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Escherichia coli Signal Peptides Direct Inefficient Secretion of an Outer Membrane Protein (OmpA) and Periplasmic Proteins (Maltose-Binding Protein, Ribose-Binding Protein, and Alkaline Phosphatase) in *Bacillus subtilis*

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Signal peptides of gram-positive exoproteins generally carry a higher net positive charge at their amino termini (N regions) and have longer hydrophobic cores (h regions) and carboxy termini (C regions) than do signal peptides of *Escherichia coli* envelope proteins. To determine if these differences are functionally significant, the ability of *Bacillus subtilis* to secrete four different *E. coli* envelope proteins was tested. A pulse-chase analysis demonstrated that the periplasmic maltose-binding protein (MBP), ribose-binding protein (RBP), alkaline phosphatase (PhoA), and outer membrane protein OmpA were only inefficiently secreted. Inefficient secretion could be ascribed largely to properties of the homologous signal peptides, since replacing them with the *B. amyloliquefaciens* alkaline protease signal peptide resulted in significant increases in both the rate and extent of export. The relative efficiency with which the native precursors were secreted (OmpA >> RBP > MBP > PhoA) was most closely correlated with the overall hydrophobicity of their h regions. This correlation was strengthened by the observation that the *B. amyloliquefaciens* levansucrase signal peptide, whose h region has an overall hydrophobicity similar to that of *E. coli* signal peptides, was able to direct secretion of only modest levels of MBP and OmpA. These results imply that there are differences between the secretion machineries of *B. subtilis* and *E. coli* and demonstrate that the outer membrane protein OmpA can be translocated across the cytoplasmic membrane of *B. subtilis*.

Most proteins destined for translocation across the cytoplasmic membrane of a bacterium are synthesized as preproteins with a cleavable amino-terminal extension known as the signal peptide. This *cis*-acting element performs several roles, including direct modulation of the preprotein's conformation (44) and targeting of the precursor to the secretion machinery (14). In *Escherichia coli*, this machinery consists of SecA (PrlD), SecD, SecE (PrlG), SecF, and SecY (PrlA) and the Lep (SPase I) and LspA (SPase II) signal peptidases (reviewed in reference 55). Molecular chaperones such as SecB (11, 29), DnaK and DnaJ (64) and GroES and GroEL (30) also participate in the secretion of certain envelope proteins (reviewed in reference 10). Homologs of *E. coli* SecA (54), SecE (26), SecY (56), and Lep (59) have been identified in *Bacillus subtilis* (reviewed in reference 40). Therefore, signal peptide-dependent protein secretion in *B. subtilis* is likely to utilize Sec components organized in a pathway similar to that of the general secretory pathway of *E. coli*.

Although signal peptides from a diverse array of organisms lack significant primary amino acid sequence identity (63), they share three structural features (61). The amino terminus (N region) usually contains one or more basic residues and is characterized by a net positive charge. Following the N region is a stretch of uncharged, largely hydrophobic residues known as the hydrophobic core (h region), and the relatively polar carboxy terminus (C region) contains a consensus processing site for the endoproteolytic removal of the signal peptide by a specific signal peptidase.

However, the structural elements of signal peptides from exoproteins of gram-positive (gm+) organisms and envelope proteins of *E. coli* are somewhat different: (i) the net positive charge of the N region for a set of bacillary exoproteins averaged +3, compared with only +2 for a set of *E. coli* envelope proteins, (ii) the h region for the *E. coli* signals averaged 12 residues, while 15 or more residues were average for the gm+ signals, and (iii) the C region of the *E. coli* signal peptides contained an average of 6 residues, two fewer than the C regions of the bacillary signal peptides (62).

Truncations in or disruption of the h region can impair or completely block secretion in both *E. coli* (reviewed in references 22 and 55) and *B. subtilis* (4, 45; reviewed in reference 40). Because the h regions of *E. coli* signal peptides are shorter than those of gm+ signal peptides, it is tempting to speculate that *E. coli* signal peptides will not function efficiently in a gm+ organism.

Heretofore there has been no systematic evaluation of *E. coli* signal peptide function in *B. subtilis*. Therefore, it is not known if *E. coli* signal peptides can mediate efficient export in *B. subtilis*. In this study, the capacity of the *E. coli* maltose-binding protein (MBP), ribose-binding protein (RBP), alkaline phosphatase (PhoA), and OmpA signal peptides to promote secretion in *B. subtilis* was examined. Hybrid precursors carrying the *B. amyloliquefaciens* alkaline protease (Apr) or levansucrase (Lvs) signal peptide (Apr_{SP} or Lvs_{SP}) were used to assess the secretion competence of each mature moiety.

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MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* BE2000 (*trpC2 lys-3 ΔaprE66 Δnpr-82*) was derived from the low-protease strain BE1010 (*trpC2 metB10 lys-3 ΔaprE66 Δnpr-82*) (46) by competent cell transformation using chromosomal DNA from the prototrophic strain 1S53 (*Bacillus* Genetic Stock Center, Ohio State University, Columbus). Selection for methionine prototrophs was on T-base minimal agar supplemented with glycerol (1%), yeast extract (0.001%), and tryptophan and lysine (each at 50 μg/ml). T base contains (per liter) 2 g of $(NH_4)_2SO_4$, 14 g of K_2HPO_4, 6 g of KH_2PO_4, 1 g of trisodium citrate · $2H_2O$, and 15 g of agar. Selection for and maintenance of BE2000 transformants carrying plasmids was on tryptose blood agar base (Difco, Detroit, Mich.) plates containing chloramphenicol (5 μg/ml). *B. subtilis* cultures were radiolabeled in S7 medium (39) modified as follows: yeast extract was added to 0.05%, glycerol (1%) was substituted for glucose, and no methionine was added. GY minimal medium agar plates containing 50 μg of XP (5-bromo-4-chloro-3-indolyl phosphate) per ml were prepared as described previously (46).

