CHARACTERIZATION OF THE SecB PROTEIN, A CHAPERONE THAT FACILITATES PROTEIN SECRETION IN *ESCHERICHIA COLI*

Thesis by

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Dedication

I would like to dedicate this thesis to my family, for without them I would not have been able to complete my Ph.D.

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I am grateful to Scott Emr for allowing me to work on *E. coli* in his lab. It undoubtedly required a great amount of patience to continue to focus on *E. coli* when the lab's interest was yeast. I am also appreciative of the rest of the lab for putting up with the odd man out, as I realize it was difficult to tolerate someone whose work was so far removed from the interest of the lab. I am also indebted to many people outside the lab for advice and assistance during my studies at Caltech. Unfortunately, this list would be too numerous to generate, and I hope that these people realize how invaluable their help was to me.

Abstract

It has become increasingly clear that in *Escherichia coli*, most exported proteins are translocated either posttranslationally or late in their synthesis, and that a component of the export apparatus, SecB, facilitates the export of a subset of the secreted proteins by maintaining them in an export-competent, unfolded form. In an effort to understand how SecB functions as an antifolding factor, we mapped and characterized the sites of SecB interaction in the outer membrane protein LamB. We found that the interaction of SecB with LamB was dependent on the LamB signal sequence as well as on a region in the mature LamB protein. The simplest interpretation of these findings is that SecB binds to both the LamB signal sequence and a mature region in LamB, and that this interaction promotes the antifolding activity of SecB.

Given the fact that several heat-shock proteins have also been shown to function as antifolding factors, we wanted to investigate whether heat-shock proteins might act in a manner analogous to SecB in facilitating the export process. We found that induction of the heat-shock response could substitute for SecB function (SecB is not a heat-shock protein), and that a basal level of heat-shock proteins was necessary for the cell to survive in the absence of SecB protein. These results suggested that heat-shock proteins might indeed be involved in the secretory process and function in a manner similar to that of SecB.

In an attempt to identify these proteins, suppressors of a *sec*B null mutation were isolated and characterized. Not unexpectedly, most of these suppressors mapped to the *rpo*H locus. Since *rpo*H encodes σ^{32}, the heat-shock transcription factor, it is likely that these suppressors affect the synthesis levels of heat-shock proteins, which can substitute for SecB function. The remaining suppressors did not map to any known heat-shock or export genes, and potentially represent unidentified heat-shock proteins or export factors that act in a manner similar to SecB in facilitating the export process in *E. coli*.

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Chapter 1

Export and Localization of *Escherichia coli* Proteins: An Introduction

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A fundamental property of all living cells is their ability to faithfully target specific proteins to unique subcellular compartments. With few exceptions, all proteins are synthesized in the cytoplasm. Yet many polypeptides are exported from this compartment to any one of a number of noncytoplasmic locations. For a eukaryotic cell, the problem of protein targeting is an extremely complex one. Such cells harbor extensive systems of membranous organelles within which the proper complement of proteins must be assembled. Prokaryotic cells are not endowed with such highly structured organellar systems and may face a somewhat simpler task in directing proteins to their correct subcellular destinations. Still, the gram-negative bacterium *Escherichia coli* targets proteins to one of at least three distinct extracytoplasmic compartments. Included are the cytoplasmic and outer membranes and the periplasm, an aqueous compartment that is bounded by these two membranes.

Although the question of how a cell sorts the various proteins to their target organelles seems more complex for eukaryotes than for prokaryotes, both types of cells must deal with the initial sorting problem. That is, how does a cell distinguish proteins that are destined for export from those that are fated to remain in the cytoplasm? Once this basic distinction has been made, the cell is confronted with the problem of translocating a generally hydrophilic polypeptide across a hydrophobic membrane. Several lines of evidence suggest that the initial steps in *E. coli* protein export from the cytoplasm to the periplasm or outer membrane may be similar to the mechanisms employed by eukaryotic cells to export proteins into the lumen of the endoplasmic reticulum. Both systems involve the synthesis of exported proteins as higher molecular weight precursor forms carrying amino terminal extensions, referred to as signal peptides, that are endoproteolytically processed to yield the mature protein. But perhaps the most compelling reasons for believing that protein export mechanisms have been conserved are derived from studies of secretion in heterologous systems. Yeast is capable of secreting and correctly processing wheat α-amylase (Rothstein *et al.*, 1984). *E. coli* has been shown to export chicken

ovalbumin (Fraser and Bruce, 1978) and rat preproinsulin (Talmadge *et al.*, 1980) to the periplasm, when the respective genes have been engineered for expression in this bacterium. Furthermore, processing of the periplasmic preproinsulin antigen to proinsulin occurs correctly.

1. MODELS FOR PROTEIN EXPORT

Any model that is formulated to describe how protein export is initiated must consider the selectivity and efficiency of the process. A number of hypotheses have been offered in an attempt to explain the mechanism of protein export. Three of these are described briefly below. These models represent the entire spectrum of current thought concerning how bacterial proteins are exported and provide wide perspectives from which the available data can be viewed.

Perhaps the most widely accepted model is the signal hypothesis (Blobel and Dobberstein, 1975; Blobel and Sabatini, 1971). It was formulated to account for data obtained from elegant biochemical studies of eukaryotic *in vitro* protein export systems (for a review see Walter *et al.*, 1984). This model invokes the presence of a signal peptide, at the amino terminus of an exported protein, which actively mediates the export process. As a secretory polypeptide is being synthesized, the signal peptide extrudes from the ribosome and is recognized by a cytosolic signal recognition particle (SRP) that functions in facilitating the exit of proteins from the cytoplasm. Interaction of the signal peptide with SRP results in translational arrest, receptor-mediated delivery of the arrested translational complex to the membrane, and finally relief of the translational block. A proteinaceous pore is then envisioned to aggregate across the membrane in response to the signal peptide. Vectorial translocation of the polypeptide through the pore ensues in a manner that is cotranslational and linear in mode, and the transmembrane pore is subsequently disaggregated upon completion of the polypeptide transfer. Finally, at some point during translocation or very shortly thereafter, the signal peptide is endoproteolytically processed,

and the mature secretory protein is released on the extracytoplasmic side of the membrane barrier. The three critical features of the signal hypothesis are (1) a cellular machinery that is obligatory for protein export; (2) the signal peptide as the source of information within a secretory protein that plays an active role in export initiation; and (3) a cotranslational and linear mode of translocation that is tightly coupled to polypeptide chain elongation.

The helical-hairpin hypothesis of Engelman and Steitz (1981) is a detailed representative of several theoretical treatments of protein export. Other related models include the loop model of Inouye and coworkers (1977) and the direct transfer model of Von Heijne and Blomberg (1979). The helical-hairpin hypothesis does not attempt to address specifically how sorting of exported proteins from cytoplasmic proteins is accomplished. It does, however, offer a detailed scenario for how proteins are translocated across the cytoplasmic membrane. The helical-hairpin hypothesis considers protein translocation across a membrane to be effected by the formation of a pair of neighboring, antiparallel helices of which the signal peptide is one. The resultant helical hairpin spontaneously partitions into the hydrophobic portion of the lipid bilayer and thereby leads the nascent secretory polypeptide across the cytoplasmic membrane. Export is suggested to be driven by the thermodynamic benefit gained from sequestering a hydrophobic helix in a nonpolar environment. The salient features of this proposal include a linear mode of polypeptide transfer across the membrane that is tightly coupled to elongation, and a proper distribution of hydrophobic and hydrophilic sequences within the secretory polypeptide so that export can properly occur. Finally, protein translocation across a membrane is considered to be a spontaneous event that does not require the participation of an export pore.

The last general proposal is the membrane trigger hypothesis of Wickner (1979). Within the framework of this model, protein export is considered to be a spontaneous process that does not involve an ancillary cellular machinery. The interaction of a secretory polypeptide with the membrane, either before or shortly after completion of synthesis, is

believed to trigger a conformational folding of the polypeptide into and across the membrane. The signal peptide is thought to participate in the formation of a "trigger-competent" structure. Processing renders the translocation event irreversible. As in the helical-hairpin hypothesis, a large portion of the polypeptide is proposed to play an intimate role in the export process. Finally, translocation and translation of the secretory polypeptide are not predicted to be coupled activities.

2. BIOCHEMICAL ASPECTS OF PROTEIN LOCALIZATION IN *E. COLI*

2.1. Precursors of Secreted Proteins in *E. coli*

Most *E. coli* proteins that are localized to noncytoplasmic compartments are initially synthesized as higher molecular weight precursor forms with short, amino terminal, signal peptides that are processed to yield the mature protein products. A number of methods have been employed to visualize these generally short-lived precursor forms. Such methods have included pulse-labeling of whole cells and subsequent analysis by immune precipitation, SDS-PAGE and autoradiography (Josefsson and Randall, 1981a), comparison of radiolabeled products observed *in vivo* with those obtained from *in vitro* translation systems (Inouye and Beckwith, 1977; Randall *et al.*, 1978), and use of *in vivo* conditions under which normal protein export, or processing, is inhibited. For instance, agents that alter membrane fluidity—such as procaine (Lazdunski *et al.*, 1979) and phenethyl alcohol (Halegoua and Inouye, 1979)—or that deenergize the membrane—for example, such energy uncouplers as carbonyl cyanamide m-chlorophenylhydrazone and dinitrophenol (Date *et al.*, 1980b; Daniels *et al.*, 1981; Palva *et al.*, 1981)—result in precursor accumulation. Fatty acid auxotrophs grown in the presence of eliadate (Pages *et al.*, 1978; DiRenzo and Inouye, 1979), ATPase mutants grown under anaerobic conditions (Enequist *et al.*, 1981), and *E. coli* strains induced for synthesis of certain classes of LacZ protein chimeras (Ito *et al.*, 1981) likewise exhibit varying degrees of precursor accumulation.

The availability of powerful techniques for determining amino acid or nucleic acid sequence has permitted the elucidation of the primary sequences for a host of bacterial signal peptides (Michaelis and Beckwith, 1982; Watson, 1984). Examination of such sequences has failed to reveal striking primary sequence homologies among them. However, several general structural homologies can be discerned, including (1) an extreme amino terminal hydrophilic segment characterized by the presence of 1-3 basic residues; (2) a hydrophobic core composed of an uninterrupted stretch of 10-15 uncharged residues that directly follow the hydrophilic segment; (3) the presence of serine or threonine residues near the processing site; and (4) the presence of an amino acid residue at the ultimate position in the signal peptide that exhibits a small, uncharged R group. Finally, signal peptides appear to assume predominantly helical conformations (Austen, 1979; Rosenblatt *et al.*, 1980; Bedouelle and Hofnung, 1981). Mutational analyses of bacterial signal peptides have lent some insight into the roles that certain of these features play in the export process (see Section 3.2).

Application of the various methods of detecting precursor forms of exported proteins has established that *E. coli* periplasmic and outer-membrane proteins are synthesized in precursor form. Paradoxically to this, however, *E. coli* cytoplasmic membrane proteins include those that exhibit precursor forms and those that clearly do not. It is possible that periplasmic and outer membrane proteins require the presence of a signal sequence and more complex delivery mechanisms, since they must be translocated across the cytoplasmic membrane to reach their final destinations. While some cytoplasmic membrane proteins require a signal sequence to aid their delivery to the membrane, other cytoplasmic membrane proteins do not and being largely hydrophobic, are simply partitioned into the membrane (Blobel, 1980; Engelman and Steitz, 1981; Michaelis and Beckwith, 1982; Silhavy *et al.*, 1983; Wickner, 1979).

2.2. Temporal Mode of Export

The demonstration of an intimate coupling between translation and protein export in eukaryotic systems (Siekevitz and Palade, 1960; Redman and Sabatini, 1966; Sabatini and Blobel, 1970; Blobel and Dobberstein, 1975; Rothman and Lodish, 1977) has stimulated similar studies in prokaryotic systems. The first data to suggest that this was also the case in *E. coli* was provided by Cancedda and Schelessinger (1974), who showed that the periplasmic alkaline phosphatase protein was synthesized preferentially on membrane-bound polysomes relative to the synthesis of cytoplasmic β-galactosidase. These basic conclusions were confirmed by Smith and coworkers (1977) and Varenne and coworkers (1978), who compared the site of synthesis of alkaline phosphatase to that of total protein or to that of the cytoplasmic protein elongation factor G. Randall and coworkers extended this relationship to include the periplasmic maltose binding protein (MBP) and obtained evidence suggesting that the periplasmic arabinose-binding protein and the outer membrane protein LamB were preferentially synthesized on membrane-bound polysomes as well (Randall *et al.*, 1978). The most convincing evidence to support the involvement of membrane-bound polysomes in *E. co*li export comes from the work of Smith *et al.* (1977). These investigators were able to externally label a class of nascent polypeptides in *E. coli* spheroplasts with a reagent incapable of penetrating the cytoplasmic membrane, and demonstrated that polysomes engaged in the synthesis of exported proteins do indeed exhibit a functional association with the membrane.

Although collectively these data implied that protein export in *E. coli* was similar to that in higher cells and occurred in a cotranslational, linear fashion, workers in several labs found that at least three proteins, M13 coat protein (Date and Wickner, 1981), TEM β-lactamase (Koshland and Botstein, 1982), and ribose binding protein (Randall, 1983) are not translocated in *E. coli* until they are completely translated. To investigate this problem, Randall and coworkers developed a two-dimensional gel assay by which they could

measure how much of a protein must be translated before translocation can occur (Josefsson and Randall, 1981a, 1981b). They found that while ribose binding protein and TEM β-lactamase are translocated 100% posttranslationally, all other proteins tested showed varying degrees of cotranslational export. MBP, arabinose binding protein, ampC β-lactamase, OmpA, LamB, and alkaline phosphatase were translocated 35%, 48%, 100%, 60%, 10%, or >60% cotranslationally, respectively. Surprisingly, however, they found that in the case of four proteins, MBP, OmpA, alkaline phosphatase, and arabinose binding protein, which are largely cotranslationally exported, translocation did not occur until 80% of the protein had been translated. Taken together these studies suggest that proteins in *E. coli* are largely translated before they are exported. Although this does not rule out the probability that proteins are committed for export well before translation is complete, it does indicate that export in *E. coli* does not occur in a simple linear fashion.

2.3. Energetics of Protein Export

The migration of a largely hydrophilic macromolecule from one aqueous compartment to another, across a hydrophobic membrane barrier, is a process that requires energy. This was experimentally demonstrated by Date and coworkers (1980b), who showed that such metabolic poisons as CCCP, cyanide, azide and dinitrophenol inhibited processing of M13 coat protein precursor to the mature form. These energy uncouplers act primarily as dissipators of the protonmotive force across the cytoplasmic membrane. Arsenate, a metabolic poison that depletes intracellular high-energy phosphate bond pools without affecting membrane potential, had no effect on procoat assembly and maturation (Date *et al.,* 1980a). These data implicated membrane potential, and not ATP, as the energy source for protein export. Subsequently, Daniels and coworkers (1981) and Enequist and associates (1981) also demonstrated the requirement of an energized membrane for export of leucine-binding protein, β-lactamase, MBP, and arabinose-binding protein to the periplasm and export of the OmpF, OmpA, and LamB proteins to the *E. coli* outer

membrane. Studies with *unc*A mutants that lack a proton-translocating ATPase also suggested that it is the protonmotive force, and not ATP, that is required for export of MBP (Enequist *et al.*, 1981).

On the basis of these collective observations Daniels and coworkers (1981) proposed that membrane potential, the major component of protonmotive force, serves to orient precursors correctly in the membrane, perhaps by charged group alignments in the electrical field, and subsequently "electrophoreses" them across the membrane. Recent experiments indicate, however, that total protonmotive force fulfills the energy requirement for export of at least β-lactamase (Bakker and Randall, 1984). This conclusion rests on the observation that either of the two components of total protonmotive force (i.e., membrane potential or the pH gradient) can be varied over wide ranges without altering the measured level of total protonmotive force at which inhibition of β-lactamase export is observed. This result cannot be explained by simple electrophoretic mechanisms such as those proposed by Daniels and associates (1981).

At present, exactly the role, be it direct or indirect, that protonmotive force plays in protein export remains unresolved. Perhaps it is required for proper function of a protein-translocating "pore" complex. Other possibilities, such as ion symport mechanisms, can also be offered (Bakker and Randall, 1984).

2.4. Processing

A great deal of effort has been directed at elucidating the mechanics of how precursors are processed in *E. coli* and related enterobacteria. This subject is of interest because of the many examples of proteins that undergo this particular modification during localization and for the observation that for at least certain proteins, processing appears to be required for correct localization (Koshland *et al.*, 1982; Fikes and Bassford, 1987). Much of what is known about processing involves the enzymology of this event. Inouye and Beckwith (1977) provided the initial description of a proteolytic activity that converted alkaline

phosphatase precursor to what appeared to be the mature form. This activity cofractionated with the *E. coli* outer membrane and was not detected in cytoplasmic membrane or periplasmic fractions. Chang and coworkers (1979) subsequently characterized an endoproteolytic activity from *E. coli* cytoplasmic membrane vesicles that correctly cleaved phage f1 procoat protein to the mature form. This processing activity operated in a cotranslational manner and was stimulated by nonionic detergent. Mandel and Wickner (1979) reported an endoproteolytic processing activity, termed leader peptidase, that fractionated with both cytoplasmic and outer membrane and matured the phage M13 coat protein precursor to coat protein in either a cotranslational or posttranslational manner. It has recently been established that leader peptidase is an ectopic cytoplasmic membrane protein and is capable of processing the precursors of the periplasmic leucine-specific and isoleucine-valine-binding proteins and the outer-membrane proteins LamB and OmpA as well (Wolfe *et al.*, 1983). Date and Wickner (1981) have identified a plasmid that carries the gene for leader peptidase (lep). This has facilitated the construction of strains exhibiting 75-fold overproduction of leader peptidase and allowed the identification of a 37,000-dalton polypeptide as the processing enzyme (Wolfe *et al.*, 1982). DNA sequence analysis indicated that lep appears to lie in an operon as it is situated promoter-distal to an open-reading frame containing sufficient genetic information to encode a 72,000-dalton polypeptide of unknown function. Sequence analysis also indicated that lep encodes a 323-amino acid polypeptide (Mr = 36,000) that is not synthesized in precursor form (Wolfe *et al.*, 1983).

The *E. coli* outer membrane lipoprotein is extensively modified (Braun, 1975), and the lipoprotein precursor, whether modified or not, is not a substrate for leader peptidase (Tokunaga *et al.*, 1982). Yamagata and coworkers (1983) have isolated a temperature-sensitive mutant for prolipoprotein maturation and cloned the prolipoprotein signal peptidase (*lsp*A) gene. The *lsp*A gene has been sequenced and found to encode a 164-residue polypeptide that does not exhibit an obvious signal peptide or any homology to the

lep gene product (Innis *et al.*, 1984). Furthermore, the *lsp*A gene product appears to be an integral membrane protein.

The maturation of secretory protein precursors is a process that is executed with remarkable fidelity. Furthermore, the nature of the signals elaborated by exported proteins that ensure the accuracy of signal peptide cleavage appear to be highly conserved throughout the biological kingdom. Consider that *E. coli* correctly processes rat preproinsulin to insulin (Talmadge *et al.*, 1980), *E. coli* leader peptidase converts mouse IgG κ chain precursor to the mature form, coliphage M13 coat protein precursor is accurately processed to mature coat protein by a eukaryotic processing activity derived from dog pancreas microsomes (Watts *et al.*, 1983), and yeast correctly matures wheat α-amylase precursor (Rothstein *et al.*, 1984). The molecular basis for how the specific cleavage catalyzed by the various signal peptidases is ensured remains unresolved. However, this question has spawned an active area of research that has generated some insight as to what the nature of the "processing signals" exhibited by precursors may be. Two general approaches have been employed to study this particular facet of the processing problem. These include the analysis of mutants that are defective in the maturation of some specific precursor and searches for general primary sequence homologies in the immediate vicinities of the processing sites for a number of bacterial and eukaryotic secretory precursor proteins.

At present, there are several examples of alterations within the primary sequence of an exported protein that affect processing. Lin and coworkers (1978) described a substitution of aspartic acid for a glycine residue within the lipoprotein signal peptide that abolished processing of the lipoprotein precursor but had little effect on export of the mutant protein (Lin *et al.*, 1980a,b). Koshland and coworkers (1982) constructed three mutants with alterations in the near vicinity of the TEM-Bla signal peptide-processing site that involved a proline residue, four amino acids proximal to the position at which signal peptide cleavage occurs. The removal of proline suggests that the processing defect in

these Bla mutants may be exerted at the level of protein secondary structure. Emr and colleagues (1980a) have reported that a deletion of 12 amino acids from the outer membrane protein LamB signal peptide abolished processing of the truncated pre-LamB when the export defect also imposed by this deletion was suppressed in an extragenic fashion. Taken together, these data indicated that at least certain processing signals reside within the signal peptide in the immediate vicinity of the processing site.

