V50E and mRBP-V50E were too unstable in BE2000 to allow meaningful data to be obtained (data not shown).

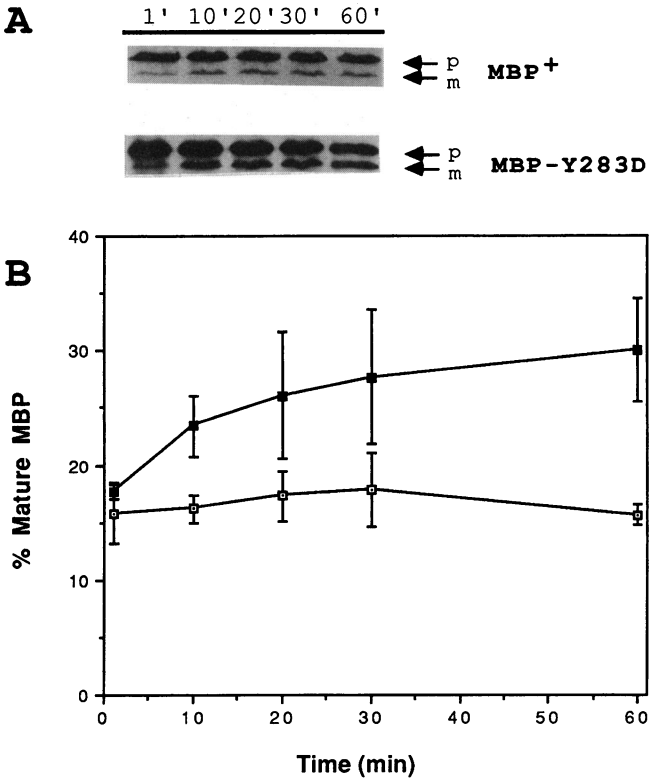

FIG. 3. Export of MBP and MBP-Y283D. (A) S7–glycerol-grown cells of strain BE2000 harboring pBE1065 (encoding MBP) (upper panel) or pBE1075 (encoding MBP-Y283D) (lower panel) were pulse-chase radiolabeled as described in the legend to Fig. 1. The positions of precursor (p) and mature (m) forms are indicated by arrows. (B) The amounts of radiolabeled MBP and MBP-Y283D recovered as the mature form were determined for each chase point (see Materials and Methods) and are given as percentages of the total (precursor plus mature) recovered at the 1-min chase point. Symbols: ⊡, MBP; ■, MBP-Y283D. The arithmetical means from three experiments (including that shown in panel A) are plotted. Error bars indicate the population standard deviation (σn).

DISCUSSION

Synthesis of SecB enhanced the rate of pMBP processing in *B. subtilis* and more than doubled the amount of mMBP recovered. Because translocation accompanies processing of MBP in *B. subtilis* (6), it can be concluded that coexpression of SecB facilitated export of MBP. SecB had no influence on secretion of MBP_{SP}-RBP. This result was expected, since RBP neither binds SecB with physiologically significant affinity (9) nor requires SecB for efficient secretion in *E. coli* (7, 9, 18). Somewhat surprising was the inability of SecB to enhance secretion of the SecB-dependent outer membrane protein, OmpA, or to rescue the small fraction of Apr_{SP}-MBP (10% of the total) that remains unprocessed in BE2000 (6). Since SecB is thought to participate in the earliest steps of the Sec pathway (15, 18, 19), SecB may be unable to rescue export of these proteins because deficiencies in their translocation occur at later steps. For OmpA, it is possible that a small increase in processing, commensurate with its low degree of SecB dependence in *E. coli* (7), could not be detected.

A portion of both the pMBP and Apr_{SP}-MBP that were secreted in BE2000 was cotranslationally processed. Although very rapid processing of certain precursors has been observed to occur in *B. subtilis* (1), this is, to the best of my knowledge, the first demonstration of cotranslational processing of any precursor in this bacterium. Because cotranslational processing of MBP in *E. coli* (15) is SecB dependent (8, 19, 20), the loss of cotranslational MBP and Apr_{SP}-MBP processing associated with SecB synthesis in *B. subtilis* was unexpected. However, high levels of SecB exert a slightly deleterious effect on MBP processing kinetics in *E. coli* (7, 19). The levels of SecB synthesized in BE2000 may be sufficiently high to cause a similar effect. Excess SecB could alter processing kinetics by competing with SecB-precursor complexes for binding to SecA (13), although it has not been determined if SecB and *B. subtilis* SecA interact. Alternatively, excess SecB could delay secretion by promoting longer-lived SecB-MBP complexes (12, 17; reviewed in reference 5).

The slowly folding pMBP-Y283D species was more efficiently secreted than $pMBP^+$. Although differences in the levels were not great (30 versus 19%), it should be noted that the amount of mature protein recovered at each chase point is expressed as a percentage of the total (precursor plus mature) recovered at 1 min. Unlike $mMBP^+$ (25), mMBP-Y283D does not assume intrinsically protease-resistant conformers (7, 10) and therefore exhibited a higher turnover in BE2000 than did $mMBP^+$. It is probable that much more than 30% of pMBP-Y283D was processed but was not recovered because of its protease sensitivity. Indeed, when expressed as a percentage of the total for the chase point, more than 50% of pMBP-Y283D

was processed—a stimulation of MBP secretion of similar magnitude to that sponsored by SecB. This result provides indirect evidence that the antifolding activity of SecB is largely responsible for stimulating MBP^+ secretion in *B. subtilis* and implies that, as for *E. coli* (7, 25; reviewed in reference 5), the secretion machinery of *B. subtilis* is sensitive to the precursor's conformation.

Randall and Hardy (25) have described secretion as a kinetic partitioning, in which the fate of the precursor is determined by a competition between folding and movement through the export machinery. If MBP_{SP} is a poor ligand for the *B. subtilis* secretion machinery, then very little pMBP is exported before it folds. Slowing the rate of MBP folding, either by coexpression of SecB or by the Y283D substitution, effectively increases the pool of translocation-competent ligands by extending the duration of competence. However, a mismatch(es) between MBP_{SP} and the Sec machinery still limits pMBP secretion. Apr_{SP} is a good ligand, moving Apr_{SP}-MBP through the system rapidly enough to render the antifolding activity of SecB superfluous. Alternatively, Apr_{SP} may serve as a more effective antifolding factor than MBP_{SP}.

These experiments demonstrate that a mismatch between the host cell's chaperones and a heterologous protein can be partially responsible for the "misbehavior" often exhibited by recombinant proteins. Here, SecB (cognate chaperone) doubled the yield of MBP (recombinant protein) secreted in *B. subtilis* (host). However, the cognate chaperone(s) may prove insufficient to elicit proper behavior, as evidenced by the inability of SecB to rectify secretion defects for all the SecB-dependent proteins tested.

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