E. coli XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*]) (Stratagene, La Jolla, Calif.) was used for plasmid manipulations. Single-stranded DNA templates were prepared in PR382 (*dut-1 ung-1 thi relA rpsE recA56 srl*::Tn*10* [F′ *lacZ*::Tn*5 lacY lacA*]), which was kindly given by J. P. Ryan. Unless stated otherwise, genes encoding *E. coli* envelope proteins were obtained from MC4100 (F$^-$ *ΔlacU169 araD139 rspL150 thi flbB5301 deoC7 ptsF25 relA1* [*malE*$^+$ *rbsB*$^+$ *ompA*$^+$ *phoA*$^+$]) (6). Preparation of 2× YT and LB has been described previously (36). When appropriate, ampicillin was included in rich medium and minimal medium at 100 and 50 μg/ml, respectively.

Plasmids. Plasmids used in this study are derivatives of the shuttle vector pBE240 (46). The ϕ(*apr-phoA*)(Hyb) gene carried on pBE240 encodes a hybrid protein consisting of the *B. amyloliquefaciens* Apr$_{SP}$ fused to the mature moiety of the *E. coli* PhoA. All precursor-encoding genes were under transcriptional control of the *B. amyloliquefaciens apr* promoter.

The unique *Nde*I site located near the 5′ end of the *apr* promoter in pBE240 was removed, resulting in pBE1013. Oligonucleotide-directed mutagenesis (ODM) was used to introduce a new *Nde*I site overlapping the initiation codon (52) of pBE1013, generating pBE1020. Replacement of the Apr$_{SP}$-coding region of pBE1020 with that of the Lvs$_{SP}$ derived from pBE350 (kindly provided by V. Nagarajan) yielded pBE1032. pBE350 (38a) was generated by converting the three bases (AAC) preceding the *lvs* initiation codon in pBE311 (41) to CAT, creating an *Nde*I site which overlaps the initiation codon.

The *malE* and ϕ(*malE-rbsB*)(Hyb) genes, encoding native MBP signal peptide (MBP$_{SP}$)-MBP and chimeric MBP$_{SP}$-RBP precursors, respectively, were obtained by PCR amplification (GeneAmp Kit and DNA Thermal Cycler; Perkin-Elmer Co.) of pJF2 (20) and pDNC197 (13) DNA, using primer pairs 45 (5′ GGACCATAGCATATGAAAATAAAAAC) plus 58 (5′ TGCATGCCTCCTCTAGATTACTTGGTGATACG) and 114 (5′ CTTAAGCTTCATATGAACATGAAAAAAC) plus 59 (5′ TGCATGCCTCCTCTAGACTACTGCTTAACAAC), respectively. Amplification by PCR resulted in the addition of an *Nde*I site overlapping the initiation codon and an *Sph*I site 3′ of the termination codon of each gene. Products of the PCR were treated with Klenow fragment and ligated with *Sma*I-cut pGEM-3Zf(+) (Promega Corporation, Madison, Wis.). *Nde*I-*Sph*I fragments carrying the *malE* or ϕ(*malE-rbsB*)(Hyb) gene from the pGEM-3Zf(+)-derived clones were ligated into pBE1020 cut with *Nde*I and *Sph*I, resulting in pBE1065 and 1064, respectively. The *malE172* (12) mutation was introduced into pBE1065 by ODM, generating pBE1074.

The *malE* and *rbsB* mature-moiety-coding regions were moved in *cis* to the Apr$_{SP}$-coding region in three steps. First, ODM was used to create a unique *Eco*RV site at the signal peptidase cleavage sites in pBE1065 and pBE1064, yielding pBE1071 and pBE1081, respectively. Then the 1,200-bp *Kpn*I-*Eco*RV fragment of pBE1020 (carries *apr* promoter-*apr$_{SP}$*) was used to replace the *Kpn*I-*Eco*RV fragments of pBE1071 and 1081, generating pBE1072 and 1082, respectively. Precise fusion junctions were generated by loop-out ODM, yielding pBE1073 and pBE1083, respectively.

The mature-moiety-coding region of *ompA* was obtained by PCR amplification of MC4100 chromosomal DNA, using primers 86 (5′ ATGCATATGGATATCGCTCCGAAAGAT ACC) and 87 (5′ TGCATGCCTCCTCTAGATTAAGCCT GCGGCTGA). The 1,213-bp *Eco*RV-*Xba*I fragment of pBE1072 was replaced with the PCR-amplified *ompA* fragment which had been cut with *Eco*RV and *Xba*I. The resultant plasmid, pBE1092, was subjected to ODM to create a precise fusion between the signal sequence and mature-moiety-coding regions, generating pBE1093. ODM introduced the *aprL16R* mutation (45) into pBE1093, resulting in pBE1141. The *rbsB*, *ompA*, and *phoA* genes were obtained by PCR amplification of MC4100 chromosomal DNA, using primer pairs 114 plus 59, 115 (5′ CTTAAGCTTCATATGAAAAAGACAGC) plus 87, and 116 (5′ CTTAAGCTTCATATGAAACAAAGCACT) plus 117 (5′ TGCATGCCTCCTCTAGATTATTTCAGCC CCAG), respectively. Replacement of the *Nde*I-*Xba*I insert of pBE1073 with *Nde*I-*Xba*I-digested PCR products yielded pBE1145, pBE1146, and pBE1147, respectively. ODM of pBE1020 to create a precise *apr$_{SP}$-phoA* fusion yielded pBE1148. Finally, replacement of the *Eco*RV-*Xba*I insert of pBE1032 with the inserts from pBE1072 and pBE1092, followed by ODM to create precise fusion joints, yielded pBE1151 and pBE1153, respectively.