The best evidence that suggests processing signals also extend beyond the limit of the signal peptide derives from studies of M13 (F1) major coat protein maturation. Russel and Model (1981) have characterized a mutant coat protein that has leucine substituted for glutamic acid at the second residue of the mature sequence. The corresponding precursor exhibited a dramatic kinetic defect for processing *in vivo* and was a poor substrate for leader peptidase *in vitro*. The membrane insertion of the mutant precursor did not appear to be affected. Nevertheless, as in the examples discussed above, this alteration resides in the immediate proximity of the signal peptide processing site.

Perlman and Halvorson (1983) and Von Heijne (1983) have independently subjected the known primary sequences for a number of prokaryotic and eukaryotic signal peptides to statistical analyses, searching for general homologies that may provide clues as to the nature of the putative signal peptidase recognition site. Perlman and Halvorson (1983) found Ala-X-Ala to constitute the most frequent sequence to immediately precede the signal peptide cleavage site and suggested a consensus signal peptidase recognition sequence of A-X-B, where A includes the large aliphatic isoleucine, leucine, and valine residues in addition to the small neutral residues (alanine, glycine, and serine) that make up the B position amino acids. The predictive rules for determining a most probable processing site as set down by Von Heijne (1983) are in general accord with the Perlman and Halvorson consensus sequence. It is interesting that none of the point or deletion mutations discussed above that elicited processing defects acted, at least not directly, at the level of the putative signal peptidase recognition site. This raises questions as to whether

the homology observed in the consensus sequence has any significance. Fikes and Bassford (1987), however, have shown that if the Ala of the -3 position of the MBP signal sequence is changed to Asp, then the MBP signal sequence is not processed. Although the unprocessed MBP protein is able to participate in the uptake of maltose, it remains bound to the cytoplasmic membrane. This result shows that the observed consensus sequence homology is significant and reaffirms that processing of the signal sequence is obligate for proper localization.

2.5. *In Vitro* Translocation Systems

Perhaps the most significant contribution that biochemical techniques have made to the study of the localization process in *E. coli* has been the development of an *in vitro* export system. Smith (1980) developed an *in vitro* assay which utilized a cytoplasmic extract and inverted cytoplasmic membrane vesicles to investigate the translocation of exported proteins across the cytoplasmic membrane in *E. coli.* In an elegant experiment he showed that when inverted cytoplasmic membrane vesicles were treated with protease, they lost their ability to transport exported proteins. This result suggested that a cytoplasmic membrane protein was necessary for translocation and was likely to be part of the cellular export machinery. Since this initial investigation, several workers have employed this system to investigate cytoplasmic components of the export machinery.

Müller and Blobel (1984a,b) isolated a 12S cytoplasmic factor which was necessary for the translocation of proteins into cytoplasmic membrane vesicles. Watanabe and Blobel (1989a,b) have subsequently shown that this 12S component is actually 7S (150 kilodaltons) and that part of the complex consists of a SecB tetramer (64 kilodaltons), which is known to be a component of the *E. coli* secretory apparatus (see below). The remaining 90 K complex has yet to be characterized, although it is interesting that its molecular weight is very close to that of SecA, another known component of *E. coli*'s export machinery (see below). Weng *et al.* (1988) have identified two additional

cytoplasmic factors that are 8S (120 kilodaltons) and 4S (60 kilodaltons) in size, and have shown that neither of these is identical to the 7S complex isolated by Müller and Blobel (see above), nor is either of them SecA. It will be interesting to see if any of these complexes represent known components of the *E. coli* export machinery (see Section 4) or represent components that have yet to be identified.

3. GENETIC ANALYSIS OF PROTEIN EXPORT: THE ACTIVE INVOLVEMENT OF THE EXPORTED PROTEIN IN LOCALIZATION

3.1. Gene Fusions and the Study of Protein Export

The efficient localization of exported proteins from the cytoplasm to their ultimate extracytoplasmic destination is obligatorily a selective process. Such proteins must exhibit some distinguishing features that direct their incorporation into the secretory pathway at the exclusion of proteins that are fated to remain in the cytoplasm. The elucidation of such export signals is the subject of intense research.

The application of the sophisticated genetic techniques afforded by the *E. coli* system to the problem of protein localization has provided major inroads toward an understanding of the mechanisms by which this bacterium exports proteins to extracytoplasmic compartments. Of central importance to the genetic dissection of *E. coli* protein export has been the development of gene fusion technology. Beckwith and colleagues took advantage of the power of lac genetics and the singular properties of the cytoplasmic enzyme β-galactosidase (LacZ) to develop general techniques for creating fusion of *lacZ* to any *E. coli* target gene (Casadaban, 1976; Weinstock *et al.*, 1983; Beckwith and Silhavy, 1984). Particularly germane to the study of protein export are gene fusions that program the synthesis of hybrid proteins with amino termini derived from a target gene product that is normally exported from the cell and an enzymatically active, carboxyl terminal LacZ moiety of essentially constant size. Such "protein fusions" have proven useful in several ways. First, the enzymatic and antigenic properties of the LacZ

domain provide specific markers for a particular amino terminal domain of an exported protein. By fusing a series of amino terminal domains derived from some particular exported protein to LacZ and using the LacZ markers to localize each "fragment" of the exported protein to some subcellular compartment, one can infer the distribution of signals within that exported protein that function in determining its ultimate extracytoplasmic destination. Second, the unusual properties exhibited by *E. coli* strains synthesizing certain classes of hybrid proteins have made possible the direct selection of mutants that are either specifically or generally defective in protein export. Such mutants represent the foundation upon which the genetic analysis of protein export in *E. coli* has been built.

Gene fusion technology has been applied most extensively to the study of MBP export to the periplasm and LamB export to the outer membrane, and the results of this analysis are reviewed below. However, this technique has also been successfully used to study the localization of the cytoplasmic membrane protein MalF (Shuman *et al.,* 1980; Silhavy *et al.*, 1977), the export of alkaline phosphatase to the periplasm (Michaelis *et al.*, 1983a,b), and the export of OmpF to the outer membrane (Hall and Silhavy, 1981).

The periplasmic MBP and the outer membrane protein LamB are encoded by the *mal*E and *lam*B genes, respectively. These genes belong to a cluster of at least five genes whose products are involved in the uptake of maltose and its polymers into the cell. The locus to which these genes map is termed the *mal*B region of the *E. coli* genome, and it is organized into two positively controlled operons whose transcription diverges from a common promoter region and is maltose-inducible (for a review see Hengge and Boos, 1983). Hence, high-level synthesis of MBP-LacZ or LamB-LacZ hybrid proteins is dependent on the presence of maltose. This property of conditional expression has proven critical to the successful application of gene-fusion technology to the problem of protein export in *E. coli.*

Perhaps the most important information obtained from studies of protein fusions is that β-galactosidase can be redirected from the cytoplasm to either of two distinct

extracytoplasmic compartments: the cytoplasmic and the outer membranes. In the case of MBP-LacZ protein fusions, the presence of an intact MBP signal peptide was sufficient to direct the hybrid to the cytoplasmic membrane. The efficiency of this membrane targeting increased with the amount of MBP present at the amino terminus. These data have been interpreted to suggest that the signal peptide initiates the cotranslational transfer of the nascent hybrid through the cytoplasmic membrane. In no case, however, was any MBP-LacZ hybrid protein ever found in the periplasm (Bassford *et al.*, 1979). This finding was of special significance, since it had originally been considered that secretion of soluble cytoplasmic proteins could be engineered simply by the attachment of a functional signal peptide. These data, along with the findings of Moreno and colleagues (1980) with LamB-LacZ protein fusions, showed that the situation is more complex. One aspect of this complexity appears to involve constraints regarding those amino acid sequences that can be translocated through membranes via the normal protein export pathway. For MBP-LacZ and LamB-LacZ hybrid proteins, it has been suggested that amino acid sequences exist within the LacZ portion of the protein chimeras that are incompatible with transfer across membranes (Bassford *et al.*, 1979; Emr *et al.*, 1980a). These sequences abort continued execution of the translocation process and leave the hybrid protein embedded in the cytoplasmic membrane. Such abortive attempts at hybrid protein export hold dire consequences for the cell (see below).

The localization of LamB-LacZ hybrid proteins exhibits some similarities to MBP-LacZ hybrid localization, but there is one important difference: LamB-LacZ fusions harboring large portions of LamB primary sequence have been observed to be efficiently exported to the outer membrane (Hall *et al.*, 1982), the normal compartment of localization for LamB. These results indicate mechanistic differences exist between protein export to the periplasm and to the outer membrane. At what level these differences are elaborated remains a matter of conjecture. However, the distribution of the signals within LamB that specify outer membrane localization has been investigated (see Section 3.7).

The induction of high-level synthesis of MBP-LacZ or LamB-LacZ hybrid proteins that are abortively localized to the cytoplasmic membrane has been observed to be lethal for the *E. coli* host cell. This lethality, termed the maltose-sensitive (Mal^S) phenotype, was shown to be manifested phenotypically by cell filamentation and eventual cell lysis and biochemically by a general accumulation of precursors for a number of *E. coli* cytoplasmic membrane, periplasmic, and outer membrane proteins (Bassford *et al.*, 1979; Ito and Beckwith, 1981; Emr and Silhavy, 1982; Herrero *et al.*, 1982). The general maturation block exerted by induced synthesis of hybrid protein has been interpreted to suggest that insertion of the fusion polypeptide into the cytoplasmic membrane results in the occupation of sites through which exported proteins exit the cytoplasm. This general block in secretion results in the inhibition of cell division and eventually cell death.

3.2. Characterization of LamB and MBP Signal Peptide Defects

The lethality associated with induction of MBP-LacZ and LamB-LacZ hybrid protein synthesis has facilitated the development of selection schemes by which mutants exhibiting export defects for a specific protein could be directly obtained. It was reasoned that maltose-resistant Mal^RLac^+ variants of Mal^SLac^+ parental strain would include mutants that are defective in hybrid protein "export". A key feature of this selection was the Lac^+ requirement, to ensure continued synthesis of hybrid protein in the Mal^R mutants. This Lac^+ criterion solved a formidable problem in designing selections for obtaining export-defective mutants: How does one easily distinguish those very rare mutants that fail to export a given polypeptide from those mutants that occur at least 100 times more frequently and are simply synthesis-defective?

The Mal^RLac^+ selection and several modifications thereof have been used to generate mutants specifically defective in LamB or MBP export (for reviews see Emr and Silhavy, 1982; Bankaitis *et al.*, 1985). It is striking that all such mutants were found to be altered in LamB and MBP signal peptide primary sequence, and not in mature protein

sequences. The corresponding LamB and MBP signal peptide alterations have been determined precisely by DNA sequence analysis. Alterations that are confined to the signal peptide sequences, and are hence most immediately relevant to protein export studies, are shown in Figure 1. These signal peptide alterations, coupled with their direct correlation to specific export defects, constituted the first genetic proof of a critical role for the signal peptide in protein export. Note that both the wild-type LamB and MBP signal peptides are structurally typical of most prokaryotic and eukaryotic signal peptides (Fig. 1). Both exhibit well-defined hydrophilic segments at their amino termini, followed by uninterrupted stretches of 18 uncharged residues that constitute the hydrophobic segments. These hydrophobic segments are predicted to assume α-helical configurations (Bedouelle and Hofnung, 1981; Emr and Silhavy, 1983). For both the LamB and MBP signal peptides, these hydrophobic regions also terminate with consensus Ala-X-Ala sequences immediately proximal to their respective processing sites. Note, however, that these signal peptides lack any meaningful primary sequence homology among them.

Since the general patterns that have emerged from mutational analyses of LamB and MBP signal peptides are very similar, we shall consider these studies together. Perhaps the two major points that have been derived from these genetic analyses are that (1) the hydrophobic segment is essential for signal peptide function, since all mutations that lead to signal peptide dysfunction alter this region; and (2) specific amino acid residues within the hydrophobic segment play particularly crucial roles in signal peptide function. All LamB and MBP signal peptide alterations that render the corresponding proteins export defective can be classified into one of two categories: those that impose major defects (class I), or those that impose minor defects (class II). The former are considered to cause a drastic perturbation in some functional component of the signal peptide, while the latter are expected to leave such a component relatively intact. Thus, comparison of class I alterations to class II alterations provides a useful means of inferring what features of signal peptide are important for proper function.

Class I LamB or MBP signal peptide alterations include both simple point substitutions and small deletions. For LamB, these include four point alterations and two deletions (Fig. 1). The results of such alterations are that greater than 95% of the corresponding mutant LamB polypeptides synthesized are blocked for localization and accumulate as cytoplasmic precursor forms (Emr *et al.*, 1980b; Emr and Silhavy, 1982). Class I MBP signal peptide alterations include five point mutations and one deletion (Bedouelle *et al.*, 1980; Bankaitis *et al.*, 1984). The corresponding mutant MBPs exhibit localization defects that range from those that exhibit greater than a 95% accumulation of cytoplasmic precursor (e.g., *mal*EΔ12-18, *mal*E18-1, and *mal*E19-1) to those that exhibit only approximately a 50% block in export (e.g., *mal*E16-1). Kinetic analyses indicate that the mutant MBP that is exported in the corresponding strains is translocated in a posttranslational manner *in vivo* (J. P. Ryan and P. J. Bassford, Jr., 1985). Class I export defects are also manifested phenotypically. *E. coli* strains that suffer class I LamB export defects cannot utilize dextrin, a maltose polymer, as a sole carbon source and are resistant to infection by coliphage w (Emr and Silhavy, 1982). Class I MBP export-defective mutants exhibit discernible Mal$^-$ phenotypes on the appropriate indicator media, and in the most extreme cases, utilize maltose as a sole carbon source only very poorly (Bassford, 1982; Emr and Bassford, 1982).

Note that all four class I LamB point alterations and four of the five class I MBP point alterations introduce charged residues at specific positions within the hydrophobic segments of the respective signal peptides. These positions correspond to amino acid residues 14, 15, 16, and 19 of the LamB signal peptide and residues 14, 16, 18, and 19 MBP signal peptide. The distribution of the class I alterations is certainly nonrandom, because of the sheer redundancy at which precisely these same alterations are encountered in mutant searches, especially in light of the fact that single-base substitutions can occur at most other LamB or MBP hydrophobic segment codons so as to introduce charged residues into this region (Emr and Silhavy, 1982; Bedouelle *et al.*, 1980). The importance

of the critical residues defined by the class I LamB and MBP export-defective point alterations is further underscored by the following: (1) two class I deletions, *lam*BS60 and *mal*EΔ12-18, remove most or all of the critical residues identified in the respective signal peptides (Fig. 1); and (2) the two alterations that do not introduce charges or otherwise directly affect the critical residues (i.e., *lam*BS78 and *mal*E10-1) appear to exert indirect effects on the critical residues of their respective signal peptides. The *lam*BS78 deletion may alter LamB signal peptide secondary structure throughout the critical region (Emr and Silhavy, 1983). Bedouelle and Hofnung (1981) have suggested that *mal*E10-1 may alter MBP signal peptide topology so as to fold back the hydrophilic segment into the vicinity of the MBP signal peptide critical residues, thereby essentially introducing a charge into that region.

Further support for the existence of critical "subsets" of hydrophobic segment residues that define functional components for the LamB and MBP signal peptides is derived from analysis of class II LamB and MBP signal peptide defects. The class II LamB and MBP signal peptide alterations are characterized as those that block LamB-LacZ or MBP-LacZ hybrid protein export, but are phenotypically silent when recombined into the wild-type *lam*B or *mal*E gene (Emr and Silhavy, 1982; Bankaitis *et al.*, 1985). Furthermore, class II mutants exhibit reduced levels of precursor accumulation. The class II LamB alterations consist of four distinct point substitutions that introduce charged amino acids at positions 12, 13, and 17 of the LamB signal peptide (Emr and Silhavy, 1982; Stader *et al.*, 1986). Note that these alterations occur at positions that lie immediately adjacent to those at which incorporation of charged amino acids leads to class I LamB export defects (Fig. 1). Of special interest in this regard are substitutions of Asp or Arg for Gly at position 17. Such mutants accumulate barely detectable amounts of LamB precursor (Emr and Silhavy, 1982). Yet incorporation of charged residues at positions 16 and 19 blocks LamB export almost completely.

The class II MBP export-defective mutants define five point alterations, all of which involve the signal peptide hydrophobic segment (Bankaitis *et al.*, 1985). Three of these involve substitutions of a hydrophilic residue for a nonpolar amino acid, and the remaining two introduce charged residues at positions 10 and 11 (Fig. 1). Note that either of the latter substitutions alters residues comprising the MBP signal peptide critical subset. The one substitution that does involve such a critical residue, an incorporation of Pro for Ala at position 14, represents the most deleterious of the class II alterations in terms of export defect caused. Nevertheless, this alteration is considerably less deleterious to signal peptide function than is the incorporation of a charge residue (Glu) at the same position.

On the basis of these collective data, two general schools of thought have emerged concerning the function of the critical residues identified by the class I LamB and MBP signal peptide alterations. One ascribes a structural role for these residues, while the other proposes a signaling role for engagement with a cellular machinery whose function is to facilitate the export of proteins from the cytoplasm. The structural model places special emphasis on the fact that these various signal peptide dysfunctions result from alterations that decrease hydrophobicity, or perhaps alter the secondary structure of, the hydrophobic segment. The recognition model places greatest weight on the position effect.

The hydrophobic axis length (HAL) hypothesis of Bedouelle and Hofnung (1981) is the best example of a structural model. These workers have attempted to relate the physical length of the hydrophobic segment to signal peptide function. They conclude that in order to be functional, a signal peptide must exhibit an uninterrupted stretch of uncharged residues existing in a periodic structure (α-helix or β-strand) that has a minimum length of 18 Å. The position effect observed is considered to reflect those residues at which the incorporation of charges reduces the "hydrophobic axis length" below the 18 Å minimum. Although this hypothesis explains the class I LamB and MBP signal peptide alterations and the class II MBP alterations fairly well, the results of introducing charges at position 17 of the LamB signal peptide are not consistent with the model. These alterations

would be predicted to exert class I LamB export defects. Yet the corresponding export defects are barely detectable.

The alternative proposal concerning the putative role of the critical residues defined by the class I LamB and MBP export-defective mutations suggests that these residues constitute a recognition site that mediates an interaction of the signal peptide with a cellular protein export apparatus (Emr and Silhavy, 1982; Silhavy *et al.*, 1983). The strong position effect observed for alterations in either of these signal peptides is consistent with such a proposal. In addition, the allele-specific suppression of these various export defects by mutant alleles of genes that most likely encode components of a cellular secretion machinery also provides compelling genetic support for such a model (see Section 4.2). The class II alteration, as these for the most part are not considered to affect the recognition site, would be predicted to have only minor effects.

3.3. Intragenic Suppressors of LamB and MBP Signal Peptide Defects

The isolation of mutants that are defective for LamB or MBP export has, in turn, made possible the isolation of mutants that are phenotypically suppressed for the original export defect. Of particular significance to genetic analyses of signal peptide function are intragenic suppressors, as these would be expected to restore certain features to the defective signal peptide that render it more efficient in facilitating protein export. The utilization of two complementary genetic strategies, the characterization of export-defective mutants followed by characterization of intragenic suppressor mutants, constitutes yet another powerful approach for identifying important functional components of a signal peptide.

Starting with a mutant deleted for 12 base pairs (bp) of LamB signal peptide coding sequence (*lam*BS78, Fig. 1), Emr and Silhavy (1983) have obtained mutants that exhibit second-site alterations within the LamB signal peptide that restore efficient export of this protein to the outer membrane. DNA sequence analysis of two such alterations (Fig. 2),

coupled with the application of predictive rules for polypeptide secondary structure of the *lam*BS78 and suppressor signal peptides, suggests that the suppressors act by restoring an α-helical conformation through the region occupied by the critical residues at positions 14, 15, and 16. This region is not predicted to assume an ordered structure in the *lam*BS78 polypeptide. Physical measurements of secondary structure assumed by "parental" and "suppressor" oligopeptides support these conclusions (Briggs and Gierasch, 1984). Emr and Silhavy (1983) have interpreted these data to indicate that the putative recognition site that is located in the LamB signal peptide hydrophobic segment must assume an α-helical conformation in order to be recognized by the cellular export apparatus.