ODM and DNA sequencing. ODM was performed with reagents from the Muta-gene Kit (Bio-Rad, Hercules, Calif.) as instructed by the manufacturer except that (i) single-stranded uracil-containing templates were prepared in PR382 and (ii) helper phage R408 (Stratagene) was used. The nucleotide sequence of each signal sequence and fusion junction was confirmed by sequencing purified double-stranded plasmid DNA (Magic Minipreps; Promega) by the method of Chen and Seeburg (7), using modified T7 DNA polymerase (Sequenase; United States Biochemical Corporation, Cleveland, Ohio) and [^{35}S]dATP (DuPont Company NEN Research Products, Boston, Mass.).

Radiolabeling, immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography. Late-logarithmic-phase cultures (A_{600} = 0.7 to 0.8) of *B. subtilis* grown in modified S7 medium at 30°C with vigorous aeration were pulse-labeled with L-[^{35}S]methionine (30 μCi/ml; DuPont). Chase was initiated by the addition of an equal volume of prewarmed S7 medium containing excess unlabeled L-methionine (10 mg/ml). At various times after initiation of chase, 1-ml samples were placed in 0.5 ml of ice-cold 15% trichloroacetic acid. Processing of radiolabeled cells for immunoprecipitation has been described elsewhere (4, 39). Rabbit polyclonal antisera specific for MBP, OmpA, PhoA, and RBP were a generous gift from P. J. Bassford, Jr.

Immunoprecipitates were resolved by SDS-PAGE (10% acrylamide–0.27% bisacrylamide gel) and visualized by fluorography of gels which had been soaked in an autoradiography enhancer (Entensify; DuPont) and dried. Dried gels were exposed to Kodak XAR X-ray film at −70°C. Where indi-

cated, the amount of radioactivity present in each precursor and mature species was determined by excising the portion of the gel corresponding to the band, rehydrating the gel slice, and then dissolving it in Solvable Tissue Solubilizer (DuPont) and counting in Formula-989 scintillation cocktail (DuPont). Counts were adjusted for the loss of methionine residues associated with signal peptide processing.

RESULTS

Genetic constructs. The *malE*, *rbsB*, *phoA*, and *ompA* genes of *E. coli*, which code for precursors of the periplasmic proteins MBP, RBP, and PhoA and the outer membrane protein OmpA, respectively, were cloned on a shuttle vector under transcriptional control of the *B. amyloliquefaciens apr* promoter (see Materials and Methods). This promoter is maximally active in *B. subtilis* during late log and stationary phases of growth (58). Chimeric genes encoding hybrid precursors consisting of the *B. amyloliquefaciens* Apr_{SP} fused to the mature moieties of MBP, RBP, PhoA, and OmpA, the *B. amyloliquefaciens* Lvs_{SP} fused to the mature moieties of MBP and OmpA, or the MBP_{SP} fused to the mature moiety of RBP were also constructed (see Materials and Methods).

Processing kinetics for periplasmic proteins synthesized in *B. subtilis*. A pulse-chase analysis (see Materials and Methods) of BE2000 cells harboring pBE1065, pBE1145, and pBE1147 was used to evaluate the ability of *B. subtilis* to process native precursors of the periplasmic proteins MBP, RBP, and PhoA (Fig. 1). The precursor and mature forms of PhoA could be visually resolved but were insufficiently separated to quantitate (Fig. 1E). Visual inspection of the autoradiogram indicated that 5% or less of $PhoA_{SP}$-PhoA was secreted. When plated on GY minimal XP indicator agar plates, BE2000 cells producing $PhoA_{SP}$-PhoA had an extremely pale blue phenotype, which is consistent with a low level of PhoA secretion (46).

During the 20-min chase period, approximately 18% of MBP_{SP}-MBP (expressed as a percentage of the total [precursor plus mature] recovered at the 1-min chase point) was recovered as the mature species (Fig. 1A). Export of MBP172, which carries a truncated h region (lacks residues T-16, T-17, and M-18), was also examined. The $MBP172_{SP}$ directs secretion in *E. coli* more efficiently than the MBP_{SP} (12) but did not direct secretion of any MBP in BE2000 (Fig. 2A). Approximately 26% of RBP_{SP}-RBP was processed (Fig. 1C). Replacing the RBP_{SP} with the MBP_{SP} resulted in processing of only about 16% of the precise RBP_{SP}-MBP hybrid (Fig. 2C).

For each envelope protein, a majority of the matured species was soluble in the culture supernatant while a majority of the precursor species was cell associated (data not shown). Hence, processing of these proteins can serve as an indirect measure of secretion. The low levels of processing suggest that export of each precursor in *B. subtilis* is inefficient when secretion is directed by the homologous signal peptide.

The limited ability of *B. subtilis* to secrete these envelope proteins could result from insufficiencies in their signal peptides and/or properties of their mature moieties that cause them to be export incompetent in the context of the *B. subtilis* secretion machinery. To determine if the mature moieties of these periplasmic proteins are intrinsically secretable in *B. subtilis*, a set of chimeric genes encoding fusions between the Apr_{SP} and the mature moieties of MBP, RBP, and PhoA was constructed. Unlike other hybrid proteins which have been used to study protein export in *B. subtilis* (4, 45, 46), these are precise fusions which retain the native signal peptidase processing site of the Apr_{SP} and native sequences within the early portion of their mature regions.

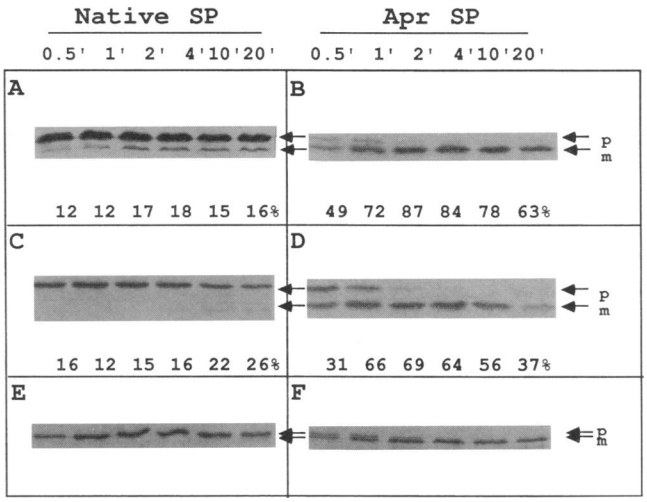

FIG. 1. Processing kinetics of native and hybrid precursors of *E. coli* periplasmic proteins synthesized in *B. subtilis*. Cultures of *B. subtilis* BE2000 cells harboring pBE1065 (A), pBE1073 (B), pBE1145 (C), pBE1083 (D), pBE1147 (E), or pBE1148 (F) were grown at 30°C in S7 medium containing glycerol; at an A_{600} of 0.7 to 0.8, they 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, samples were processed, and the relevant proteins were immunoprecipitated with appropriate rabbit polyclonal antisera and analyzed by SDS-PAGE and autofluorography. Time of chase (minutes) is given above each lane. The positions of the precursor (p) and mature (m) species are indicated by arrows. The amount of mature species, expressed as a percentage of the total recovered at the 1-min chase point, is shown below each chase point (except PhoA [see Materials and Methods]). Secretion of MBP (A and B), RBP (C and D), and PhoA (E and F) directed by their native signal peptides (A, C, and E) or Apr_{SP} (B, D, and F) is shown.

A pulse-chase analysis indicated that the Apr_{SP}-PhoA fusion was readily secreted in *B. subtilis* (Fig. 1F). At the 1-min chase point, about 70% had been matured, and by 4 min of chase, there was no detectable precursor. These results are consistent with the efficient secretion of imprecise Lvs_{SP}-PhoA and Apr_{SP}-PhoA fusions previously demonstrated in *B. subtilis* (41, 46). Unlike the pale blue phenotype of cells synthesizing $PhoA_{SP}$-PhoA, BE2000 cells synthesizing the Apr_{SP}-PhoA hybrid were dark blue on XP agar plates (data not shown). This observation supports the notion that secretion of PhoA accompanied processing.

When fused to the Apr_{SP}, up to 87% of MBP was secreted (Fig. 1B). The maximum extent of processing was achieved by 2 min of chase, with no increase in the amount of mature MBP recovered after 2 min of chase. Overexposure of the autoradiogram, and quantitation of the precursor bands indicated that a majority of the precursor present at the 2-min chase point remained unprocessed at the 10-min chase point, and a fraction remained trapped even after 20 min of chase (not shown).

Secretion of Apr_{SP}-RBP (Fig. 1D) was slightly less efficient than that of Apr_{SP}-MBP: 66% was processed by 1 min of chase, with a maximum of 69% processing having occurred by 2 min of chase. Although all of the Apr_{SP}-RBP precursor disappeared during the chase period, it could not be determined if this was due to export or degradation, since a significant percentage of the total RBP was degraded during the chase period.