Bankaitis and coworkers (1984) have characterized 12 mutants that are intragenically suppressed for the *mal*EΔ12-18 export defect. Six unique suppressor alterations were recognized on the basis of strength of suppression and DNA sequence analysis (Fig. 2). The three most efficient suppressors exhibited an almost complete alleviation of the *mal*EΔ12-18 export defect (Bankaitis *et al.*, 1984). Note that the five most efficient suppressors of *mal*EΔ12-18 represent second-site alterations that map within the MBP signal peptide. Each of these appears to function by lengthening the truncated hydrophobic segment by one of three mechanisms: (1) duplication of hydrophobic sequences (suppressors R1 and R4); (2) extension of the amino terminal boundary of the hydrophobic segment by substitution of an uncharged residue for Arg at position 8 (suppressors R2 and R3); and (3) the predicted introduction of a pentapeptide in β-strand conformation into the α-helical hydrophobic segment (suppressor R5). The net effect of the R5 suppressor may be to physically extend the hydrophobic segment without increasing the number of residues that make it up. The analysis of intragenic suppressors R1-R5 strongly suggests that hydrophobicity is a major determinant of MBP signal sequence function. In a subsequent study in which intragenic suppressors of several class I export-defective MBP proteins were analyzed, Ryan *et al.* (1986) obtained similar results and

showed that it is the overall hydrophobicity of the MBP signal sequence, and not the hydrophobic length per se, which is important for its function.

Both Bankaitis *et al.* (1984) and Ryan *et al.* (1986) have identified mutations at amino acid 19 of the mature MBP protein that can suppress export defective MBP proteins, albeit weakly. How this suppression occurs is as yet unknown, but these results suggest that this portion of the mature MBP protein may participate in the export process. Interestingly, this region of the LamB protein has been shown to be necessary for the efficient export of LamB from the cytoplasm (see Section 3.7).

3.4. Characterization of Signal Peptide Defects for Other *E. coli* Exported Proteins

A variety of *in vivo* and *in vitro* techniques have been employed to generate mutants defective in signal peptide function for several other exported proteins. These include the periplasmic alkaline phosphatase (Michaelis *et al.*, 1983b), the periplasmic TEM β-lactamase (Koshland *et al.*, 1982) in *S. typhimurium*, the outer membrane OmpF protein (Sodergren *et al.*, 1985), and the outer membrane lipoprotein (Inouye *et al.*, 1984; Vlasuk *et al.*, 1984). The data from these proteins is in general accord with the pattern observed for both LamB and MBP- that reducing hydrophobicity or altering secondary structure of the hydrophobic segment leads to signal peptide dysfunction. Whether these various signal peptides exhibit well-defined critical regions is an unresolved question that awaits characterization of larger sets of mutants. One important caveat of the β-lactamase studies is that a systematic effort was made to isolate mutants defective in β-lactamase export. Literally hundreds of mutations mapping throughout the gene were characterized, yet none of the corresponding mutant polypeptides were localization-defective (Koshland *et al.*, 1982). These results, in concert with the LamB and MBP mutant data presented above, underscore the restricted nature of the distribution of export information exhibited by bacterial secretory proteins.

The application of site-directed mutagenesis to the problem of generating mutants defective in lipoprotein export has yet to yield a concrete pattern. Although small deletions and relatively subtle point substitutions have been incorporated in the lipoprotein signal peptide, the only conclusion reached so far is that this structure can absorb a number of alterations and remain largely functional (Inouye *et al.*, 1984; Vlasuk *et al.*, 1984).

3.5. The Role of the Hydrophilic Segment in Signal Peptide Function

On the basis of the observation that all of the signal peptide alterations leading to the export defects discussed in Section 3.3 alter the hydrophobic segment of the signal peptide, one might conclude that the hydrophilic segment does not play an important role in initiating protein export. However, other data suggest that it may. Hall and colleagues (1982, 1983) have converted Arg at position 6 of the LamB signal peptide to Ser (Fig. 1). Although this substitution does not elicit a measurable LamB export defect, a translational block is imposed on "export-competent" LamB-LacZ hybrid proteins, and the translational efficiency of lamB mRNA is depressed some four- to fivefold. Inouye and associates (1982) have generated numerous alterations in the lipoprotein signal peptide hydrophilic segment by site-directed mutagenesis. This segment normally exhibits a net charge of +2. Reduction of this charge to +1 or 0 had no effect on lipoprotein export but did result in a progressive attenuation of lipoprotein synthesis that was directly proportional to the reduction in net basicity of the hydrophilic segment. When the net charge of the hydrophilic region was reduced to -1, very little lipoprotein was produced, and that which was synthesized was observed to be predominantly in the unmodified precursor form. The LamB and lipoprotein data are consistent and Hall and coworkers (1983) have proposed that the basic residues of the signal peptide hydrophilic segment are involved in an obligate coupling of translation to export.

3.6. Other Intragenic Information that is Required for the Initiation of LamB and MBP Export

The overwhelming genetic evidence supporting a critical role for the signal peptide in different export of proteins from the cytoplasm has raised the question of what role, if any, mature protein sequences play in the export process. Ito and Beckwith (1981) have addressed this problem genetically. They followed the localization of two MBP amber fragments, one that represented 90% and one that represented 30% of the primary *mal*E translation product. Both fragments were observed to undergo at least some measure of processing, and the larger fragment was recovered from the periplasm as a soluble species. The smaller amber fragment did not fractionate as a soluble periplasmic component, but trypsin accessibility experiments suggested that this polypeptide had been translocated across the cytoplasmic membrane, albeit inefficiently. On the basis of these data, Ito and Beckwith (1981) concluded that the carboxyl terminal two-thirds of the MBP primary sequence does not play an essential role in the export of maturation of this polypeptide. Similar data have been reported from analogous studies with other exported proteins. It does not appear that the carboxyl termini of β-lactamase (Koshland *et al.*, 1982), the periplasmic arginine-binding protein (Celis, 1981), and the outer membrane protein OmpA (Bremer *et al.*, 1980) are required for the export and processing of their corresponding proteins.

An observation made by Bankaitis and Bassford (1984) however, strongly suggested that export information was present in the mature portions of the MBP and LamB proteins. They found that the synthesis of export defective MBP or LamB proteins interfered with the export of other normally secreted proteins and caused precursors to accumulate. The simplest interpretation of these results is that some component(s) of the export machinery (see below) was sequestered by the export defective MBP and LamB proteins. Several observations have made it clear that the interference exerted by the synthesis of export defective MBP and LamB proteins is due to the limitation of a single

component of the export machinery, SecB. First, only a subset of exported proteins are affected by interference and this subset is identical to the subset of proteins that require SecB for export (Collier *et al.*, 1988). Second, overproduction of SecB suppresses interference (Collier *et al.*, 1988). Third, interference does not increase the export defect observed in SecB mutant cells (Collier *et al.*, 1988). Finally, interference is caused by the limitation of a single export component (see Chapter 2, this thesis).

The regions of the mature MBP and LamB proteins that interact with SecB have been mapped. In MBP this region lies between amino acids 151-186 of the mature protein (Collier *et al.*, 1988), while in LamB this region lies between amino acids 320-380 of the mature protein (see Chapter 2, this thesis). A detailed comparison of these two regions has not revealed any obvious homology, however, which is not necessarily surprising since the signal sequences of these two proteins are also dissimilar.

The importance of these mature regions in the export process has been intensely investigated over the past few years. Workers in several labs have shown that a functional interaction between SecB and a mature region of the exported protein is necessary in order to maintain the protein in an export-competent, unfolded form (Collier *et al.*, 1988; Kumamoto and Gannon, 1989; Liu *et al.*, 1989). This antifolding activity of SecB is promoted by an interaction between SecB and the exported protein, which is dependent on the presence of both the mature region and the signal sequence of the exported protein (see Chapter 3, this thesis). The implications that are suggested by the fact that SecB interacts with both the signal sequence and a mature region of the exported protein are discussed in Section 4.1.

3.7. Intragenic Signals that Target LamB to the Outer Membrane

Data from a number of studies suggest that the pathways for localization of periplasmic proteins and outer membrane proteins share one or more steps (Ito and Beckwith, 1981; Emr and Bassford, 1982; Bankaitis and Bassford, 1984, 1985). Yet the localization

pathways of these classes of proteins must diverge at some point. In a series of studies, Silhavy and coworkers have determined that the LamB protein exhibits discrete signals within the mature protein sequence that direct (1) LamB export from the cytoplasm, and (2) LamB localization to the outer membrane.

The distribution of LamB export information was first inferred from localization studies with four classes of LamB-LacZ hybrid proteins (Hall *et al.*, 1982). Hybrid proteins carrying only a portion of the LamB signal peptide, or even the entire LamB signal peptide plus the first 15 residues of the mature LamB, were observed to fractionate as cytoplasmic species (Moreno *et al.*, 1980). Furthermore, the cell was able to tolerate high-level synthesis of these small fusion proteins, as evidenced by the Mal^R phenotype exhibited by the corresponding *lam*B-*lac*Z fusion strains. Both the phenotypic and the biochemical data are consistent with the notion that such fusion polypeptides are not export-competent. LamB-LacZ polypeptides that carry more substantial amounts of LamB sequence exhibit very different properties. A fusion protein carrying the LamB signal peptide plus some 173 mature LamB residues was found to exhibit an efficiency of localization to the outer membrane of about 40%. The remainder appeared to be equally distributed between the cytoplasmic and cytoplasmic membrane fractions. A LamB-LacZ hybrid protein carrying the signal peptide and approximately 241 mature LamB residues was localized to the outer membrane at an efficiency approaching 90% (Hall *et al.*, 1982). High-level synthesis of these large fusion proteins is debilitating to the host cell and leads to a Mal^S phenotype.

From these studies, it was concluded that the LamB signal peptide and some region between residues 15 and 173 of the 421-residue mature LamB protein are required for export initiation and targeting of LamB to the outer membrane. In addition, some information was suggested to lie between residues 173 and 240 of the mature LamB that somehow affects the efficiency of LamB targeting to the outer membrane. Using a more extensive series of LamB-LacZ fusion proteins, Benson and Silhavy (1983) showed that

two regions of the mature LamB protein were indeed necessary for the efficient export of LamB to the outer membrane and that these regions mapped to between amino acids 1-70 and 220-235 of the mature protein.

To characterize more precisely the targeting information contained between amino acids 1-70 of the mature LamB protein, Benson *et al.* (1984) developed an *in vivo* selection with which they were able to generate a series of additional LamB-LacZ fusion proteins. An analysis and characterization of these novel LamB-LacZ fusion proteins revealed the following information:

1. A hybrid protein that includes an intact signal peptide and 27 residues of the mature LamB protein remains cytoplasmic, and the corresponding fusion strain is Mal^R.
2. A hybrid protein that includes 39 residues of the mature LamB protein is inserted into the cytoplasmic membrane but is translocated to the other membrane at an efficiency of about only 1%. In this case, the corresponding fusion strain is Mal^S.
3. When the hybrid protein includes the first 49 residues of the mature LamB protein, the protein is translocated to the outer membrane with an efficiency of some 20%. Again, the corresponding strain is Mal^S.

These studies strongly suggest that the initiation of LamB protein export requires an intact signal peptide and information contained within the first 39 residues of the mature protein. In addition, specific targeting to the outer membrane requires information residing between residues 39 and 49 of the mature protein. The latter corresponds to a region of sequence homology noted to exist among various major outer membrane proteins (Nikaido and Wu, 1984) and has been termed by Benson and coworkers (1984) the outer membrane signal. Although the results of Benson *et al.* (1984) implied that the first 27 amino acids of mature LamB are not necessary to target LamB to the outer membrane, a more recent study

by Rasmussen and Silhavy (1987) has shown that information contained in this region is necessary if LamB is to be efficiently exported from the cytoplasm.

4. GENETIC ANALYSIS OF PROTEIN EXPORT: COMPONENTS OF THE *E. coli* PROTEIN EXPORT MACHINERY

Although several of the models that have been proposed to describe protein export in *E. coli* suggest that the cell does not require a specialized apparatus to facilitate protein export, it now seems clear that a number of proteins are synthesized by the cell for that specific purpose. Over the last years, data accumulated from several laboratories have permitted the identification of new genetic loci believed to encode elements of a cellular export machinery. Two general strategies have been employed to genetically dissect the *E. coli* protein export apparatus: searching for sec mutants that exhibit pleiotropic defects in protein export, and searching for prl mutants that restore proper localization to export-defective proteins. The success of these two approaches is due largely, either directly or indirectly, to the power of gene fusion technology. Section 4 reviews what is known about those elements of the *E. coli* protein export machinery whose identification has emerged from these genetic analyses.

4.1. Mutants that Are Pleiotropically Defective in Protein Export

It has been observed that *E. coli* strains synthesizing MBP-LacZ hybrid proteins and efficiently incorporating them into the cytoplasmic membrane are phenotypically Lac^- because of abnormally low uninduced levels of β-galactosidase activity (Bassford *et al.*, 1979). These low uninduced levels of β-galactosidase activity are thought to reflect the inability of hybrid protein monomers to form enzymatically active tetramers when inserted into the cytoplasmic membrane at a low density. Oliver and Beckwith (1981) exploited this phenotype to select for mutants that are defective in membrane localization of the hybrid protein. By selecting for Lac^+ mutants and screening those for conditional growth

properties, it was reasoned that mutants exhibiting general protein export defects could be obtained. Many Lac$^+$ mutants were isolated at 30°C, and two genetic loci (*sec*A and *sec*B) were identified that encode gene products that appear to be involved in protein export.

SecA. Two of the Lac$^+$ mutants obtained proved to be temperature-sensitive (*ts*) for growth on all media. Each mutant harbored a single lesion that affected MBP-LacZ hybrid protein localization in trans and was responsible for both the Lac$^+$ and the *ts* phenotypes. These two lesions identified a previously uncharacterized gene that maps at 2.5 min on the *E. coli* chromosome and has been designated *sec*A (Oliver and Beckwith, 1981). Mutants carrying a *sec*A^{ts} allele accumulate cytoplasmic precursors for a number of exported proteins when shifted to the nonpermissive temperature (42°C). Examples of proteins whose export is impaired in a *sec*A^{ts} mutant incubated at 42°C include the periplasmic MBP, ribose-binding protein and alkaline phosphatase, and the outer membrane proteins LamB and OmpF. As predicted in the selection, *sec*A^{ts} mutants exhibit partial export defects at the permissive temperature (30°C) as well. However, functional secA gene product is not universally required for export of *E. coli* proteins. The proper localization of certain proteins does not appear to be affected in *sec*A^{ts} mutants at any of the temperatures tested (Oliver and Beckwith, 1981). Nevertheless, *sec*A has been shown to be a gene that is essential for *E. coli* viability (Oliver and Beckwith, 1982b).

By the use of sophisticated genetic techniques, a *sec*A(Am) strain was constructed in which the suppression of the *sec*A nonsense allele was conditional (Oliver and Beckwith, 1982b). When SecA synthesis was shut off in this strain, it was observed that not only did precursors of exported proteins start to accumulate, but that their synthesis levels were drastically reduced. The additional observation that signal sequence mutations suppressed the synthesis block caused by the lack of SecA led to a proposal that SecA was involved in the coupling of translation and export in *E. coli* in a manner analogous to that of SRP in higher cells (Kumamoto *et al.*, 1984). Subsequent studies however, have revealed

that this was not the case and that the reduced synthesis levels of exported proteins were the result of an indirect affect (Strauch *et al.*, 1986).

The *sec*A gene has been cloned (Oliver and Beckwith, 1982a) and its DNA sequence determined (Schmidt *et al.*, 1988). SecA is a large (102 kilodalton) soluble protein that is associated peripherally with the cytoplasmic membrane (Oliver and Beckwith, 1982b). Interestingly, it was observed that SecA synthesis exhibits at least a tenfold increase in *sec*A^{ts} strains at the nonpermissive temperature and in *sec*A^{+} strains induced for the Mal^{S} response. Since both of these conditions are considered to stress the export capacity of the cell, Oliver and Beckwith (1982b) suggested that SecA regulation may be sensitive to cellular secretion needs. While details of this regulatory response have not yet been elucidated, Schmidt and Oliver (1989) have shown that SecA possesses an autoregulatory mechanism that acts at the level of translation. The development of *in vitro* translocation assays have recently allowed workers to demonstrate that SecA protein is required for exported proteins to be translocated (Cabelli *et al.*, 1988), and that SecA protein possesses an ATPase activity (Cunningham *et al.*, 1989; Lill *et al.*, 1989). Collectively, the studies on SecA have shown that this protein is intimately involved in the export process and may be involved in an energy requiring step that drives the exported protein across the cytoplasmic membrane.

SecB. The second locus that was genetically defined in the Lac^{+} selection has been termed *sec*B, and it maps at 80.5 min on the *E. coli* linkage map (Kumamoto and Beckwith, 1983). *sec*B is an unusual gene for two reasons. First *sec*B is the only sec gene that has been isolated which is not truly essential. While null mutations in *sec*B fail to grow on rich media, they survive on minimal media where growth is less rapid (Kumamoto and Beckwith, 1985). Additionally, SecB is required only for the efficient export of a subset of the exported proteins that are dependent on other sec genes (Kumamoto and Beckwith, 1983, 1985).

The *sec*B gene has been cloned (Kumamoto and Beckwith, 1985) and its DNA sequence determined (Kumamoto and Nault, 1989). SecB is a small (17 kilodalton) cytoplasmic protein. Several workers have shown that SecB is an antifolding factor that maintains SecB-dependent proteins in an export competent unfolded form (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Liu *et al.*, 1989). The use of *in vitro* translocation systems has demonstrated that SecB is necessary for SecB-dependent proteins to be translocated across the cytoplasmic membrane (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989; Watanabe and Blobel, 1989b). SecB appears to facilitate the export of SecB-dependent proteins by an interaction that requires the presence of both the signal sequence and a mature region of the exported protein (Chapter 3, this thesis; Collier *et al.*, 1988; Gannon *et al.*, 1989; Liu *et al.*, 1989; Watanabe and Blobel, 1989c). Since SecB is a cytoplasmic factor that interacts with the signal sequence of exported proteins, Watanabe and Blobel (1989c) have proposed that SecB is the signal recognition particle of *E. coli*. Although this is a very tantalizing idea, it is inconsistent with the fact that *sec*B is a nonessential gene and is necessary only for the efficient export of a subset of the secreted proteins in *E. coli*. Nonetheless, genetic evidence exists that supports a functional relationship between SecB and two other components of the *E. coli* export apparatus, SecA and PrlA. A synergistic response is seen at the permissive temperature when a *sec*B null mutation is combined with the *sec*A^{ts} allele (Kumamoto and Beckwith, 1983), and the suppressor function of the prlA4 allele is disabled in a *sec*B null background (Trun *et al.*, 1988). Since other factors have been shown to act in a manner similar to SecB (Lecker *et al.*, 1989), it appears more likely that SecB acts as a chaperone that recognizes and presents a certain subset of the exported proteins to the rest of the secretory apparatus (see Chapter 3 and 4, this thesis).

SecD. The *sec*D gene which maps to 9.5 minutes on the *E. coli* chromosome was isolated using an analogous selection scheme to the one that yielded the *sec*A and *sec*B genes except that *pho*A-*lac*Z and *lam*B-*lac*Z fusions were utilized (Gardel *et al.*, 1987). While *sec*A mutations that forced the MBP-LacZ hybrid protein to remain in the cytoplasm

were temperature sensitive, *sec*D mutations that forced PhoA-LacZ and LamB-LacZ hybrid proteins to remain in the cytoplasm were cold-sensitive. This preliminary study has shown that SecD is required for the export of the same proteins that are dependent on SecA.

SecE. Since it was known that SecA protein levels increased when the export machinery was limited (see above), Riggs *et al.* (1988) employed a selection scheme to look for mutations that caused an increase in SecA protein through the use of a SecA-LacZ hybrid protein. A new sec gene *sec*E, which maps to 90 min on the *E. coli* chromosome, was identified by this selection. Interestingly, the *sec*E mutations that were isolated were cold sensitive and affected the export of the same proteins that were dependent on SecA. The *sec*E gene has been cloned and its DNA sequence determined (Schatz *et al.*, 1989). This analysis predicts a 127 amino acid protein with three membrane spanning domains that shares homology with PrlA (see Section 4.2).