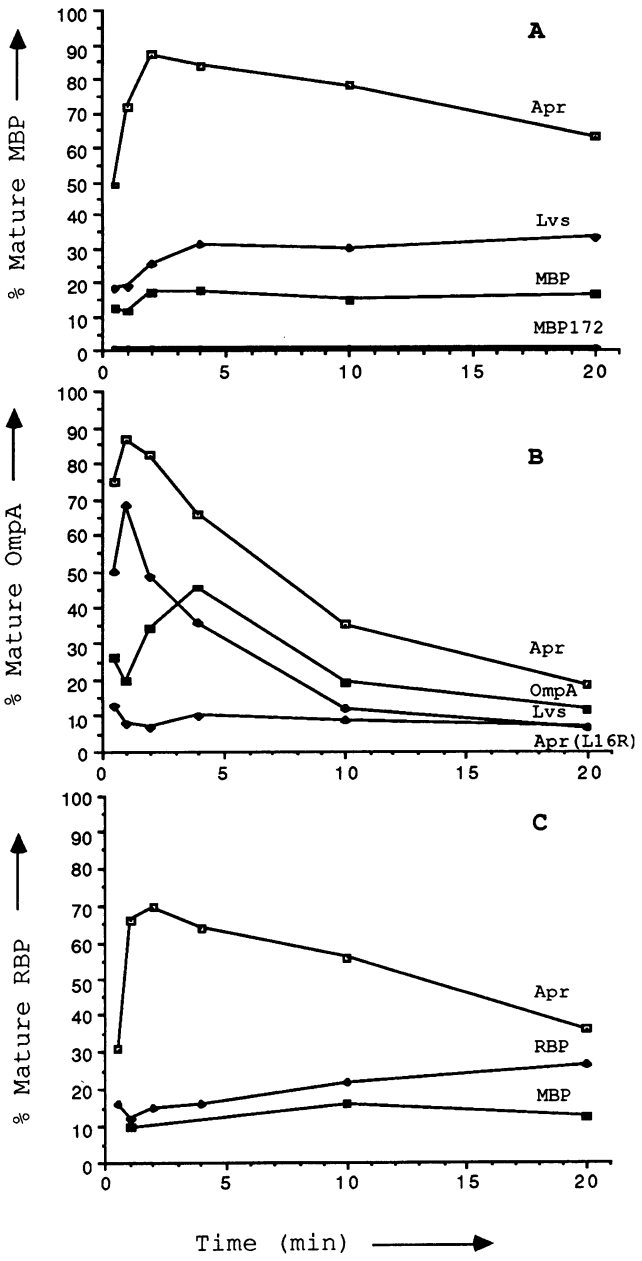

FIG. 2. Secretion of Lvs_{SP} and MBP_{SP} hybrids. Cells of BE2000 carrying pBE1074 (encoding $MBP172_{SP}$-MBP), pBE1151 (encoding Lvs_{SP}-MBP), pBE1153 (encoding Lvs_{SP}-OmpA), or pBE1064 (encoding MBP_{SP}-RBP) were subjected to a pulse-chase analysis as described in the legend to Fig. 1. The amount of mature protein, expressed as a percentage of the total recovered at the 1-min chase point, is plotted as a function of time (minutes). The signal peptide directing export is given above its corresponding curve [except below Apr(L16R)$_{SP}$-MBP (B)]. (A) Secretion of MBP. The data for Apr_{SP}-MBP and MBP_{SP}-MBP are from Fig. 1. (B) OmpA. The data for Apr_{SP}-OmpA, $OmpA_{SP}$-OmpA, and Apr(L16R)$_{SP}$-OmpA are from Fig. 3. (C) RBP. The data for Apr_{SP}-RBP and RBP_{SP}-RBP are from Fig. 1.

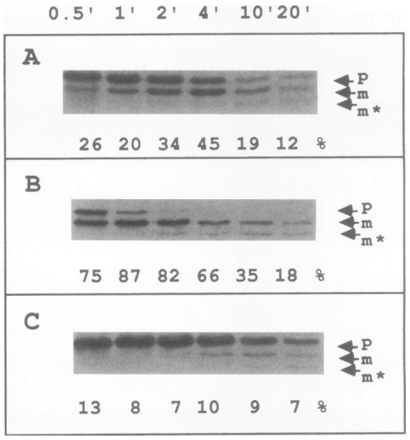

FIG. 3. Processing kinetics for various OmpA precursors. A pulse-chase analysis as described in the legend to Fig. 1 was performed on cells of BE2000 carrying pBE1146 (encoding $OmpA_{SP}$-OmpA), pBE1093 (encoding Apr_{SP}-OmpA), or pBE1141 [encoding Apr (L16R)$_{SP}$-OmpA]. Time of chase (minutes) is given above each lane. The positions of the precursor (p), mature (m), and mature* (m*) species are indicated by arrows. The amount of mature species, including m*, expressed as a percentage of the total recovered at the 1-min chase point, is shown below each chase point (see Materials and Methods). Secretion of $OmpA_{SP}$-OmpA (A), Apr_{SP}-OmpA (B), and Apr(L16R)$_{SP}$-OmpA (C) is shown.

The Lvs of *B. amyloliquefaciens* is an exoprotein that is efficiently secreted by *B. subtilis* (4). Interestingly, only 35% of a precise Lvs_{SP}-MBP hybrid was processed by 20 min of chase (Fig. 2A). Although both the initial rate and extent of Lvs_{SP}-MBP secretion were greater than those of MBP_{SP}-MBP, they were significantly lower than those of Apr_{SP}-MBP (Fig. 2A). Hence, when fused to MBP, the Lvs_{SP} behaved more like an *E. coli* signal peptide.

Secretion of the outer membrane protein OmpA. Several reports have indicated that OmpA and other outer membrane proteins cannot be secreted in *B. subtilis* (27, 47, 48) even if fused to an efficient bacillary signal peptide (43). To determine if low levels of secretion could be detected by radioimmunoprecipitation, BE2000 cells synthesizing the $OmpA_{SP}$-OmpA precursor were subjected to a pulse-chase analysis. Unexpectedly, the OmpA precursor was converted into two lower-molecular-weight forms (Fig. 3A). One form, present after only 0.5 min of chase, comigrated with mature OmpA which had been immunoprecipitated from radiolabeled *E. coli* (data not shown). The presence of this form, which is most likely properly processed mature OmpA (designated mOmpA), suggested that a fraction of the native $OmpA_{SP}$-OmpA was secreted. The intensity of the mOmpA band reached a maximum at the 4-min chase point. A second species, designated m*OmpA, had an apparent molecular weight lower than that of mOmpA and appeared somewhat later in the chase period than mOmpA.

In cells of BE2000 synthesizing an Apr_{SP}-OmpA hybrid (Fig. 3B), there was significantly more mOmpA present at the early chase points, and less precursor remained after 20 min of chase, than in cells synthesizing the $OmpA_{SP}$-OmpA precursor (Fig. 3A versus B). Overexposure of the film indicated that some precursor persisted at the 20-min chase point. Although the increase in mOmpA associated with the Apr_{SP}-OmpA hybrid is most likely due to the ability of the Apr_{SP} to efficiently direct secretion in *B. subtilis*, the small percentage of mOmpA and m*OmpA that was recovered from the supernatant fraction (data not shown) does not support this hypothesis.

To determine if secretion of OmpA precursors was required to generate mOmpA and/or m*OmpA, an arginine codon was substituted for the leucine codon at position 16 of the Apr_{SP}

TABLE 1. Characteristics of the signal peptides used

Peptide (reference)	Predicted amino acid sequence and secondary structure[a]	Net charge of early mature moiety	h-region parameter			
			No.[b]	H*[c]	<H>[d]	H_p[e]
Apr (60)	+ ++ MRGKK**VWISLLFALALIFTMAFG**STSSAQA ·····**BBBBBBBBBBBBBBBB**·tTTt···	+2	18	15.2	0.84	274
Lvs (57)	++ + MNIKKIVKQ**ATVLTFTTALLAGGA**TQAFA ··HHHHHHH**HBBBBBBBBBBB**··**H**HHHHH	+1	15	8.7	0.58	131
OmpA (38)	++ MKKT**AIAIAVALAGFA**TVAQA HHHH**HHHHHHHHHHHH**HHHHH	0	12	10.3	0.86	124
RBP (23)	++ MNMKK**LATLVSAVALSATV**SANAMA ··HHH**HHHHHHHHHHHHHH**HHHHHH	0	14	8.4	0.60	118
MBP (17)	+ + + MKIKTGAR**ILALSALTTMMF**SASALA ·····HH**HHHHHHHHHHHH**HHHHHH	0	12	8.0	0.67	96
PhoA (28)	+ + MKQST**IALALLPLLF**TPVTKA ····B**BBBBBBBBBB**B·····	−1	10	9.2	0.92	92
MBP172 (12)	+ + + MKIKTGAR**ILALSAL**---**MF**SASALA HHHHHHHH**HHHHHH** **HH**HHHHHH	0	9	7.5	0.83	68

[a] Basic residues are indicated by plus signs, and deleted residues are indicated by dashes. The amino acids in the h region are shown in boldface. The h-region borders were defined essentially as described previously (61) except that (i) when the most carboxy-terminal charged residue of the N terminus was followed by one or a stretch of hydrophilic residues (Q, N, S, or T), the h region was defined as starting after the last hydrophilic residue, and (ii) the h region was defined as ending either at a predicted β turn or at the first charged or hydrophilic residue encountered within seven residues of the signal peptidase cleavage site. Predictions of secondary structure were performed with the algorithm of Chou and Fasman (9) as modified for use by the PeptideStructure program of the Genetics Computer Group package (15). Conformational preferences are indicated below the primary amino acid sequence: H, high probability of α helix; h, probability of α helix; B, high probability of β structure; b, probability of β structure; T, high probability β turn; t, probability of β turn; ·, probability of random coil. The first 10 residues of the mature moiety were used to calculate the net charge of the early mature moiety.

[b] Number of residues assigned to the h region.

[c] Net hydrophobicity (H*) is the sum of hydrophobicities for the h-region residues based on the normalized consensus scale of Eisenberg et al. (18).

[d] <H>, average hydrophobicity per h-region residue.

[e] The hydrophobic product (H_p) is defined as the product of the number of residues assigned to the h region and the net hydrophobicity.

(L16R). The L16R substitution has been shown to impart a moderately strong block in the export of Apr_{SP}-Apr and an Apr_{SP}-PhoA hybrid (45). The low level (about 10%) of mOmpA and m*OmpA generated in BE2000 cells synthesizing the $Apr(L16R)_{SP}$-OmpA hybrid precursor (Fig. 3C) is compatible with the notion that mOmpA and m*OmpA result from processing associated with secretion.

Irrespective of the signal peptide used, mOmpA accumulated earlier in the chase period than m*OmpA, and a significant portion of the accumulated mOmpA disappeared during subsequent incubation (Fig. 3). The concomitant increase in the amount of m*OmpA recovered suggests that there is a precursor-product relationship between mOmpA and m*OmpA and that m*OmpA probably results from proteolysis of secreted mOmpA. Therefore, m*OmpA was included with mOmpA in calculating the extent of OmpA secretion.

As was the case for MBP, the Lvs_{SP} directed secretion of OmpA at a level intermediate between those of the Apr_{SP} and its own signal peptide. Processing kinetics for the four OmpA precursors are compared in Fig. 2B.

DISCUSSION

When precursors of the *E. coli* envelope proteins MBP, RBP, PhoA, and OmpA were synthesized in *B. subtilis*, both the rate and extent of their processing were low. Inefficient secretion of these proteins could be ascribed largely to characteristics of their native signal peptides, since replacing them with the Apr_{SP} resulted in a significant enhancement in the export kinetics for each of these four envelope proteins.

The h regions of signal peptides from *E. coli* are significantly shorter than those of *Bacillus* species and other gm+ organisms (12 versus 15 or more residues [62; reviewed in reference 40]). Given the central role(s) that the h region plays in sponsoring translocation of precursors across the cytoplasmic membrane in *E. coli* (2, 19, 20, 53; reviewed in references 22 and 55) and *B. subtilis* (4, 45, 52), it is likely that the poor secretion of these *E. coli* proteins in *B. subtilis* is related to their comparatively short h cores rather than properties of their N or C regions. Indeed, with the exception of PhoA, the relative efficiency with which the native precursors were secreted in *B. subtilis* (OmpA >> RBP > MBP > PhoA) correlated well with the net hydrophobicity (H*) of their h regions (Table 1).