Other approaches that have been employed in an attempt to isolate mutants pleiotropically defective in protein export have included selections for mutants with reduced amounts of properly localized envelope proteins. A number of loci, such as *per*A (Wanner *et al.*, 1979), *tpo* (Wandersman *et al.*, 1980), *exp*A (Dassa and Boquet, 1981), and *cpx*A and *cpx*B (McEwen *et al.*, 1983), have been identified using such approaches. It is not yet known, however, whether the localization effects observed in these mutants are the result of export defects, or synthesis defects, or both.

4.2. Mutants that Restore Proper Localization to Export-Defective Proteins

The availability of *E. coli* strains that synthesized export-defective LamB and MBP proteins allowed for the selection of suppressor mutants that relieved the particular export-effective phenotype. Of special interest with regard to the genetic analysis of the *E. coli* export machinery were extragenic suppressor mutations, since they might define loci that coded for components of the cellular secretory apparatus. To date, five such genes have been

isolated by a genetic analysis of mutants that are extragenically suppressed for LamB or MBP export defects.

PrlA. The overwhelming majority of extragenic suppressor mutations that have been obtained map to *prl*A. This locus was originally described by Emr and coworkers (1981) in their analysis of mutants suppressed for the *lam*BS60 export defect. Subsequent studies have shown that suppressor *prl*A alleles suppress other LamB signal peptide defects as well as MBP and alkaline phosphatase signal peptide defects (Emr *et al.*, 1981; Emr and Bassford, 1982; Bankaitis and Bassford, 1985; Michaelis *et al.*, 1983b). Suppression of the various export defects is manifested by the proper localization, and except in the case of the LamBS60 protein, proper processing of a greater fraction of the export-defective polypeptide than that measured in the corresponding prl^+ strain. *prl*A mediated suppression of LamB and MBP export defects is allele specific and this has been used to argue for a direct interaction between the prlA gene product and the mutant signal peptides (Emr *et al.*, 1981; Emr and Bassford, 1982).

Fine structure genetic mapping has assigned *prl*A to the extreme promoter-distal end of the *spc* operon at 72 min on the *E. coli* chromosomal map (Emr *et al.*, 1981; Shultz *et al.*, 1982). It is noteworthy that all the remaining 10 genes, that along with *prl*A, make up the *spc* operon, encode ribosomal proteins. Although *prl*A does not code for a ribosomal protein, its inclusion in the spc operon is intriguing from the standpoint of the proposed coupling between protein synthesis and export in *E. coli.*

Sequencing of the *spc* operon revealed that *prl*A coded for a 443 amino acid protein that was largely hydrophobic (Cerretti *et al.*, 1983). Ito and associates (1983) employed localized mutagenesis of the *prl*A region of the genome and obtained two temperature sensitive conditional mutants that affected *prl*A, which were named *sec*Y. The first mutant (*ts* 215) was found to carry an amber mutation in *rpl*O, the gene that lies immediately promoter-proximal to *prl*A (*sec*Y), and exerts a strong polar effect on *prl*A(*sec*Y) expression at the nonpermissive temperature (Ito *et al.*, 1983, 1984). The second mutant

(*ts* 24) exhibits a Gly to Asp substitution in the middle of the open-reading frame that defines *prl*A (*sec*Y) (Shiba *et al.*, 1984b). At the nonpermissive temperature, both mutants accumulate precursors for several exported proteins, and cell death eventually ensues.

Consistent with the predictions from DNA sequence analysis, Akiyama and Ito (1985, 1987) have shown that the PrlA protein is an integral membrane protein that spans the cytoplasmic membrane 10 times. Interestingly, two of the strong *prl*A suppressors alter amino acids within the seventh and tenth transmembrane segments of the PrlA protein (Sako and Iino, 1988). Because of its cellular location and the fact that prlA suppressors appear to interact directly with the signal sequence of exported proteins, it is likely that PrlA is part of the translocator apparatus of the *E. coli* export machinery. Using *in vitro* export assays, it has been shown that the PrlA protein is necessary for the translocation of proteins across the cytoplasmic membrane (Fandl and Tai, 1987; Cabelli *et al.*, 1988) and appears to act together with SecA in facilitating this process (Lill *et al.*, 1989).

PrlB. The *prlB* suppressor was isolated in the same screen that yielded *prl*A (Emr et al., 1981). Subsequent analysis, however, has revealed that the *prl*B allele is unusual in that it suppresses only LamB signal peptide defects, and export of the mutant LamB proteins is achieved without detectable processing (Emr and Bassford, 1982). Furthermore, the suppressor *prl*B allele represents a deletion within the structural gene for the periplasmic ribose-binding protein (J. Garwin and S. Emr, personal communication). It is therefore clear that *prl*B does not alter a component of the protein export machinery and, as a result, illustrates a difficulty with the suppressor approach. This case emphasizes that the correlation of an altered gene product with some anomaly in export of wild-type envelope proteins is essential to a convincing argument for that gene product's playing a role in facilitating normal protein export. How prlB suppresses LamB export defects is unknown.

PrlC. The original *prl*C suppressors were isolated in the same screen that yielded *prl*A (Emr *et al.*, 1981). Although *prl*C acts in a manner similar to *prl*A in that *prl*C

suppresses both LamB and MBP export defects (Emr and Bassford, 1982), the isolation of additional *prl*C suppressors has shown that *prl*C suppressors are much more discriminating than *prl*A (Trun and Silhavy, 1989). *prl*C affects only *lam*B signal sequence mutations that alter the amino terminal region of the hydrophobic core, and most of the LamB protein that is suppressed is not translocated.

PrlD. The *prl*D suppressors were isolated as mutants that suppressed the Δ12-18 MBP export defect (Bankaitis and Bassford, 1985). Subsequent characterization of *prl*D has revealed that it functions in a manner very similar to *prl*A and suppresses export defects in both MBP and LamB proteins. As seen with *prl*A, *prl*D also exhibits an allele-specific suppression of LamB and MBP export defects, which supports a direct interaction of *prl*D with the signal sequence. Interestingly though, *prl*D suppressors are less potent than *prl*A in suppressing signal sequence mutations that affect the hydrophobic core, yet export detects caused by charge alterations in the amino terminal portion of the signal sequence are suppressed by *prl*D but not by *prl*A (Puziss *et al.*, 1989).

It was first suggested that although *prl*D mapped very close to *sec*A, *prl*D was nonetheless a separate gene (Bankaitis and Bassford, 1985). The isolation and characterization of additional *prl*D alleles, however, have shown that *prl*D is, in fact, *sec*A (Fikes and Bassford, 1989). This result is very intriguing since it argues that SecA like PrlA interacts directly with the signal sequence of the exported protein. Given the fact that SecA is a soluble protein and that PrlA is an integral membrane protein, it is appealing that SecA preferentially interacts with the hydrophilic portion of the signal sequence, while PrlA preferentially interacts with the hydrophobic portion of the signal sequence. Additional evidence suggests that PrlA and SecA are functionally interconnected, since Bankaitis and Bassford (1985) have demonstrated that *prl*D strains harboring certain suppressor *prl*A alleles exhibit marked growth defects. Pulse-labeling experiments show that such double mutants accumulate significant amounts of precursors of the periplasmic MBP and ribose-binding proteins and of the outer membrane proteins LamB and OmpA. Furthermore,

these general growth and export defects are observed only with combinations of *prl*D and specific suppressor *prl*A alleles. Collectively, the studies on the *prl*A and *prl*D suppressors indicate that SecA and PrlA may interact sequentially with the signal sequence during the export process.

PrlE. *prl*E was an "in-house" name originally given to the *sec*D mutations that were isolated using LamB-LacZ fusions (S. Benson, personal communication). Since the term *prl* had already been defined to describe suppressor mutations, of which *prl*E clearly was not, the name was later changed to *sec*D (Gardel *et al.*, 1987).

PrlF. As discussed above, the Mal^S phenotype faithfully reflects the efficiency at which MBP-LacZ or LamB-LacZ fusion proteins initiate translocation across the cytoplasmic membrane. If the interpretation is correct, that the Mal^S phenotype is the result of an irreversible occupation by hybrid protein molecules of cytoplasmic membrane sites that serve as "gates" through which proteins must pass enroute to the cell envelope (see above), then suppressor mutations that relieve the Mal^S response in trans without reducing levels of fusion protein synthesis may alter components of such "export sites." Using a selection for Mal^R mutants that exhibit reduced levels of β-galactosidase activity, Kiino and Silhavy (1984) have identified one such suppressor mutation, which maps to 69 min on the *E. coli* chromosome, and designated the corresponding locus *prl*F. The *prl*F1 mutation suppresses both MBP-LacZ and LamB-LacZ fusion protein-mediated lethality. The *prl*F1 strains induced for hybrid protein synthesis do not accumulate precursors of wild-type envelope proteins, nor do these strains exhibit significant reduction in the rate of hybrid protein synthesis. In these mutants, hybrid protein localization is not altered, since efficient incorporation of these polypeptides into the cell envelope still occurs. In fact, the membrane localization of the hybrids is so efficient that very little fusion protein is detected in the cytoplasm. This results in a 50- to 100-fold reduction in cellular β-galactosidase activity without significant reduction in hybrid protein synthesis. The *prl*F1 strains are slightly cold sensitive for growth but exhibit no measurable defect in wild-type envelope

protein export kinetics. Subsequent analysis has revealed that *prl*F encodes a bifunctional protein whose autoregulatory domain is derepressed by the *prl*F suppressor mutation (Kiino *et al.*, 1990). Thus, prlF suppression is caused by overproduction of the PrlF protein. Interestingly, *prl*F, like *prl*D, suppresses the export defects caused by charge alterations in the hydrophilic amino acid portion of the signal sequence (Puziss *et al.*, 1989; Iino and Sako, 1988). Exactly what *prl*F's function is during the export process, however, remains to be elucidated.

PrlG. Since the isolation of suppressor mutations using the export-defective LamBS60 protein turned up only two types of suppressors, *prl*A and *prl*C, Stader *et al.* (1989) isolated additional suppressor mutations using the export-defective LamB14D protein. A new suppressor, *prl*G, which is allelic to *sec*E, was uncovered. Although *prl*G suppresses both export -defective LamB and MBP proteins, it is a much weaker suppressor than *prl*A. More interesting, however,is that the identification of *prl*G brings the total of export components to three that have been uncovered both by screens for pleiotropic export defects and by suppressor studies. By virtue of PrlG's being amongst export components such as PrlD/SecA and PrlA/SecY, the implication is that PrlG is an integral component of the export machinery awaiting further characterization.

4.3. Suppressors of Temperature Sensitive SecA and SecY Mutations

Since it was well established that the isolation of cold sensitive suppressors of temperature sensitive genes often leads to the identification of interacting components (Jarvik and Botstein, 1975), several workers have isolated cold sensitive suppressors of the *sec*A(*ts*) and *sec*Y(*ts*) alleles. Suppressors of *sec*A(*ts*) named *sec*C, which is allelic to *rps*O (Ferro-Novick *et al.*, 1984), and *ssa*D, *ssa*E, *ssa*F, *ssa*G, and *ssa*H, which stand for suppressor of *sec*A, have been isolated (Oliver, 1985). Suppressors of *sec*Y(*ts*) were also isolated and named *ssy*A, *ssy*B, *ssy*C, *ssy*D, *ssy*E, *ssy*F, and *ssy*G, which stands for suppressor of *sec*Y (Shiba *et al.*, 1984a, 1986). Subsequent studies have shown, however, that most if

not all of these mutations act by slowing down the rate of protein synthesis (Lee and Beckwith, 1986). In fact, low levels of chloramphenicol can effectively suppress the temperature sensitive mutations of *sec*A and *sec*Y. Thus, none of the cold sensitive suppressors appear to represent additional components of the *E. coli* export machinery.

5. OVERVIEW OF *E. coli* PROTEIN EXPORT

From the accumulated biochemical and genetic data reviewed here, it is clear that no single model will be able to explain completely how exported proteins are localized in *E. coli*. The difficulties of incorporating these various data into a unified model are equally evident. Nonetheless, great strides have been made toward understanding how the export process occurs in *E. coli*. It is apparent that most exported proteins contain multiple export signals (both the signal sequence and mature regions of the exported proteins contribute to the export process) that interact with components of the export machinery in delivering the protein to its proper extracytoplasmic compartment. Interestingly, cumulative studies on how the export components interact with secreted proteins allow the localization process to be divided into five conceptually, but perhaps not temporally, distinct steps. These five steps and the components characterized to date that are known to interact at each step are as follows: 1) recognition (SecB), 2) membrane delivery (SecA), 3) translocation (PrlA), 4) processing (Lep), and 5) sorting (?). As indicated by this analysis, however, although the basic process is beginning to be unravelled, larger questions such as how cytoplasmic-membrane, periplasmic, and outer-membrane proteins are delineated from each other and properly delivered to their extracytoplasmic destination, are far from being answered.

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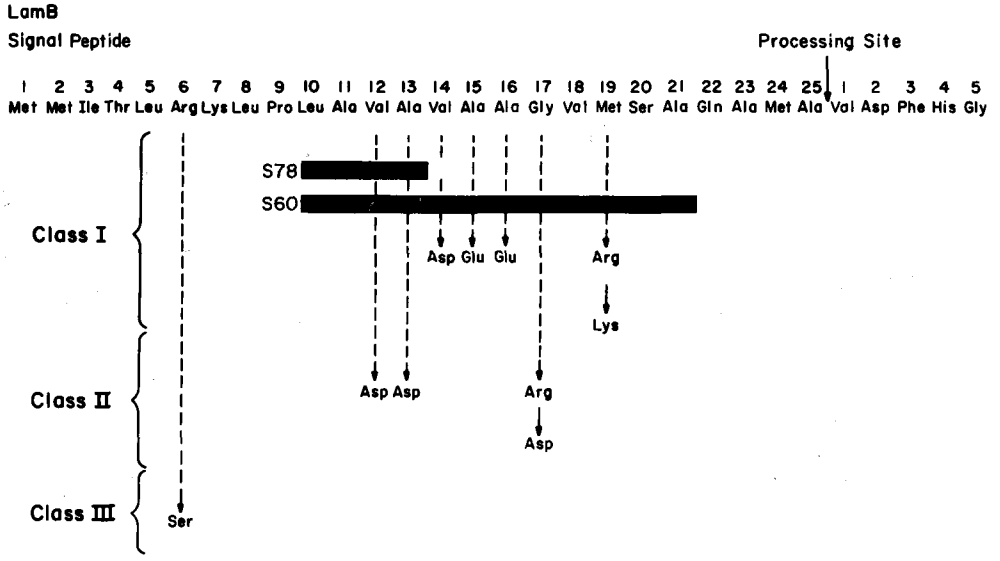
Figure 1. Primary sequence of wild-type and mutant LamB and MBP signal peptides. The 32 amino terminal residues of the LamB and MBP precursors are given, including the signal peptides and processing sites. Single amino acid substitutions that have been characterized for each are indicated by arrows. Deletions are indicated by solid bars. In the case of the deletions and the MBP point alterations, the corresponding allelic designations are also given. Further details concerning these alterations and their classification are provided in the text.


LamB
Signal Peptide
Processing Site
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 1 2 3 4 5
Met Met Ile Thr Leu Arg Lys Leu Pro Leu Ala Val Ala Val Ala Ala Gly Val Met Ser Ala Gln Ala Met Ala Val Asp Phe His Gly
S78
S60
Class I
Asp Glu Glu
Arg
Lys
Class II
Asp Asp
Arg
Asp
Class III
Ser

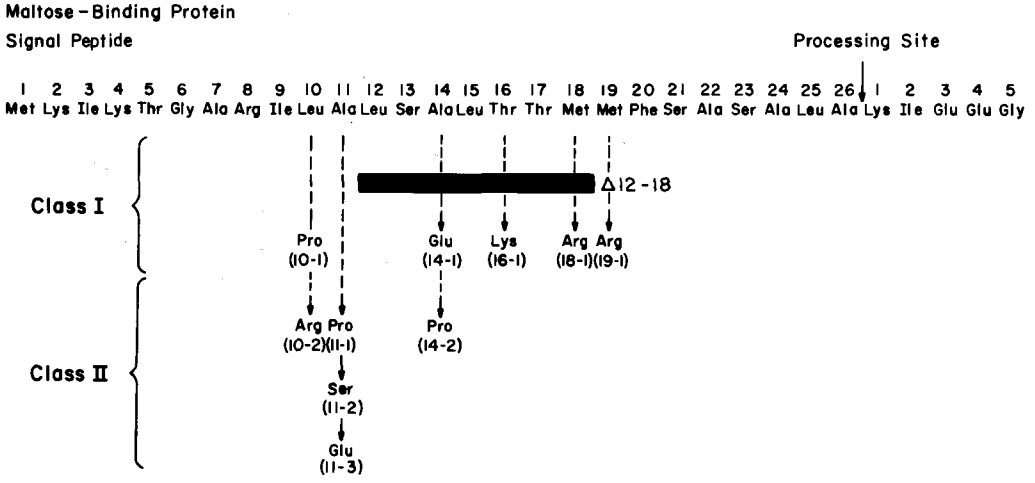
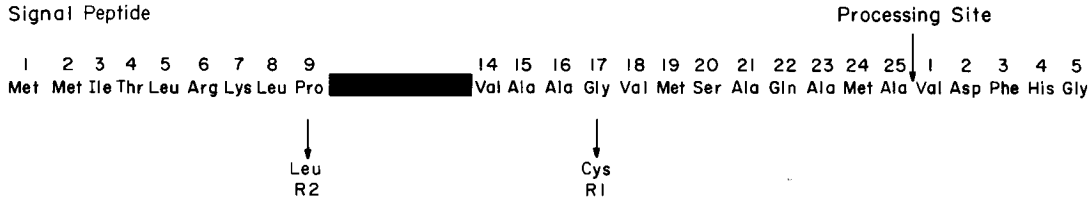

Maltose-Binding Protein
Signal Peptide
Processing Site
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 1 2 3 4 5
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly
Δ12-18
Class I
Pro (10-1)
Glu (14-1)
Lys (16-1)
Arg (18-1)
Arg (19-1)
Class II
Arg Pro (10-2)(11-1)
Pro (14-2)
Ser (11-2)
Glu (11-3)

Figure 2. Intragenic suppressors of the export defects elicited by the *lam*BS78 and *mal*EΔ12-18 signal peptide alterations. The truncated LamB and MBP signal peptides are shown, along with the alterations incorporated into the primary sequence by intragenic suppressor mutations. Point substitutions are indicated by a downward-pointing arrow. Duplications are identified by an upward-pointing arrow. The corresponding allelic designations are given. See the text for additional details.

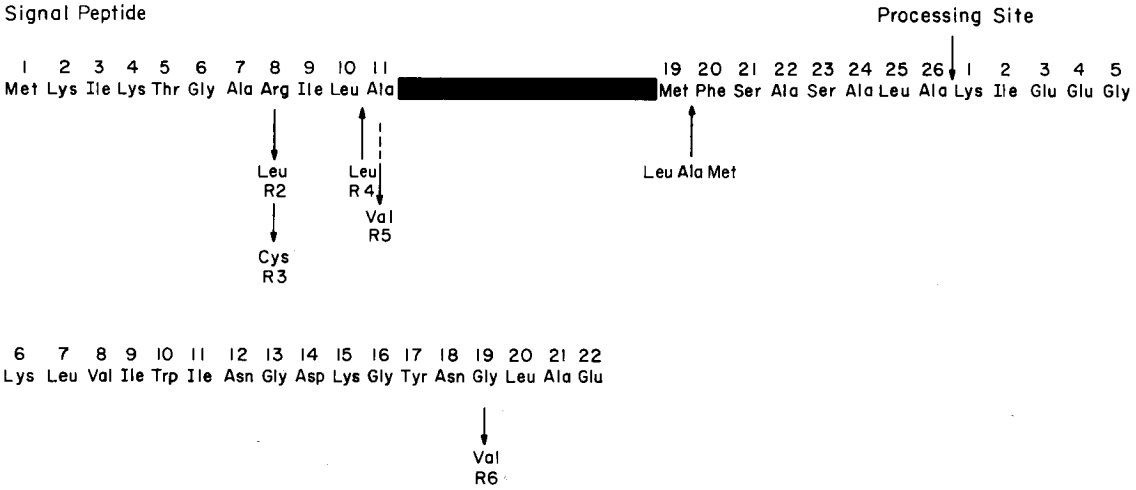
LamBS78

Signal Peptide



Maltose-Binding Protein Δ12-18

Signal Peptide



Chapter 2

Characterization of a Region in Mature LamB Protein that Interacts with a Component of *Escherichia coli*'s Export Machinery

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Summary

It has been shown that the synthesis of an export-defective protein can interfere with the normal export process in *Escherichia coli* by limiting the availability of SecB protein, a component of the export apparatus (Collier *et al.*, 1988). Consistent with this observation, we find that the interference elicited by an export-defective LamB protein is a titratable response resulting from the limitation of a single ligand. We have mapped the interfering region in LamB to between amino acids 320 and 380 of the mature protein. Expression of this sequence in the form of a LacZ-LamB-LacZ fusion protein elicits the export interference phenotype. Deletion of the sequence from an export-defective LamB protein eliminates the ability of this protein to interfere with the export of other secreted proteins. Together, these findings show that this sequence is both necessary and sufficient to cause export interference. Surprisingly, deletion of this sequence from an otherwise wild-type LamB protein does not cause the mutant LamB product to exhibit any obvious export defect. Based on our results, we propose that SecB interacts with both amino acids 320-380 of mature LamB and the LamB signal sequence during initiation of the export process.