However, it is not clear if secretion in *B. subtilis* requires an h region that is simply longer than those of *E. coli* signal peptides, if the extended h regions actually reflect a requirement for a higher net hydrophobicity that generally accompanies longer cores, or both. The comparatively high percentage of the polar, hydroxylated serine and threonine residues found in the h regions of *B. subtilis* signal peptides (40) suggests that length itself, rather than simply hydrophobicity, is a significant parameter. An expression accommodating contributions made by both length and hydrophobicity is the hydrophobic product (H_p), defined here as the product of the length and the net hydrophobicity of the h region. There is a better correlation between the extent to which these *E. coli* preproteins were

secreted in *B. subtilis* (OmpA >> RBP > MBP > PhoA > MBP172) and their H_p values (Table 1) than with their H* values or any other readily identifiable parameters. Hence, the lengths of the PhoA and MBP172 h regions may fall below a functional threshold that, in the case of PhoA, cannot be rescued by its high H* value. The relative secretion efficiency for each MBP (Apr_{SP}-MBP >> Lvs_{SP}-MBP > MBP_{SP}-MBP > $MBP172_{SP}$-MBP), RBP (Apr_{SP}-RBP >> RBP_{SP}-RBP > MBP_{SP}-RBP) and OmpA (Apr_{SP}-OmpA > Lvs_{SP}-OmpA > $OmpA_{SP}$-OmpA) precursor was also correlated with the H_p value of its signal peptide (Table 1).

In addition to its low H_p value, the $PhoA_{SP}$ (21 residues) is much shorter than the average gm+ signal peptide (29 to 31 residues [62]). However, this alone cannot explain its poor function, since the 21-residue $OmpA_{SP}$ performed better than the other *E. coli* signal peptides tested, and the 23-residue $MBP172_{SP}$ was incapable of sponsoring MBP secretion. In contrast to the average net charge of +3 carried by the N region of gm+ signal peptides (62), the $PhoA_{SP}$ carries a net charge of only +1. In this respect, it is similar to the β-lactamase signal peptide, which carries a single basic residue (22) and also is incapable of directing secretion in *B. subtilis* (H. Smith, personal communication cited in reference 37). However, a net charge of +1 is sufficient to support efficient export of Lvs in *B. subtilis* (4), while MBP172, which carries a charge of +3, did not direct secretion. Therefore, it is not likely that the charge of the N region is the principal weakness of the $PhoA_{SP}$. It should be noted that in *E. coli*, secretion of MBP species with a truncated but functional h core is significantly more sensitive to N-region charge reductions than are the corresponding full-length core species (49). Hence, addition of positive charges to the N region of the $PhoA_{SP}$ might improve its function in *B. subtilis.*

The early mature region can affect secretion efficiency in *E. coli* by influencing the charge balance flanking the h region (32, 50). PhoA, with a single positive charge in its N region and a net charge of 0 on the carboxy-terminal side of the h region (+/0; Table 1), has a relatively poor charge balance that might also contribute to its poor secretion. However, charge balance is not likely a principal cause of defective secretion for species such as MBP (3+/0), since Apr (3+/2+) and even a 1+/1+ Lvs derivative (4) are efficiently secreted. Furthermore, for a given mature moiety, secretion efficiency was not strictly correlated with charge balance (Fig. 2).

The h regions of many *E. coli* signal peptides, including those of MBP, RBP, and OmpA, are predicted (9) to assume an α-helical conformation. Both genetic (3, 19) and biophysical (5, 34) evidence suggests that the export competence of certain *E. coli* signal peptides depends on the ability of their h regions to adopt this secondary structure. Although introduction of helix-breaking proline residues into the h region of MBP (3, 13), RBP (13, 25), or OmpA (22) impairs secretion of these proteins in *E. coli*, proline residues occur naturally in the h regions of some *E. coli* signal peptides, including those of PhoA and β-lactamase. Consequently, the h regions of these two signal peptides are not expected to favor helix formation. However, it is not likely that the poor performance of the $PhoA_{SP}$ is due strictly to this proline residue or the predicted lack of helical content, since proline residues are also found in the h regions of the *B. subtilis* α-amylase and alkaline phosphatase III precursors (reviewed in reference 40). Furthermore, the h regions of Apr, Lvs (Table 1), and α-amylase are predicted to have little or no helical content.

Under certain circumstances, the C region (20) and even the first residues of the mature region (2, 53) may also function as part of the h region in *E. coli*. If *E. coli* signal peptides are, in effect, truncated core derivatives of gm+ signals, then secretion in *B. subtilis* may require recruitment of the *E. coli* signal peptide's C region for h-region function. Recruitment of PhoA's C region would not be productive since it contains a lysine residue at the −2 position, and lysine substitutions in the h region are known to impair secretion in *B. subtilis* (45). Interestingly, insertion of a glutamic acid residue into the C region of the *B. subtilis* α-amylase signal peptide diminishes its function in *B. subtilis* (42), which suggests that the h and C regions of this signal peptide may overlap. However, insertion of a lysine residue at the same position had no visible effect on secretion (42). The glutamine and asparagine residues found in the C regions of the OmpA and RBP signal peptides, respectively, may also limit the effectiveness of these signal peptides. The effect should be less severe than that caused by a lysine, since a leucine-to-asparagine substitution in the h region of the Apr_{SP} has only modest effects on secretion (45). While the effects of glutamine substitutions on secretion in *B. subtilis* have not been reported, glutamine may not have the same strong effect on secretion in *B. subtilis* as it does in *E. coli* (22), since glutamine is frequently found in the h regions of bacillary signal peptides (40).