All cells are faced with the problem of faithfully sorting proteins that must be targeted to specific subcellular compartments from those destined to remain in the cytoplasm. For many noncytoplasmic proteins this process is facilitated through an interaction of an amino-terminal signal (or leader) sequence with cellular export machinery (for general reviews, see Briggs and Gierasch, 1986; Silhavy *et al.*, 1983). Three of the components of *Escherichia coli*'s export machinery, SecA (Oliver and Beckwith, 1981; Cabelli *et al.*, 1988), SecB (Kumamoto and Beckwith, 1983; Weiss *et al.*, 1988), and PrlA/SecY (Emr *et al.*, 1981; Ito *et al.*, 1983; Fandl and Tai, 1987), have been well characterized, both genetically through the isolation of mutants and biochemically through the use of *in vitro* assays. While *sec*A and *prl*A/*sec*Y are essential genes, *sec*B is not (Bankaitis *et al.*, 1986). SecB is also unusual in that it appears to be necessary for the efficient export of only a subset of the proteins which require SecA and PrlA/SecY (Kumamoto and Beckwith, 1983; Collier *et al.*, 1988).

Although export in *E. coli* is absolutely dependent on the interaction of a functional signal sequence with the export machinery (Bankaitis *et al.*, 1986), signal sequences of exported proteins share no common consensus sequence (Michaelis and Beckwith, 1982). This raises the possibility that other signals present in the mature portion of exported proteins may also be necessary for export recognition. Consistent with this idea, it has been shown that the export of several proteins is not initiated until most of the mature protein has been translated (Randall and Hardy, 1984). In addition, it has been observed that the synthesis of an exported protein harboring a defective signal sequence interferes with normal export and causes precursor accumulation of other secreted proteins (Bankaitis and Bassford, 1984). The simplest interpretation of this phenomenon is that some region of the mature protein sequesters a component of the export machinery, thus limiting its ability to function in the secretory process. On the basis of three findings, Collier *et al.* (1988) have shown that SecB is the export component which is limited by interference: (1) Only a subset of the exported proteins were affected by interference and this subset was

identical to the subset of proteins that required SecB for export; (2) overproduction of SecB suppressed interference; and (3) interference did not increase the export defect observed in *sec*B mutant cells.

SecB is a cytoplasmic tetrameric protein complex, composed of identical 17 kilodalton SecB subunits, which has been shown to stimulate the translocation of exported proteins across inverted vesicles in reconstituted translocation systems (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989; Watanabe and Blobel, 1989a). SecB facilitates the export of the periplasmic maltose binding protein, MBP, by maintaining the protein in an export-competent, unfolded form (Collier *et al.*, 1988; Kumamoto and Gannon, 1988). This antifolding activity appears to be due to an interaction between SecB and the mature portion of the MBP protein (Collier *et al.*, 1988; Gannon *et al.*, 1989; Liu *et al.*, 1989).

It is easy to envision how a SecB binding site in the mature region of an exported protein would facilitate SecB's antifolding function. In this study we have examined and mapped the interfering region in LamB, an *E. coli* outer membrane protein that is dependent on SecB for its efficient export.

Materials and Methods

Bacterial strains—All strains used in this study are isogenic derivatives of MC4100: F$^-$Δ*lac*U169 *ara*D139 *rps*L150 *thi rel*A1 *flb*B5301 *deo*L1 *pts*F25 (Casadaban, 1976). SE2078 is MC4100 *lam*BS78 (Emr and Silhavy, 1980), SE3001 is MC4100 *mal*K'Δ1' *lam*B (Emr and Silhavy, 1980), CK1953 is MC4100 *sec*B::Tn5 (Kumamoto and Beckwith, 1985), and SE9100 is MC4100 *rps*L$^+$ *rps*D F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ (this study). The F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ episome was obtained from Niels Fiil and moved into strains by mating with SE9100, while the *sec*B::Tn5 lesion was moved into SE3001 by P1 transduction.

Parental lamB plasmids—pSE1 was constructed by replacing the 750 bp EcoRI PstI *bla* fragment of pHC6 (Hedgepeth *et al.*, 1980) with the 950 bp EcoRI PstI *bla* fragment from pBH20 (Itakura *et al.*, 1977), thus placing LamB under *lac* transcriptional

control. pSE60 was constructed by recombining the *lam*BS60 mutation from λapSE60 into pSE1 (Emr and Silhavy, 1980). To simplify additional plasmid constructions, we moved the *lam*B gene into pUC8. pUC*lam*Bwt and pUC*lam*BS60 were constructed by moving the EcoRI StuI *lam*B fragment from pSE1 or pSE60, respectively into pUC8 that had been restricted with EcoRI and SmaI.

Fusion plasmids—To construct p*lac*Z-5-*lam*B and p*lac*Z-90-*lam*B, pUC*lam*BS60 was restricted with FspI, then treated with Bal31 (slow Bal31 was used to construct p*lac*Z-5-*lam*B). After filling in with DNA polymerase and then restricting with BamHI, the *lam*B fragment was moved into pUC8, which had been restricted with BamHI and SmaI. p*lac*Z-220-*lam*B was constructed exactly as p*lam*Z-5-*lam*B, except that pUC*lam*BS60 was restricted with NdeI instead of FspI.

To construct p*lam*BS60-420-*lac*Z, p*lam*BS60-380-*lac*Z, and p*lam*BS60-320-*lac*Z, pUC*lam*BS60 was restricted with BamHI, then treated with Bal31 (slow Bal31 was used to construct p*lam*BS60-420-*lac*Z). After filling in with DNA polymerase and then restricting with EcoRI, the *lam*B fragment was moved into pORF5 (Shultz *et al.*, 1982), which had been restricted with EcoRI and SmaI. p*lam*BS60-180-*lac*Z construction was identical to p*lam*BS60-420-*lac*Z except that pUC*lam*BS60 was restricted with SmaI instead of BamHI. To construct p*lac*Z-[320-*lam*B-420]-*lac*Z, p*lam*BS60-420-*lac*Z was restricted with NcoI, then treated with slow Bal31. After restricting with EcoRI and filling in with DNA polymerase, the vector was reannealed. p*lac*Z was constructed by reannealing pORF5 after it had been restricted with EcoRI and SmaI.

All of the fusions were sized to within 10 base pairs, using convenient restriction sites located throughout the *lam*B gene (Clément and Hofnung, 1981). *E. coli* strains harboring each of the plasmid constructs were then assayed on SDS-PAGE for the presence of the expected fusion protein.

Additional lamB plasmids—To remove the coding sequence for the interfering region from mature LamB, an EcoRI BamHI fragment from pUC*lam*S60 containing the

*lam*BS60 gene was moved into M13mp18 that had been restricted with EcoRI and BamHI. Oligonucleotide-directed mutagenesis was employed using a 24-mer consisting of the sequence TGGACGCACAATCAACGCTGATAAC (Neuman *et al.*, 1985). This allowed us to precisely delete the coding sequence for amino acids 320 through 382 of the mature LamB protein. pUC*lam*BS60ΔIR was constructed by moving the mutated EcoRI and BamHI fragment from M13mp18 back into pUC8 that had been restricted with EcoRI and BamHI. By exchanging an EcoRI SmaI fragment that included the coding sequence for LamB's signal peptide between pUC*lam*Bwt and pUC*lam*BS60ΔIR, pUClamBΔIR was easily constructed.

We found it difficult to control the synthesis levels of LamB protein in pUC*lam*Bwt or pUC*lam*BS60. To overcome this problem in experiments where it was necessary to quantitatively regulate the synthesis levels of the LamB protein, we moved the *lam*B clones back into a pBH20 background, where regulation of the LamB protein could be easily controlled. p*lam*Bwt and p*lam*BΔIR were constructed by restricting either pUC*lam*Bwt or pUC*lam*BΔIR with EcoRI and BamHI and moving the resulting EcoRI BamHI *lam*B fragment into pBH20 that had been restricted with EcoRI and BamHI. p*lam*BS78 was constructed by recombining p*lam*Bwt with λapSE78 (Emr and Silhavy, 1980).

Pulse-chase experiments—Vigorously aerated cultures were grown to logarithmic phase (.3OD_{550} to .4OD_{500}) in M63 minimal medium (Miller, 1972) at 30°C, and where appropriate, ampicillin and kanamycin were added to a final concentration of 50 μg/ml and 40 μg/ml, respectively. Cultures containing lamB plasmid constructs which required IPTG induction were induced at an OD_{550} of .1 so that they would be in logarithmic growth when labeled. Chromosomal levels of LamB protein were obtained by inducing cultures with 20 μM IPTG, while maximal LamB synthesis was obtained by inducing cultures with 1 mM IPTG. Samples were pulsed with the addition of 10 μCi of ^{35}methionine per ml of culture. Chase was initiated by the addition of unlabeled methionine to a final concentration of .5% wt/vol. To terminate the chase, 1 ml aliquots were removed and immediately

dispensed into 150 µl of ice-cold 50% TCA. Samples with no chase time were obtained by dispensing 150 µl of 50% TCA into 1 ml of cells after pulsing with ^{35}S methionine for the desired length of time.

Antigen extracts and subsequent immune precipitations were performed as described by Bankaitis and Bassford (1984) except that ProteinA-Sepharose was used instead of IgGsorb. 10 µl of the antigen extracts were counted in 10 mls of Safety-Solve so that samples could be quantitatively loaded in subsequent SDS-PAGE analysis. 5 µl of the final sample were also counted so that degradation could be measured if necessary.

Polyacrylamide gel electrophoresis and autoradiography—Immune precipitates were resolved by SDS-PAGE and autoradiography as previously described (Altman *et al.*, 1985), except that all resolving gels were 10%. Quantification of bands on autoradiograms was achieved using a LKB 2202 Ultrascan laser densitometer interfaced with an Apple II computer. Percent precursor protein was determined as a ratio of precursor protein/(precursor + mature protein), while percent exported protein was determined as a ratio of mature protein/(mature + precursor protein). In the $SecB^-$ experiment (Fig. 5B), some of the LamB protein was degraded before it could be exported. To correct for this, the values given for the percent of LamB protein exported were adjusted by subtracting the amount of LamB protein that had been degraded.

Reagents—ProteinA-Sepharose CL-4B and IPTG were purchased from Sigma. DNA restriction and modifying enzymes were obtained from New England BioLabs, Inc., except for Slow Bal31, which was from International Biotechnologies Inc. ^{35}S methionine came from Amersham, while Safety-Solve came from Research Products International Corp. Anti-LamB and MBP rabbit serum were the generous gifts of Tom Silhavy and Phil Bassford, respectively.

Results

Interference is titratable—It had been observed that the synthesis of export-defective LamB protein interfered with the export of wild-type MBP, resulting in the accumulation of precursor MBP. Cell fractionation experiments with the interfered MBP protein demonstrated that mature MBP was localized to the periplasm, while precursor MBP remained in the cytoplasm (Bankaitis and Bassford, 1984). Thus, precursor accumulation serves as an accurate measurement of the export rate.

Initially, we evaluated the effect that export-defective LamB protein had on the export of wild-type MBP. If the interference caused by an export-defective LamB protein was due to limiting an export component, it should be a titratable response, since limiting an export component would decrease the rate of export. To quantitatively measure this, we placed a *lam*B mutant gene, *lam*BS78, deleted for part of its signal sequence, under the control of the lac promoter. In this way we could vary the production level of the LamBS78 export-defective protein by varying the concentration of IPTG used to induce expression. Figure 1 shows that interference is proportional to the amount of export-defective LamBS78 protein that is synthesized.

Export-defective LamB protein binds a single ligand—Since the export rate of MBP decreased proportionally to the increasing amounts of export-defective LamB protein synthesized, we were able to determine whether interference involved the limitation of a single or multiple ligands. If export-defective LamB protein bound a single ligand, then a plot of MBP export rate versus increasing amounts of export-defective LamB protein would obey Henri-Michaelis-Menten kinetics. However, if interference was the result of binding multiple ligands, then the hyperbolic kinetics predicted by the Henri-Michaelis-Menten equation would not be observed (Segel, 1975).

The data from Fig. 1 was subjected to Henri-Michaelis-Menten analysis. As shown in Fig. 2A, the theoretical values obtained using V_{max} and K_m, calculated from a Hanes-Woolf plot of S/V versus S of the experimental data (Fig. 2B), are virtually

indistinguishable from the experimental values. Linear regression analysis of the Hanes-Woolf plot gave a variance of 0.99, indicating that the plot was linear. To confirm the results obtained from the Hanes-Woolf plot, the experimental data was also subjected to a Woolf-Augustinsson-Hofstee plot of V versus V/S, an Eadie-Scatchard plot of V/S versus V, as well as a Lineweaver-Burk plot of 1/V versus 1/S. All three of these plots were linear with a variance of 0.99. Additionally, a Hill analysis of the data indicated that both the η_H and η_{app} values are equal to 0.99 (data not shown). The results of these analyses are consistent with the export-defective LamBS78 protein binding a single ligand (Segel, 1975). It should be noted that these data do not rule out the possibility that the bound ligand is part of a complex involving other proteins.

The interfering region maps to between amino acids 320-380 of the mature LamB protein—To map the residues of the LamB protein that caused export interference, we constructed a series of *lam*B fusions that contained different intervals of the *lam*B gene fused to *lac*Z. By scoring whether the encoded hybrid proteins elicited an interference phenotype, we were able to map the interfering region in the LamB protein (Fig. 3). Initially, *lac*Z-*lam*B fusions were constructed by fusing different amounts of the 3' coding sequences of *lam*B to the promoter and coding sequence for the first seven amino acids of the *lac*Z gene. The interference caused by the p*lac*Z-5-*lam*B fusion, which encodes a LacZ-LamB hybrid protein that lacks only the signal peptide and approximately the first five amino acids of the mature LamB protein, is identical to the interference caused by pUC*lam*BS60, which encodes the LamBS60 protein deleted for amino acids 10-21 in the signal peptide (the wild-type LamB signal peptide is 25 amino acids long). Since the expression levels of these two interfering proteins were within 5% of each other and both caused equivalent amounts of interference, we reasoned that sequences mapping entirely in the mature portion of the LamB protein could cause export interference. The construction of additional *lac*Z-*lam*B fusions placed the interfering region beyond amino acid 220 of the mature LamB protein (Fig. 3A). *lac*Z-*lam*B fusions that removed more than 60% of the

*lam*B gene encoded unstable hybrid proteins and the interference effects of such fusions were not analyzed (data not shown).

To position the interfering region in LamB more precisely , we employed *lam*BS60-*lac*Z fusions that were constructed by fusing different amounts of the 5' coding sequence of *lam*BS60 to a large 3' fragment of the *lac*Z gene. All of the *lam*BS60-*lac*Z fusions encoded stable hybrid proteins. Hybrid proteins that contained 380 or more amino acids from mature LamB interfered with MBP export, while hybrid proteins that contained less than 320 amino acids of mature LamB did not interfere with MBP export (Fig. 3B). These observations indicated that the interfering region maps between amino acids 320 and 380 of the mature LamB protein. The lack of interference seen by deleting amino acids 320-380 could result from an altered folding pattern in the truncated protein, which causes the actual interfering region to become inaccessible. To rule out this possibility and to provide further support for our mapping data, we inserted a fragment of the *lam*B gene encoding the C terminal 100 amino acids of the LamB protein (amino acids 320-420) into the *lac*Z gene, such that the interfering region was expressed as part of the *lac*Z gene product. This *lac*Z-*lam*B-*lac*Z fusion coded for a stable hybrid protein that also strongly interfered with MBP export (Fig. 3C).

Deletion of the interfering region suppresses the interference caused by an export-defective LamB protein—If amino acids 320-380 corresponded to the interfering region in LamB, then deletion of this sequence alone should suppress the interference caused by an export-defective LamB protein. Using oligonucleotide-directed mutagenesis we constructed a deletion in the export-defective *lam*BS60 gene that removed the coding sequence for amino acids 320-382 of mature LamB. Figure 4 shows that while the LamBS60 protein interferes strongly with MBP export, removal of amino acids 320 through 382 completely suppresses the interference. This result cannot be attributed to the instability of the mutant LamB protein, since the stability of the LamBS60 protein lacking

its interfering region was found to be identical to that of the normal LamBS60 protein (data not shown).

Wild-type LamB export kinetics are unaltered when the interfering region is deleted—It is well documented that the export of LamB protein is SecB-dependent and that at early chase times in a pulse-chase labeling experiment, the export of LamB protein is totally blocked in a *sec*B null background (Kumamoto and Beckwith, 1985; Trun *et al.*, 1988). If the single ligand bound by the interfering region corresponded to the SecB binding site, we reasoned that removal of the interfering region should cripple LamB's export, since SecB would no longer interact with the mutant LamB protein. Using oligonucleotide-directed mutagenesis, we deleted the interfering region (coding sequence for amino acids 320-382 of mature LamB) from an otherwise wild-type *lam*B gene. Export of the resulting mutant LamB protein lacking its interfering region was indistinguishable from that of wild-type LamB (Fig. 5A). This unexpected result could be explained if deletion of amino acids 320-382 had caused LamB to become SecB-independent. However, this was not the case, as the export kinetics of the mutant LamB protein lacking amino acids 320-382 was as defective as wild-type LamB in a secB null background (Fig. 5B).

Discussion

An observation has been made in *E. coli* that the synthesis of export-defective proteins can interfere with the normal export process and cause precursors of other exported proteins to accumulate in the cell (Bankaitis and Bassford, 1984). The simplest explanation for this phenomenon is that the mature portion of the export-defective protein binds a component of the export machinery, thereby limiting its availability to the export process. If this were the case, we reasoned that interference should be a titratable response, since limiting an export component would decrease the rate of export, and precursor accumulation would increase as increasing amounts of interfering protein were

synthesized. By placing an export-defective LamB protein under *lac* control, we were able to vary the synthesis levels of the interfering protein, and found that the interference of MBP was indeed titratable and increased proportionally to the increasing synthesis levels of the export-defective LamB protein.

Because precursor accumulation serves as an accurate measurement of the export rate (Bankaitis and Bassford, 1984), we were able to employ a kinetic analysis to determine whether the interference caused by the export-defective LamB protein involved the limitation of single or multiple ligands. A plot of MBP export rate versus increasing amounts of export-defective LamB protein was found to obey Henri-Michaelis-Menten kinetics which is consistent with the export-defective LamB protein binding a single ligand (Segel, 1975). This result suggests that interference is caused by the limitation of a single component of the export machinery.

We have mapped the interfering region in the export-defective LamBS60 protein to between amino acids 320 and 380 of the mature protein on the basis of the following observations: (1) LamBS60-LacZ hybrid proteins that contain the first 380 amino acids of the mature LamB protein interfere, while LamBS60-LacZ hybrid proteins that contain only the first 320 amino acids of the mature LamB protein do not interfere; (2) insertion of the interfering region into LacZ converts LacZ into an interfering protein; (3) deletion of the interfering region from the export-defective LamBS60 protein eliminates the ability of this protein to interfere.

Collectively our results indicate that a single component of the *E. coli* export machinery interacts with a site mapping between 320-380 amino acids of the mature LamB protein. Since Collier *et al.* (1988) have shown that the export component which is limited by interference is SecB, it seemed likely that SecB interacted with the interfering region of the mature LamB protein. It is well documented that LamB's export is SecB-dependent and at early chase times in a pulse-chase labeling experiment, the export of the LamB protein is totally blocked in a *sec*B null background (Kumamoto and Beckwith, 1985; Trun

et al., 1988). We reasoned that removal of the interfering region from an otherwise wild-type LamB protein would eliminate LamB's ability to interact with SecB and LamB export would be crippled. Surprisingly, however, the export of a LamB protein lacking its interfering region was indistinguishable from that of wild-type LamB.

This unexpected result could be explained if removal of amino acids 320-382 from mature LamB bypassed the need for SecB by altering LamB's folding. This would be consistent with the results of Collier *et al.* (1988), who found that deletions in mature MBP protein altered its folding properties such that the requirement for SecB was bypassed. If this were true for LamB protein, then a wild-type LamB protein lacking amino acids 320-382 should be exported in a SecB-independent manner. This was not the case. Wild-type LamB and wild-type LamB lacking amino acids 320-382 exhibited the same slow export kinetics in a *sec*B null background.

One explanation for why removal of the interfering region from the LamB protein causes no export defect is that SecB may have a second site of interaction with some other region of the LamB protein. Since our mapping data rule out multiple sites of interaction with the mature protein, we propose that SecB also may interact with the signal sequence. The suggestion that SecB interacts with the signal sequence is not an unreasonable one, since interference can be measured only if the interfering protein lacks a functional signal sequence. Indeed, mapping of the LamB interfering region was accomplished using an export-defective LamB protein that contained a large deletion in the signal sequence. This mutation may have disrupted an interaction between the signal sequence and the SecB protein. In fact, recent studies by Watanabe and Blobel (1989b) have shown that SecB may interact directly with the signal sequence of MBP during the export process. In the next chapter we provide direct biochemical evidence that the interaction of SecB with the LamB protein is dependent on the presence of both the LamB signal sequence and the interfering region between amino acids 320-380 of mature LamB.

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FIG. 1. **Synthesis of export-defective LamBS78 interferes with the export of wild-type MBP.** Maltose-grown cells of MC4100 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ (*lam*B$^+$), SE2078, F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ (*lam*BS78), or MC4100 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ containing the plasmid plamBS78 were induced with various amounts of IPTG 2 h prior to pulse labeling with ^{35}S Met for 60 s. The cells were then TCA precipitated, immunoprecipitated using MBP antisera, and subjected to SDS-PAGE and autoradiography. Because LamBS78 protein migrated in a region of the gel that lacked any other comigrating proteins, LamBS78 protein levels could be easily quantified. To measure the amount of LamBS78 protein that was synthesized, an aliquot of each sample was removed prior to the addition of anti-MBP rabbit serum and analyzed by SDS-PAGE and autoradiography. LamBS78 synthesis levels were then normalized to the chromosomal level of LamBS78 protein expressed in the control strain SE2078.

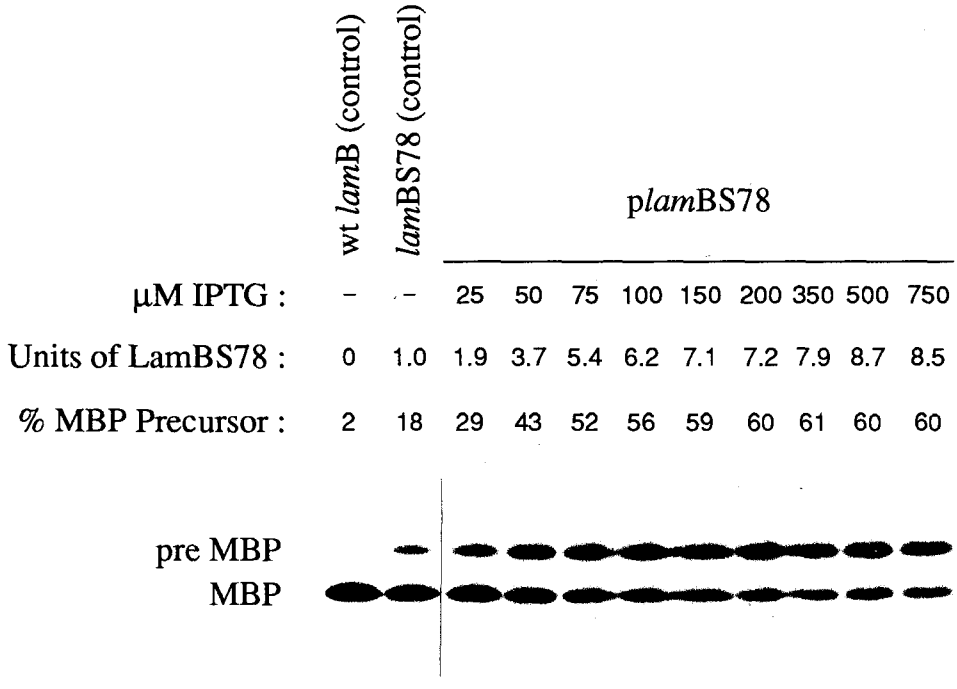
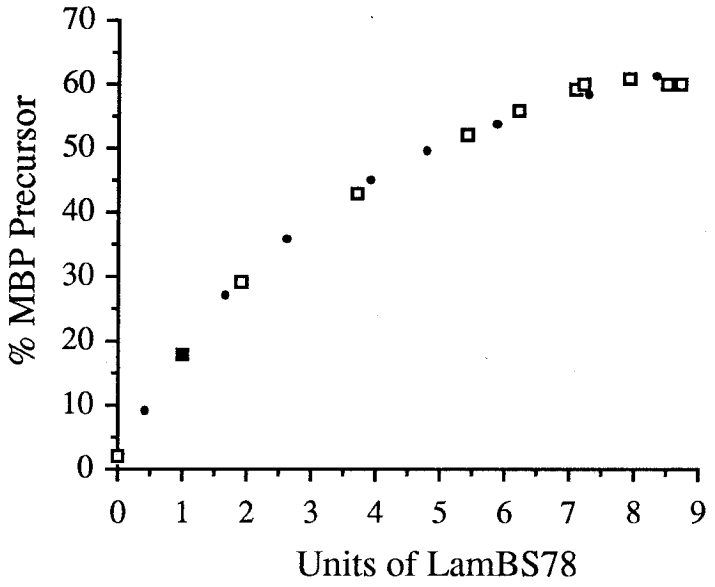

wt lamB (control)
lamBS78 (control)
plamBS78
μM IPTG : – – 25 50 75 100 150 200 350 500 750
Units of LamBS78 : 0 1.0 1.9 3.7 5.4 6.2 7.1 7.2 7.9 8.7 8.5
% MBP Precursor : 2 18 29 43 52 56 59 60 61 60 60
pre MBP
MBP

FIG. 2. **Kinetic analysis of the MBP export rate as synthesis of export-defective LamBS78 protein is varied.**

A. V versus S plot. Since the % MBP precursor represents an equivalent measure of the rate of MBP export, Henri-Michaelis-Menten analysis could be performed, assuming that the units of LamBS78 protein reflected a titration of some export component that influenced the rate of MBP export. Thus, a V versus S plot was obtained by plotting % MBP precursor versus units of LamBS78 protein synthesized. These points are shown as open squares in the figure. The data was replotted using theoretical V' and S' values obtained from V_{max} and K_m (Fig. 2B) to verify that the V versus S plot was a rectangular hyperboloid, which is expected from a single ligand reaction. V' values were calculated as a percentage of V_{max}, while S' values were extrapolated from K_m, where $[S']_x = 1$, since S' = [V']/[1-V']. These data points are shown as closed circles in the figure.

B. Hanes-Woolf plot of S/V versus S. [units of LamBS78 protein]/[% MBP precursor] was plotted versus units of LamBS78 protein. These points are shown as open squares in the figure. As indicated in the figure, V_{max} and K_m were calculated to be 90% MBP precursor and 3.9 units of LamBS78 protein, respectively.

A


% MBP Precursor
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9
Units of LamBS78

B

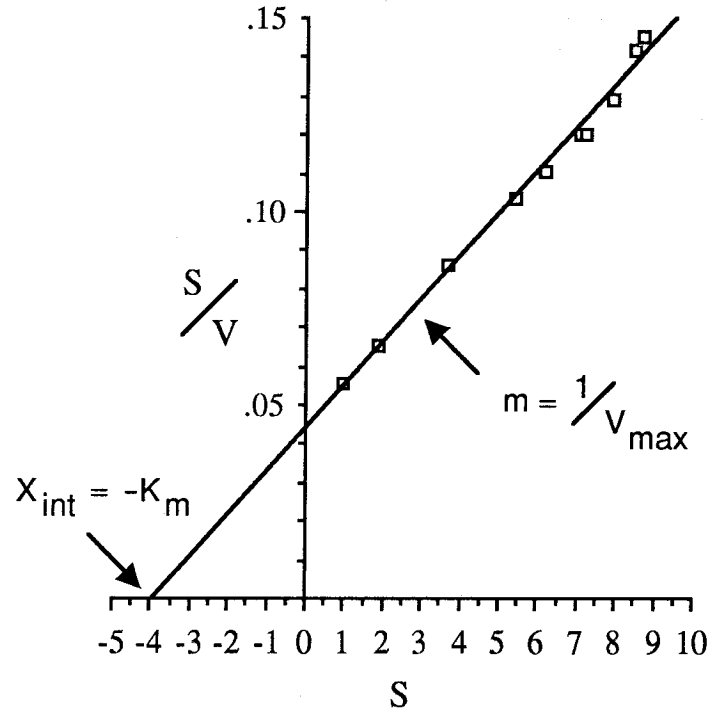

.15
.10
.05
S/V
m = 1/Vmax
Xint = -Km
-5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
S

FIG. 3. **Mapping the interfering region in LamB.** Maltose grown cells of MC4100 F'*lac*iQ^{1}Z^{+}Y^{+}A^{+} (*mal*E^{+}) containing the indicated plasmids were induced with 1 mM IPTG 2 h prior to pulse labeling with ^{35}S Met for 30 s. The cells were then TCA precipitated, immunoprecipitated using MBP antisera, and subjected to SDS-PAGE and autoradiography. Since the amount of interference increases as the synthesis level of the export-defective protein increases (Fig. 1), it was important to quantify the synthesis levels of the encoded proteins used in this experiment to map the interfering region. To accomplish this, an aliquot of each sample was removed prior to the addition of anti-MBP rabbit serum and analyzed by SDS-PAGE and autoradiography. All proteins encoded in part A were found to be within 5% of the synthesis level of the LamBS60 protein encoded by pUC*lam*BS60, while all proteins encoded in parts B and C were found to be within 5% of the synthesis level of the LacZ protein encoded by p*lac*Z (data not shown). Proteins encoded by pUC*lam*BS60, p*lam*BS60-420-*lac*Z, p*lam*BS60-380-*lac*Z, p*lam*BS60-320-*lac*Z, and p*lam*BS60-180-*lac*Z, contain an export-defective LamBS60 signal sequence, while proteins encoded by p*lac*Z-5-*lam*B, p*lac*Z-90-*lam*B, p*lac*Z-220-*lam*B, and p*lac*Z-[320-*lam*B-420]-*lac*Z, utilize the translational start of LacZ. Where appropriate, fusion joints are indicated as approximate amino acid positions. See Materials and Methods for additional information.

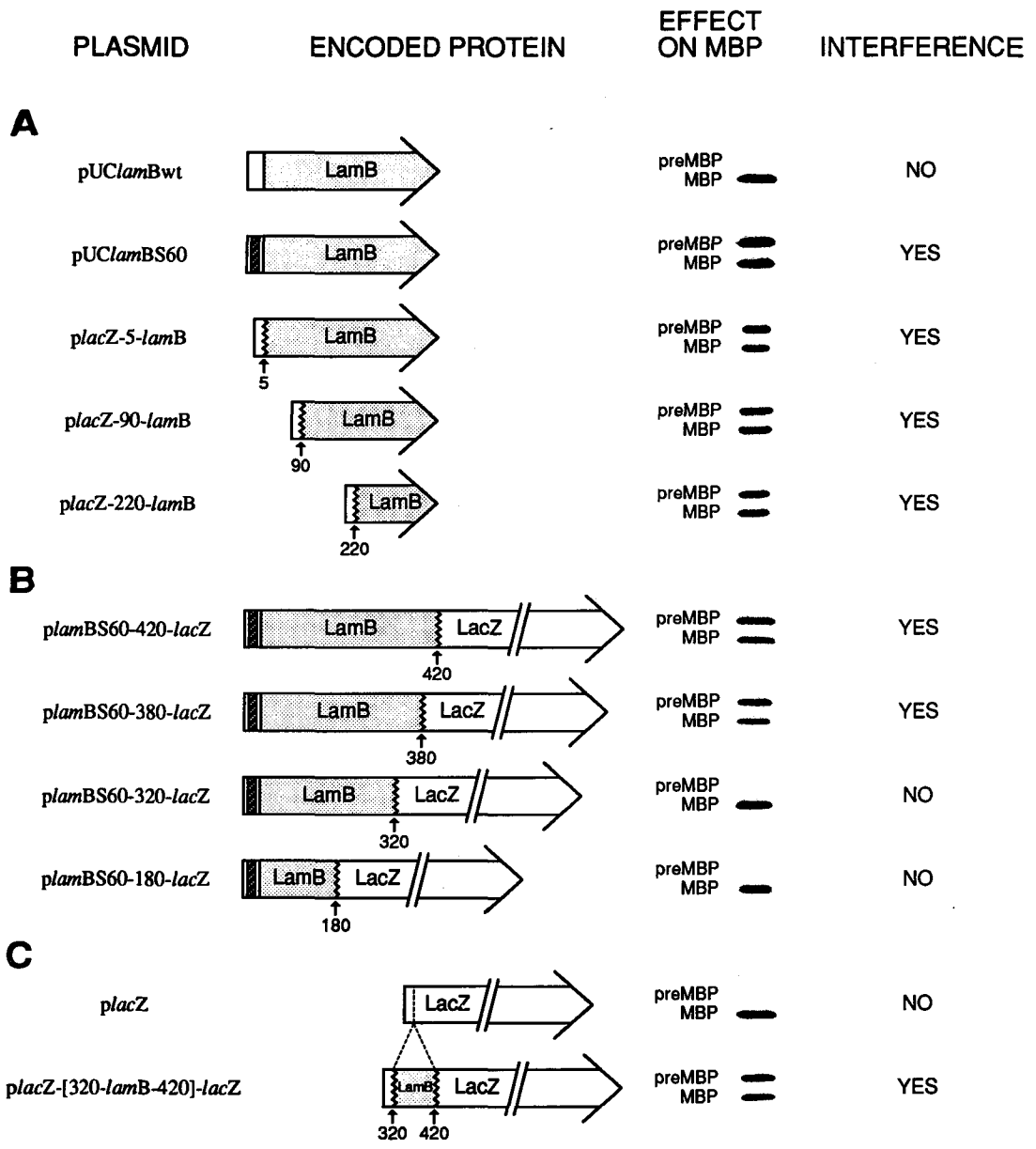
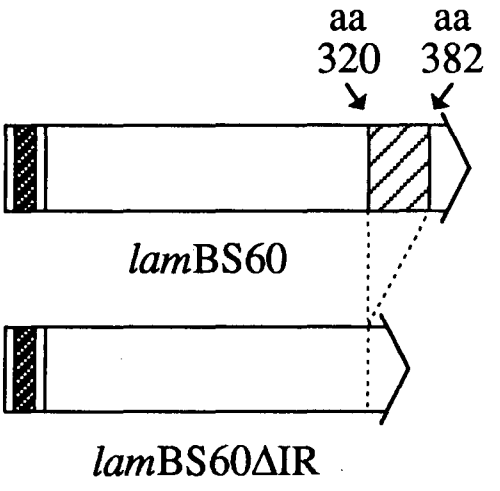

PLASMID
ENCODED PROTEIN
EFFECT ON MBP
INTERFERENCE
A
pUClamBwt
LamB
preMBP
MBP
NO
pUClamBS60
LamB
preMBP
MBP
YES
placZ-5-lamB
LamB
5
preMBP
MBP
YES
placZ-90-lamB
LamB
90
preMBP
MBP
YES
placZ-220-lamB
LamB
220
preMBP
MBP
YES
B
plamBS60-420-lacZ
LamB
LacZ
420
preMBP
MBP
YES
plamBS60-380-lacZ
LamB
LacZ
380
preMBP
MBP
YES
plamBS60-320-lacZ
LamB
LacZ
320
preMBP
MBP
NO
plamBS60-180-lacZ
LamB
LacZ
180
preMBP
MBP
NO
C
placZ
LacZ
preMBP
MBP
NO
placZ-[320-lamB-420]-lacZ
LamB
LacZ
320
420
preMBP
MBP
YES

FIG. 4. **Interference caused by LamBS60 can be suppressed if amino acids 320 through 382 are deleted.**

A. Using oligo-directed mutagenesis the coding sequence for amino acids 320 through 382, which represented the interfering region in the mature LamB protein, was deleted from the export-defective *lam*BS60 gene. The new construct was called *lam*BS60ΔIR.

B. Maltose grown cells of MC4100 F'*laci*$Q^1Z^+Y^+A^+$ (wt control), MC4100 F'*laci*$Q^1Z^+Y^+A^+$ pUC*lam*BS60, and MC4100 F'*laci*$Q^1Z^+Y^+A^+$ pUC*lam*BS60ΔIR were induced with 1 mM IPTG 2 h prior to labeling. The cells were pulse labeled for 30 s with no chase. See Materials and Methods for complete experimental details.

A


aa
320
aa
382
lamBS60
lamBS60ΔIR

B

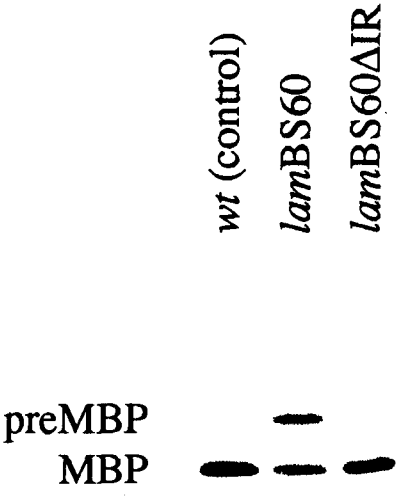
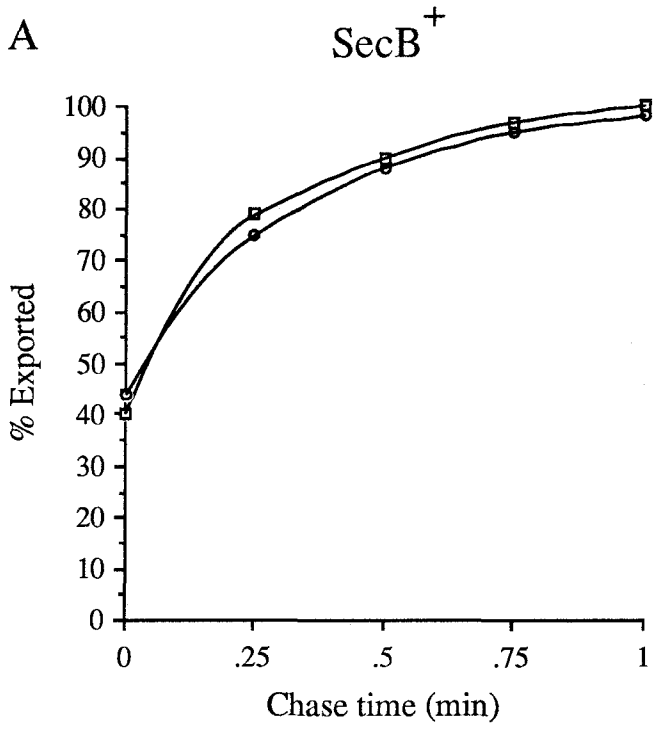
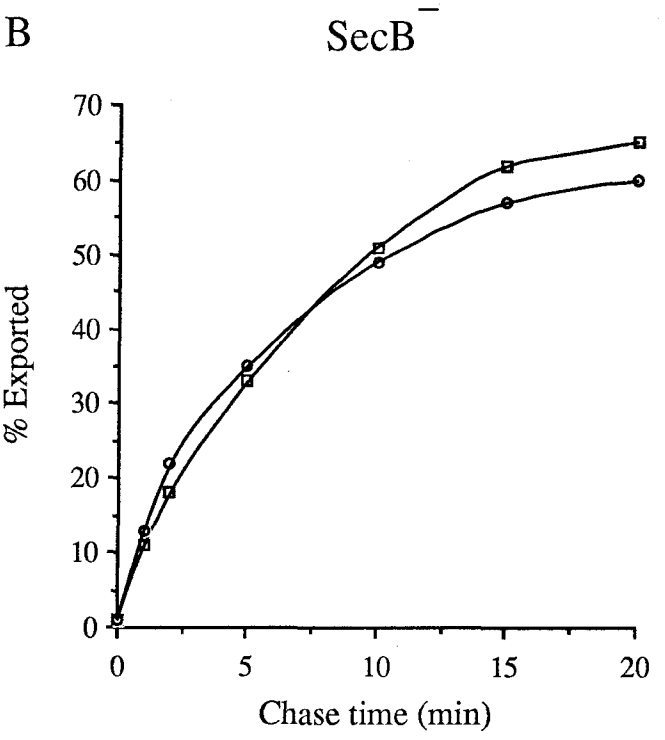

wt (control)
lamBS60
lamBS60ΔIR
preMBP
MBP

FIG. 5. **Export kinetics of wild-type LamB versus LamBΔIR in SecB$^+$ and SecB$^-$ backgrounds.**

A. SecB$^+$. Glycerol grown cells of SE3001 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ (Δ*lam*B), containing either plasmid plamBwt or plamBΔIR were induced with 20 μM IPTG 2 h prior to labeling. The cells were pulsed for 15 s and chased as indicated. Wild-type LamB data points are shown as open squares, while LamBΔIR data points are shown as open circles. See Materials and Methods for complete experimental details.