The correlation between H_p and secretion efficiency in *B. subtilis* could be coincidental rather than causal, with unrecognized characteristics of the signal peptides and/or properties of the mature proteins actually determining secretion efficiency. However, when fused to the Apr_{SP}, the relative efficiencies with which the mature moieties were exported (PhoA > OmpA > MBP > RBP) were different from those of the native precursors (OmpA >> RBP > MBP > PhoA). If the mature moieties alone dictated efficiency, then the relative secretion efficiencies should remain the same regardless of the signal peptide directing export. Hence, the mature moieties are not the principal determinants of export competence for secretion of these native proteins in *B. subtilis*. However, the Lvs_{SP}, which directs the rapid and complete secretion of Lvs (4) and PhoA (38b), directed secretion of only 68% of OmpA and 33% of MBP, indicating that the mature moiety can influence the secretion proficiency of signal peptides. This has been previously noted in both *B. subtilis* (8, 24, 42, 45) and *E. coli* (11, 13, 31, 33).

Molecular chaperones, such as SecB (11, 29), DnaK and DnaJ (64), and GroEL and GroES (30), modulate precursor conformation and actively participate in protein targeting in *E. coli* (reviewed in reference 10). Interestingly, the overall secretion efficiency for the four Apr_{SP} fusion proteins in *B. subtilis* (Apr_{SP}-PhoA > Apr_{SP}-OmpA > Apr_{SP}-MBP > Apr_{SP}-RBP) reflects the degree to which their secretion in *E. coli* relies on DnaK and DnaJ, suggesting that DnaK and DnaJ also participate in secretion of these proteins in *B. subtilis.*

Although a significant percentage of each native precursor disappeared during the last half of the chase period, RBP_{SP}-RBP and $PhoA_{SP}$-PhoA were the only species with a significant percentage of their secretion ascribable to late posttranslational chase. Maximum measurable levels of secretion for all other precursors, including $Apr(L16R)_{SP}$-OmpA, were achieved with similar half-times (0.5 to 2 min). This finding suggests that all precursors are maintained in translocation-competent states (11, 51) for similar periods and that the extent of secretion is largely determined by the rate with which the secretion machinery acts on the signal peptide during this period of competency. These results differ significantly from the extensive late posttranslational secretion exhibited by mutationally altered MBP (2, 53) and PhoA (16) precursors in *E. coli*. In *E. coli*, secretion of MBP172 is essentially SecB independent, presumably the result of enhanced cotransla-

tional secretion that sponsors translocation of MBP172 before it forms export-incompetent conformers (12). MBP172 was not secreted in *B. subtilis*, indicating that the features of the $MBP172_{SP}$ that have been optimized for interaction with the *E. coli* secretion machinery do not interact properly with the export apparatus of *B. subtilis*.

The ability of the Apr(L16R) mutation to severely reduce the conversion of precursor OmpA to mOmpA indicates that mOmpA arises from the cleavage of OmpA precursors by *B. subtilis* signal peptidase during translocation. This result contrasts sharply with several reports concluding that OmpA and other outer membrane proteins cannot be secreted by *B. subtilis* even when fused to the *B. amyloliquefaciens* α-amylase signal peptide (27, 47, 48). It is probable that these disparate conclusions are the result of the methods used to measure secretion. Using a pulse-chase analysis, I have found that precursors of OmpA are more stable than secreted OmpA. Because of its relative instability, secreted OmpA will be underrepresented during steady-state conditions. Factors which enhance the stability of the precursor, such as the reported aggregation of intracellular OmpA (48), or destabilize the secreted species, such as the nonnative amino termini (1) that could result from signal peptidase processing of imprecise fusions, will exacerbate the underestimation of secretion that can result from steady-state measurements such as immunoblotting. Recently Meens et al. (35) have also demonstrated that *B. subtilis* can secrete $OmpA_{SP}$-OmpA. In their report, more than half of the OmpA detected by Western blot (immunoblot) analysis was in the precursor form, while a pulse-chase analysis of the same strain indicated that a majority of the precursor was processed and/or degraded by 10 min of chase.

Therefore, it can be concluded that there is no mechanistic barrier preventing translocation of OmpA, and perhaps other outer membrane proteins, across the cytoplasmic membrane of *B. subtilis*. However, *B. subtilis* is a poor host for accumulating extracellular OmpA, presumably because in the absence of lipopolysaccharide, secreted OmpA is limited to conformers (21) that are especially sensitive to proteolysis.

Although the secretion machineries of *E. coli* and *B. subtilis* are in many respects similar, the inability of *B. subtilis* to efficiently secrete proteins fused to signal peptides from *E. coli* demonstrates that the exaggerated structural features of bacillary signal peptides are functionally significant and imply that one or more steps in the secretion pathway of *B. subtilis* differ from those of *E. coli* in requiring these more pronounced features. The capacity of *B. subtilis* to secrete four *E. coli* envelope proteins was apparently related to the H_p value of the signal peptide directing export. A titration of signal peptide function by varying H_p values, as has been done for H^* values and signal peptide function in *E. coli* (16), is needed to demonstrate the generality of this concept. Finally, it should be noted that idealization of the wheat α-amylase signal peptide by increasing the net positive charge of its n region, extending its h region, or generating a consensus signal peptidase site did not improve its function in *B. subtilis* (52). Hence, it is possible that unrecognized properties of the signal peptide are required to efficiently mediate the entire multistep process of protein secretion in *B. subtilis*.

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