B. SecB$^-$. Glycerol grown cells of SE3001 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ *sec*B::Tn5 (Δ*lam*B, *sec*B$^-$) containing either plasmid p*lam*Bwt or p*lam*BΔIR were induced with 20 μM IPTG 2 h prior to labelling. The cells were pulsed for 30 s and chased as indicated. Wild-type LamB data points are shown as open squares, while LamBΔIR data points are shown as open circles. See Materials and Methods for complete experimental details.


A
SecB+
% Exported
100
90
80
70
60
50
40
30
20
10
0
0
.25
.5
.75
1
Chase time (min)


B
SecB−
% Exported
70
60
50
40
30
20
10
0
0
5
10
15
20
Chase time (min)

Chapter 3

The Presence of Both the Signal Sequence and a Region of Mature LamB Protein is Required for the Interaction of LamB with the Export Factor SecB

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In press in *The Journal of Biological Chemistry*

Summary

In the preceding chapter a putative SecB binding site was identified in the mature LamB protein. The export of wild-type LamB was unperturbed when this region was removed, however, suggesting the presence of a second site of interaction between SecB and LamB. In this chapter we show that the interference caused by export-defective LamB proteins is influenced by the amount of signal sequence that is present. If a large portion of the signal sequence is deleted, then the interference levels are significantly reduced. This result suggests that a region of the signal sequence contributes to the interaction of SecB with the LamB protein. Using anti-SecB affinity chromatography, we demonstrated directly that the association of SecB protein with precursor LamB is dependent on the presence of both the LamB signal sequence and the interfering region, which maps to amino acids 320-380 of mature LamB.

Although the interfering region is not necessary for the export of wild-type LamB under normal conditions, when the signal sequence is mutationally altered, the interfering region is required to promote the efficient export of LamB protein. Also, deletion of the interfering region eliminates the ability of wild-type LamB precursor to be maintained in an export-competent conformation *in vivo*. Collectively, our results indicate that efficient export of the LamB protein is achieved by an interaction with SecB that involves both the LamB signal sequence and the interfering region in mature LamB.

It has become increasingly clear that the *sec*B gene codes for a component of the *Escherichia coli* export machinery. However, unlike other export components which are encoded by the *sec*A and *prl*A/*sec*Y genes, *sec*B is a nonessential gene (for a general review, see Bankaitis *et al.*, 1986). How SecB interacts with exported proteins remains an intriguing question since SecB appears to be necessary only for the efficient export of a subset of the secreted proteins that require SecA or PrlA/SecY. While the periplasmic maltose binding protein (MBP) and outer membrane proteins LamB, OmpA, and OmpF are absolutely dependent on SecB for their efficient export, the periplasmic ribose binding protein (RBP), alkaline phosphatase, β-lactamase and M13 coat protein, as well as the outer membrane lipoprotein can be efficiently exported independent of SecB (Kumamoto and Beckwith, 1983, 1985; Collier *et al.*, 1988).

Several observations have made it clear that the synthesis of export-defective SecB-dependent proteins interferes with the normal export process, and that this interference is due to the limitation of a single component of the export machinery, SecB. First, the subset of exported proteins affected by interference is identical to the subset of proteins that require SecB for export (Collier *et al.*, 1988). Second, overproduction of SecB suppresses interference (Collier *et al.*, 1988). Third, interference does not increase the export defect observed in *sec*B mutant cells (Collier *et al.*, 1988). Finally, interference is caused by the limitation of a single export component (see chapter 2, this thesis).

In the preceding chapter, the interfering region of an export-defective LamB protein was mapped and found to reside between amino acids 320-380 of the mature protein. Surprisingly, when this region was deleted from a wild-type LamB, protein export was unperturbed even though it is well documented that LamB export is crippled in a *sec*B null background (Kumamoto and Beckwith, 1985; Trun *et al.*, 1988). This result suggested that LamB contained an additional site of SecB interaction. In this study we show that the efficient interaction of SecB with LamB protein is dependent on the presence of both the LamB signal sequence and the interfering region in mature LamB.

Materials and Methods

Bacterial strains—All strains used in this study are isogenic derivatives of MC4100: F$^-$ Δ*lac*U169 *ara*D139 *rps*L150 *thi rel*A1 *flb*B5301 *deo*L1 *pts*F25 (Casadaban, 1976), and the relevant genotypes are listed in Table 1. When necessary, the *sec*B::Tn5 lesion was moved into strains by P1 transduction, while the F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ episome was moved into strains by mating with SE9100 (see chapter 2, this thesis).

LamB plasmids—The construction of p*lam*Bwt, p*lam*BΔIR, pUC*lam*BS60, and pUC*lam*BS60ΔIR is described in the preceding chapter. p*lam*B17D was constructed by replacing the EcoRI SmaI fragment containing *lam*B's signal sequence in p*lam*BS78 with the equivalent EcoRI SmaI fragment from pSE73. pSE73 was constructed by replacing the SalI fragment containing *lam*B's signal sequence in pSE60 with the equivalent SalI fragment from λapSE73 (Emr and Silhavy, 1980). By exchanging a SmaI BamHI fragment that included the *lam*B interfering region between p*lam*BΔIR and p*lam*B17D, p*lam*B17DΔIR was constructed.

Anti-SecB affinity chromatography—Bacterial cells were labeled for 15 sec at 37°C with TranSLabel (ICN), 60 uCi/ml [^{35}S]methionine, 90 nM methionine. Cells were extracted as described previously (Kumamoto, 1989), and the extract was divided into three equal portions. One portion was precipitated with TCA and immunoprecipitated with anti-LamB antiserum (Kumamoto and Gannon, 1988), to determine the total amount of radioactive LamB precursors in the sample. The second portion was applied to an affinity column containing anti-SecB antibody that was prepared as described (Kumamoto, 1989). The third portion was mixed with an excess of purified SecB protein (prepared as described previously; Kumamoto, 1989) and then applied to an anti-SecB column. After washing with PBS, 0.5% Tween-20, the columns were eluted, (Kumamoto, 1989) and the LamB precursor that bound to the columns in association with SecB was detected by immunoprecipitation, SDS polyacrylamide gel electrophoresis, and fluorography

(Kumamoto and Gannon, 1988). Quantification of the relative amounts of precursor LamB that bound to the columns was determined by densitometry of fluorograms as described in the previous chapter.

Posttranslational assay—By varying the amount of carbonyl cyanide m-chlorophenylhydrazone and β-mercaptoethanol used in the assay conditions described by Zimmerman and Wickner (1983), we were able to reverse the carbonyl cyanide m-chlorophenylhydrazone block directly with the addition of β-mercaptoethanol without the need of a prior wash step to remove excess carbonyl cyanide m-chlorophenylhydrazone. This allowed us to easily monitor posttranslational export in *E. coli*. Vigorously aerated cultures were grown to logarithmic phase (.3 OD_{550} to .4 OD_{550}) in M63 minimal medium (Miller, 1972) at 30°C. Carbonyl cyanide m-chlorophenylhydrazone was added to a final concentration of 50 μM 1 min prior to pulse labelling (a stock solution of carbonyl cyanide m-chlorophenylhydrazone was prepared at a concentration of 5 mM in 95% ethanol and stored at -20°C). Samples were then pulse-labeled for 1 min with the addition of 10 μCi of ^{35}S methionine per ml of culture and chase was initiated by the addition of unlabeled methionine to a final concentration of .5% wt/vol. One min later the carbonyl cyanide m-chlorophenylhydrazone block was released by the addition of β-mercaptoethanol to a final concentration of .05% wt/vol. To terminate the assay, 1 ml aliquots were removed and immediately dispensed into 150 μl of ice-cold 50% TCA.

We find that under the assay conditions described, the translocation of both MBP and LamB can be blocked, but that when the block is released, 100% of the proteins are exported. If higher concentrations of carbonyl cyanide m-chlorophenylhydrazone are used, however, the translocation block cannot be released by β-mercaptoethanol. Although degradation of unexported LamB is sometimes a problem in experiments involving long chase times, we find that in this assay the unexported LamB protein is 100% stable and that no degradation occurs.

Radiolabeling, immunoprecipitation, polyacrylamide gel electrophoresis, and autoradiography—With the exception of the SecB binding study, pulse-chase experiments, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described in the preceding chapter. While unexported MBP and OmpA protein are stable, unexported LamB protein is subject to degradation in the cytoplasm. Therefore, degradation of unexported LamB protein had to be considered when calculating the percentage of exported LamB protein. Although degradation was not a problem in most of the LamB experiments reported here, some LamB protein did get degraded in experiments employing long chase times. In these cases the values given for the percent of LamB protein exported were corrected by subtracting the amount of LamB protein that had been degraded. We found that unexported wild-type LamB protein was degraded at an average rate of 2% per minute of chase, while unexported LamB17D protein was degraded at an average rate of 3% per minute of chase.

Reagents—DNA restriction and modifying enzymes were obtained from New England BioLabs, Inc. ^{35}S methionine came from Amersham, TranSLabel came from ICN, while Safety-Solve came from Research Products International, Corp. Carbonyl cyanide m-chlorophenylhydrazone and β-mercaptoethanol used in the posttranslational assay were purchased from Sigma. Anti-LamB, MBP, and OmpA rabbit serum were the generous gifts of Tom Silhavy, Phil Bassford, and Paul Ray, respectively.

Results

Interference is influenced by the LamB signal sequence—In the preceding chapter, the interfering region of the export-defective LamBS60 protein was mapped to between amino acids 320-380 of the mature protein. We suggested that this region might be responsible for SecB binding. However, deletion of the interfering region from an otherwise wild-type LamB protein did not affect its export, suggesting that SecB was still able to associate with the deleted derivative. Because the interfering region was mapped

using export-defective LamB proteins that contained a large deletion in the signal sequence, it is possible that normally there is a signal sequence dependent interaction between LamB and SecB that was not detected in the mapping study. To test this, we scored the interference caused by six different export-defective LamB proteins (Fig. 1). Since Bankaitis and Bassford (1984) had shown that interference was directly proportional to the strength of an export-defective mutation (weaker signal sequence mutations that cause incomplete export blocks do not interfere as much as stronger signal sequence mutations that cause complete export blocks), we examined only LamB proteins containing signal sequence mutations that caused total export blocks as judged by their inability to secrete any LamB protein after a 4 min chase (Stader *et al.*, 1986). Although all of the export-defective LamB proteins examined were totally export-incompetent and synthesized LamB at equivalent rates, their interference levels varied. The export-defective proteins showed a general pattern in that the interference levels decreased as increasing amounts of the signal sequence was removed from the export-defective protein.

Previous analysis of LamB's signal sequence has indicated that a core of four residues are absolutely critical for export, since all point mutations which abolish LamB's export lie in either amino acids 14, 15, 16, or 19 of the signal sequence (Emr and Silhavy, 1982). Closer examination of the data in Figure 1 indicates that maximal interference occurs when amino acids 14, 15, and 16 of the signal sequence are preserved (interference caused by *lam*B19R or *lam*BS87). Amino acid 19 can be either mutated (*lam*B19R) or deleted (*lam*BS87) and interference is still maximal. However, when amino acids 14, 15, or 16 are either mutated or deleted, only a reduced level of interference is observed (interference caused by *lam*B14D, *lam*BS60, or *lam*BS68). These results could be explained if the region around amino acids 14-16 contributed to the interaction between SecB and the LamB protein.

SecB association with intracellular LamB precursors is dependent upon the presence of both the LamB signal sequence and the interfering region between amino acids

320-380 of mature LamB—The results described above and in the preceding chapter suggests that the interaction of SecB with the LamB protein might depend upon multiple regions of LamB. To test this hypothesis directly, we used anti-SecB affinity chromatography to determine whether SecB was capable of association with precursor LamB molecules containing or lacking the interfering region, and containing or lacking a functional signal sequence.

It had previously been demonstrated that SecB complexes containing SecB and wild-type precursor LamB could be detected using anti-SecB affinity chromatography of labeled cell extracts (Kumamoto, 1989). To determine the effect of the deletions that were used in the previous chapter, we labeled *sec*B$^+$ cells harboring plasmids that encoded either wild-type LamB (wtLamB), wild-type LamB lacking the interfering region (LamBΔIR), export-defective LamB containing a deletion in the signal sequence (LamBS60), and export-defective LamB containing a deletion in the signal sequence as well as a deletion of the interfering region (LamBS60ΔIR). Extracts of the labeled cells were prepared and split into three equal portions. One portion was immunoprecipitated directly to determine the total amount of labeled precursor LamB present in the extract. The remaining two portions were applied to anti-SecB columns in the presence or absence of excess SecB competitor. After elution, the amount of precursor LamB that was complexed with SecB was determined by immunoprecipitation of the bound fraction and compared to the total amount of labeled precursor LamB that was present in the extract. We observed that consistent with previous results, 50% of the intracellular precursor could be obtained as a SecB complex, when extracts containing wild-type precursor LamB were analyzed (Fig. 2). When excess SecB was present during chromatography to compete with the binding of SecB complexes, precursor LamB was not detected in the bound fraction, demonstrating that binding of precursor LamB to the affinity column was dependent upon its association with SecB.

Deletion of either the LamB interfering region or the signal sequence resulted in a marked decrease in the amount of precursor that was recovered as a SecB complex, and deletion of both regions almost completely eliminated the ability of LamB to associate with SecB (Fig. 2). When excess SecB was present during chromatography of the extracts, LamB species were not detected in the anti-SecB bound fraction (Fig. 2), demonstrating that LamB binding occurred by virtue of association with SecB. We conclude from these studies that under physiological conditions, both the signal sequence and the interfering region are necessary for the efficient association of SecB with precursor LamB.

LamB17D's export kinetics are altered when the interfering region is removed or when SecB is absent—The above results suggested that the SecB association mediated by the interfering region might be more critical for export if the signal sequence was compromised. Although it is not possible to demonstrate the importance of the interfering region using signal sequence mutations that prevent export, we reasoned that the interfering region might be important for the export of a LamB protein containing a weak signal sequence mutation.

All of the LamB signal sequence mutants were isolated by employing *lam*B-*lac*Z fusions that resulted in a maltose-sensitive phenotype upon induction with maltose. Maltose resistant mutants were obtained and then crossed from the *lam*B-*lac*Z fusion back to wild-type *lam*B to examine the effect of the mutation on LamB protein export. While most of the mutations rendered both the LamB-LacZ hybrid protein and the wild-type LamB protein export-defective, mutations that introduced a charged residue at amino acid 17 of the LamB signal sequence caused only the LamB-LacZ hybrid protein to become export-defective (Emr and Silhavy, 1980, 1982). Interestingly, the *lam*B-*lac*Z gene fusion used in the selection process coded for a LamB-LacZ hybrid protein that lacked the interfering region of mature LamB. It is possible that silent mutants such as LamB17D , which contains an aspartic acid residue at amino acid 17 instead of the wild-type glycine residue, were isolated for this reason. To test this, the export kinetics of the LamB17D

protein with or without the interfering region was examined. As shown in Figure 3, the export kinetics of the LamB17D protein without the interfering region was two-fold slower than that of the LamB17D protein containing the interfering region; furthermore, 25% of the LamB17D protein lacking the interfering region appears to be export-incompetent, yet no such defect is seen with the LamB17D protein containing the interfering region. Thus, a significant defect in the export of LamB protein can be seen if removal of the interfering region is combined with a weak signal sequence mutation such as *lam*B17D.

This result suggests that the LamB17D protein is extremely dependent on the presence of SecB for its efficient export. To analyze this further, we compared the export kinetics of LamB17D with wild-type LamB in both *sec*B$^+$ and *sec*B null backgrounds (Fig. 4A and 4B). Although the export rate of LamB17D was only three times slower than wild-type LamB in a *sec*B$^+$ background, the export rate of LamB17D was more than thirty times slower than wild-type LamB in a *sec*B null background. After a 20 min chase in a *sec*B null background 65% of the wild-type LamB protein was exported, while less than 2% of the LamB17D protein was exported. This synergistic effect demonstrates that the efficient export of LamB17D is absolutely dependent on the presence of SecB. When SecB is limited, either by removing the interfering region or by removing SecB, the export kinetics of LamB17D are drastically affected. Collectively these data indicate that if the signal sequence is altered, interaction of SecB with the interfering region is necessary for the efficient export of the LamB protein.

If the interfering region is removed, LamB precursor is not maintained in an export-competent conformation—Although the export of wild-type LamB is unperturbed under normal conditions when the interfering region is removed, our results indicate that the presence of the interfering region is necessary if proper association of SecB with precursor LamB is to occur. We reasoned that if wild-type LamB's export could be temporally delayed, a dependence on the interfering region might be uncovered. To accomplish this, we employed a posttranslational export assay. Cells containing wild-type LamB with or

without the interfering region were pulse-labeled in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone, which causes a translocation block (Randall, 1986). Chase was then initiated and the translocation block was released by the addition of β-mercaptoethanol. As shown in Figure 5, while wild-type LamB can be efficiently exported posttranslationally, LamB protein that lacks the interfering region can not. Thus, it appears that an interaction between SecB and the interfering region is necessary if the LamB precursor is to be maintained in an export-competent form.

Role of SecB in the export of three SecB-dependent proteins—SecB appears to interact with the exported protein in a complex manner, as evidenced by the presence of two sites of interaction in the LamB protein. Towards a better understanding of how SecB promotes the export of SecB-dependent proteins, we compared the export rates of LamB, MBP, and OmpA in both *sec*B$^+$ and *sec*B null backgrounds (Fig. 6). While all three proteins are exported with swift kinetics in a *sec*B$^+$ background, there is a severe export block in a secB null background at early chase points. At later chase points, however, there is a clear difference in the three proteins' dependence on SecB; OmpA is 100% exported, LamB is 65% exported (this number might be greater, except that 35% of LamB was degraded before it could be exported), yet only 40% of MBP ever gets exported. This result indicates that the requirement for SecB differs from protein to protein.

Discussion

It is now apparent that the export interference phenomenon in *E. coli* is caused by the limitation of SecB (Collier *et al.*, 1988; chapter 2, this thesis). In the preceding chapter, we mapped the interfering region in an export-defective LamB protein to between amino acids 320-380 of the mature protein. However, when the interfering region was removed from the wild-type LamB protein, no export defect was observed, even though it has been shown that export of the wild-type LamB is crippled in a *sec*B null background (Kumamoto and Beckwith 1985; Trun *et al.*, 1988). These results raised the possibility

that SecB might also interact with the LamB signal sequence. Consistent with this hypothesis, we found that the interference caused by export-defective LamB proteins was weakest when a large portion of the signal sequence was deleted. Previous analysis of mutations in the LamB signal sequence that rendered the LamB protein export-defective have indicated that amino acids 14-16 might be part of a critical core of amino acids within the LamB signal sequence (Emr and Silhavy, 1982). Interestingly, we found that interference is greater when the export-defective LamB protein contains amino acids 14-16 of the signal sequence than when it lacks them. Taken together, these observations suggest that a region of the LamB signal sequence is necessary for the interaction of LamB with SecB.

Using anti-SecB affinity chromatography, it was possible to test directly whether the efficient interaction of SecB with LamB precursor was dependent on the presence of either the LamB signal sequence or the interfering region in mature LamB. Consistent with previous results (Kumamoto, 1989), we observed that SecB/precursor LamB complexes were efficiently formed *in vivo*. However, if the LamB precursor contained a deletion in the signal sequence or a deletion of the interfering region, the association of LamB with SecB was reduced 5-fold. When both the signal sequence and the interfering region were removed from the LamB protein, the association of LamB with SecB was reduced 25-fold. The results of these binding studies show that the interaction of SecB with the LamB protein requires that both the signal sequence and the interfering region be intact. The simplest interpretation of our data is that SecB protein binds directly to both the LamB signal sequence and the interfering region between amino acids 320-382 of mature LamB, and that maximum binding depends on the presence of both sites.

Although deletion of the interfering region does not alter the normal export kinetics of wild-type LamB, two observations demonstrate that the interfering region is required for the efficient export of the LamB protein: 1) the export of a LamB protein harboring a weak signal sequence mutation is significantly slower when the interfering region is removed,

and 2) the removal of the interfering region drastically reduces the ability of wild-type LamB precursor to be maintained in an export-competent conformation. Thus, the efficient action of SecB appears to require the presence of both the LamB signal sequence and the interfering region in mature LamB. It is well documented that SecB functions as an antifolding agent during the export process (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Liu *et al.*, 1989) and that SecB may form a tetrameric complex in the cytoplasm (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989; Watanabe and Blobel, 1989a). In light of our results, it is possible that a single SecB tetramer acts simultaneously at both the signal sequence and a region in the mature portion of the exported protein. This would provide a simple model to explain how the SecB protein acts as an antifolding factor during the export process.

The data from several labs suggest that MBP may interact with SecB in a manner similar to LamB. First, it is apparent that SecB interacts with the mature region of MBP (Collier *et al.*, 1988; Gannon *et al.*, 1989; Liu *et al.*, 1989), and that this interaction promotes the antifolding activity of SecB (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Liu *et al.*, 1989). Second, Watanabe and Blobel (1989b) have demonstrated that while precursor MBP species that contain an intact signal sequence are bound by SecB, precursor MBP species that contain a deletion in the signal sequence are not bound by SecB. Together, these data indicate that the interaction of SecB with MBP involves both the signal sequence and a region of mature MBP. Considerable controversy exists, however, as to whether the signal sequence is directly or indirectly involved in the binding of SecB to the exported protein. While Watanabe and Blobel (1989b) have proposed that SecB binds directly to the signal sequence of the exported protein, Randall and colleagues favor a model in which SecB does not bind to the signal sequence of the exported protein, and have proposed that the signal sequence plays an indirect role in modulating the binding of SecB to the mature protein (Liu *et al.*, 1989; Randall *et al.*, 1990). Although it will be extremely difficult to delineate between these two possibilities, the data presented in this

and the preceding chapter favor a more direct role for the signal sequence during the association of SecB with the exported protein.

The larger question of how SecB facilitates the export of the bound protein remains to be answered. It could be simply that SecB functions as an antifolding agent and maintains SecB-dependent proteins in an export competent unfolded form. In this scenario, SecB's only function would be to keep the protein unfolded so that it could interact productively with the export complex represented by other components, such as SecA and PrlA/SecY. However, two observations suggest that SecB interacts directly with both SecA and PrlA/SecY; first, a synergistic response is seen at the permissive temperature when a secB mutant is combined with the *sec*A^{ts} allele (Kumamoto and Beckwith, 1983), and second, the suppressor function of the *prl*A4 allele is disabled in a *sec*B null background (Trun *et al.*, 1988). Because of these data, we believe that SecB has two functions in the export process: 1) a recognition function in which SecB presents the exported protein to the SecA:PrlA/SecY complex, a process that might involve an interaction with the signal sequence, and 2) an antifolding function that retards the folding of the exported protein into an export-incompetent form.

Our finding that three SecB-dependent proteins vary widely in their dependence for SecB is also consistent with SecB's having two functions. Although the export of MBP, LamB, and OmpA is blocked shortly after synthesis in a *sec*B null background, the final export fates of these proteins are different, as OmpA and LamB are eventually exported, but MBP is not. Very similar results have been obtained when SecB is limited due to the interfering effects of synthesizing an export-defective MBP protein. At early chase times precursors of MBP, LamB, and OmpA accumulate as a result of interference. At later chase times, however, all of the LamB and OmpA precursor is exported, while a significant portion of the MBP precursor is rendered export-incompetent (Collier *et al.*, 1988; Bankaitis and Bassford, 1984). While MBP, LamB, and OmpA would all be dependent on SecB to recognize and deliver them to the SecA:PrlA/SecY complex, MBP would also be

dependent on SecB's antifolding function for export, since MBP folds rapidly into an export-incompetent form (Randall and Hardy, 1986; Collier *et al.*, 1988; Kumamoto and Gannon, 1988). OmpA and LamB, although requiring SecB for efficient delivery to the export complex might not have the same requirements for SecB's antifolding function, since being largely hydrophobic membrane proteins, they may not tend to fold into export-incompetent forms.

It appears likely that SecB affects the interaction of SecA:PrlA/SecY with the signal sequence by stabilizing a productive interaction of the signal sequence with the export complex and/or triggering the active translocation of the bound, exported protein. In fact, Watanabe and Blobel (1989b) have proposed that SecB is the signal recognition particle of *E. coli*. Although this is consistent with our data, we feel it is more likely that SecB belongs to a family of chaperones that facilitate the export of certain proteins, for the following reasons. First, trigger factor and GroEL, two chaperones not as well characterized as SecB, have been shown to function in a manner similar to SecB by maintaining OmpA in an export-competent form (Lecker *et al.*, 1989). Second, SecB is required only for the efficient export of a subset of the secreted proteins that are dependent on the *E. coli* export apparatus (Kumamoto and Beckwith, 1983, 1985; Collier *et al.*, 1988). Third, one of the SecB-independent proteins, β-lactamase, has been shown to be dependent on GroEL for its efficient export (Kusukawa *et al.*, 1989). Collectively, these data suggest that multiple export factors such as SecB, trigger factor, and GroEL act in concert to facilitate the recognition and delivery of secreted proteins. Some proteins such as OmpA might utilize all of the factors for their efficient export, while the export of other proteins might require only some of the factors. Clearly, further work will be needed to elucidate precisely how SecB and the rest of the export machinery of *E. coli* efficiently translocates secreted proteins across the cytoplasmic membrane.

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Table 1

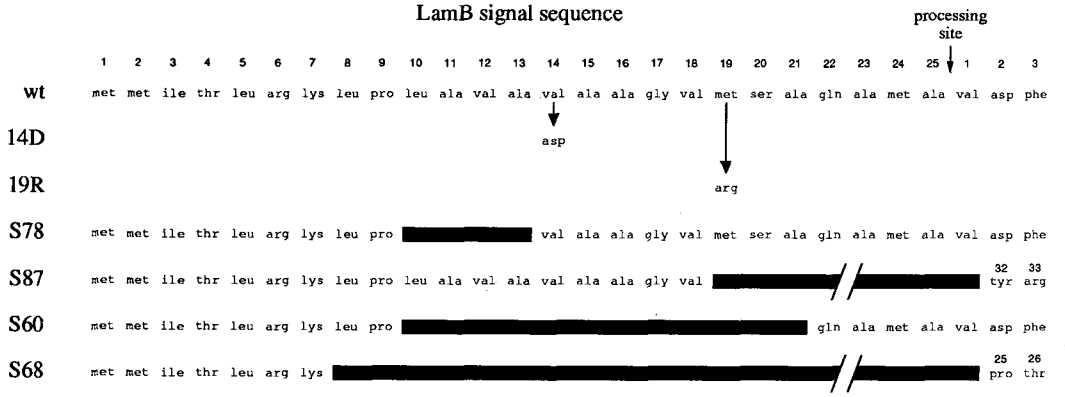
Bacterial Strains

Strain	Relevant Genotype	Reference
MC4100	Wild-type, *rps*L150, F^-	Casadaban (1976)
SE2060	*lam*BS60[a]	Emr and Silhavy (1980)
SE2068	*lam*BS68[a]	Emr and Silhavy (1980)
SE2069	*lam*B19R[b]	Emr and Silhavy (1980)
SE2071	*lam*B14D[b]	Emr and Silhavy (1980)
SE2073	*lam*B17D[b]	Emr and Silhavy (1980)
SE2078	*lam*BS78[a]	Emr and Silhavy (1980)
SE2087	*lam*BS87[a]	Emr and Silhavy (1980)
SE3001	malK'Δ1'lamB	Emr and Silhavy (1980)
SE9100	*rps*L^+*rps*D F'*lac*$I^q Z^+ Y^+ A^+$	Altman *et al.* (In press)
CK1953	*sec*B::Tn5	Kumamoto and Beckwith (1985)

[a] The original nomenclature of Emr and Silhavy (1980) is used in this study to describe export-defective LamB proteins that result from deleting parts of the LamB signal sequence.

[b] The nomenclature suggested by Stader *et al.* (1986) is used in this study to describe export-defective LamB proteins that are the result of single amino acid changes in LamB's signal sequence.

FIG. 1. **Interference of MBP caused by different export-defective LamB proteins.** Maltose grown cells of MC4100 (*lam*B$^+$), SE2071 (*lam*B14D), SE2069 (*lam*B19R), SE2078 (*lam*BS78), SE2087 (*lam*BS87), SE2060 (*lam*BS60), or SE2068 (*lam*BS68), were pulse labeled for 30 s with no chase, and the interfered MBP protein was assayed by immunoprecipitation usng anti-MBP serum. The signal sequence alteration for each of the export-defective proteins is indicated in the figure. A reduced level of interference is caused by *lam*B14D, *lam*BS60, or *lam*BS68, and occurs at an average of 14% MBP precursor, while an increased level of interference is caused by *lam*B19R, or *lam*BS87, and occurs at an average of 40% MPB precursor. The interference caused by *lam*BS78 occurs at an intermediate level, which is consistent with the model proposed by Emr and Silhavy (1983), that the effect of the *lam*BS78 mutation is an indirect one, and is due to the proximal location of the deletion to the critical amino acid core defined by residues 14, 15, 16, and 19 of the LamB signal sequence (reviewed in Silhavy *et al.*, 1983).


LamB signal sequence
processing site
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 1 2 3
wt met met ile thr leu arg lys leu pro leu ala val ala val ala ala gly val met ser ala gln ala met ala val asp phe
14D asp
19R arg
S78 met met ile thr leu arg lys leu pro val ala ala gly val met ser ala gln ala met ala val asp phe
S87 met met ile thr leu arg lys leu pro leu ala val ala val ala ala gly val 32 33 tyr arg
S60 met met ile thr leu arg lys leu pro gln ala met ala val asp phe
S68 met met ile thr leu arg lys 25 26 pro thr

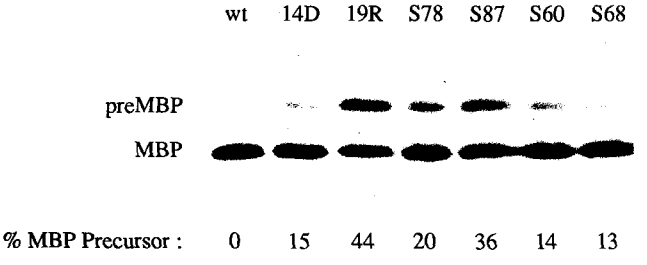

wt 14D 19R S78 S87 S60 S68
preMBP
MBP
% MBP Precursor : 0 15 44 20 36 14 13

FIG. 2. **Association of LamB precursors with SecB *in vivo*.** The LamB protein in glycerol grown cells of SE3001 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ (Δ*lam*B), containing either plasmids p*lam*Bwt, p*lam*BΔIR, pUC*lam*BS60, or pUC*lam*BS60ΔIR, was induced to chromosomal levels by the addition of IPTG 2 h prior to labeling. Cells were then pulse-labeled for 15 sec and extracted as described in Materials and Methods. The total amount of labeled LamB precursor in each extract (TOTAL) was determined by immunoprecipitation of a portion of the extract prior to affinity chromatography. After affinity chromatography of the labeled extract on an anti-SecB column, the amount of total LamB precursor was determined by elution and immunoprecipitation (BOUND). As a control, chromatography was also performed in the presence of excess SecB competitor and the amount of bound LamB precursor under these conditions was determined as above (CONTROL). Equivalent amounts of all immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis and fluorography. In order to accurately quantify the amount of bound precursor, 1/10 or 1/5 dilutions of the total samples were also analyzed (data not shown).

A map of the four *lam*B constructs analyzed in this experiment is shown in the upper part of the figure. The signal sequence is indicated by SS, while IR denotes the sequence coding for the interfering region, which maps to between amino acids 320-382 of the mature protein. Deletions in either the signal sequence or the interfering region are indicated by a striped box.

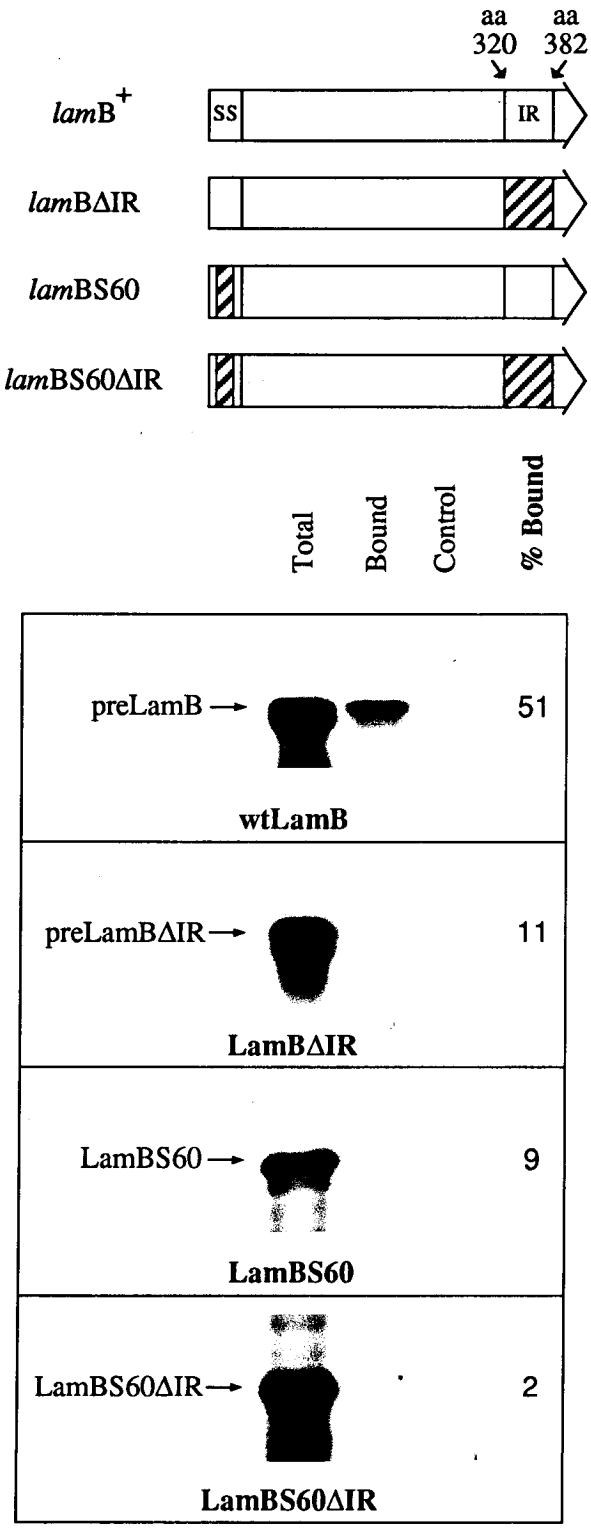

aa 320
aa 382
lamB+
SS
IR
lamBΔIR
lamBS60
lamBS60ΔIR
Total
Bound
Control
% Bound
preLamB
51
wtLamB
preLamBΔIR
11
LamBΔIR
LamBS60
9
LamBS60
LamBS60ΔIR
2
LamBS60ΔIR

FIG. 3. **Export kinetics of LamB17D versus LamB17DΔIR.** Glycerol grown cells of SE3001 F'*lac*iQ^{1}Z^{+}Y^{+}A^{+} (Δ*lam*B), containing either plasmid p*lam*B17D or p*lam*B17DΔIR, were induced with 20 μM IPTG 2 h prior to labeling. The cells were pulse labeled for 30 s and chased as indicated. LamB17D data points are shown as open squares, while LamB17DΔIR data points are shown as open circles.

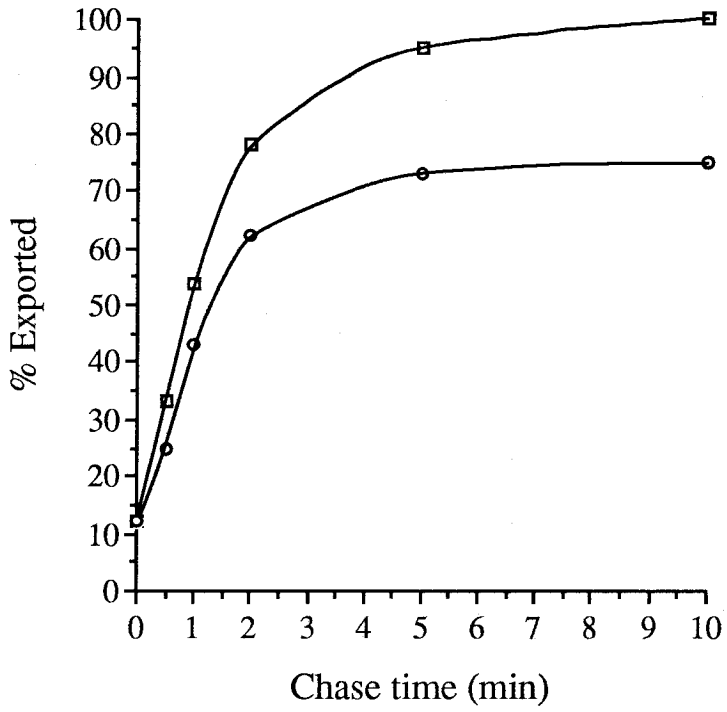
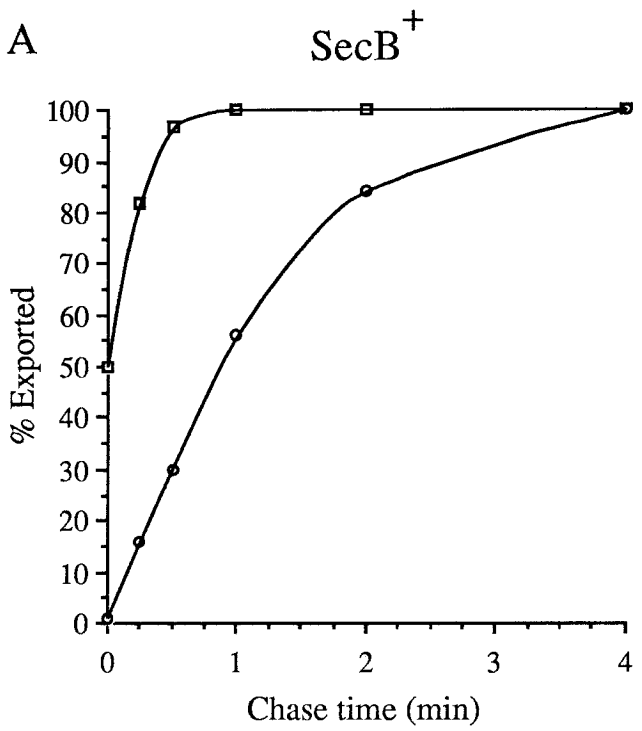

100
90
80
70
60
50
40
30
20
10
0
% Exported
0 1 2 3 4 5 6 7 8 9 10
Chase time (min)

FIG. 4. **Export kinetics of wild-type LamB versus LamB17D in SecB$^+$ and SecB$^-$ backgrounds.**

A. SecB$^+$. Maltose grown cells of MC4100 (*lam*B$^+$) or SE2073 (*lam*B17D) were pulse labeled for 15 s and chased as indicated. Wild-type LamB data points are shown as open boxes, while LamB17D data points are shown as open circles. If the slopes of the linear region of the export curves of these two proteins are examined, wild-type LamB's slope is three times greater than LamB17D's slope. Since wild-type LamB is completely exported by 1 min of chase, the slope comparison would predict that LamB17D should be completely exported by 3 min of chase. LamB17D appears to be completely exported somewhere between 2 min and 4 min of chase (LamB17D is 82% exported at the 2 min chase time and 100% exported at the 4 min chase time).

B. SecB$^-$. Maltose grown cells of MC4100 *sec*B::Tn5 (*lam*B$^+$, *sec*B$^-$) or SE2073 *sec*B::Tn5 (*lam*B17D, *sec*B$^-$) were pulse labeled for 30 s and then chased as indicated. Wild-type LamB data points are indicated with open squares, while LamB17D data points are indicated with open circles. The predicted data points (based on the fact that export of LamB17D is three times slower than wild-type LamB) for LamB17D are indicated with open triangles.


A
SecB+
% Exported
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Chase time (min)

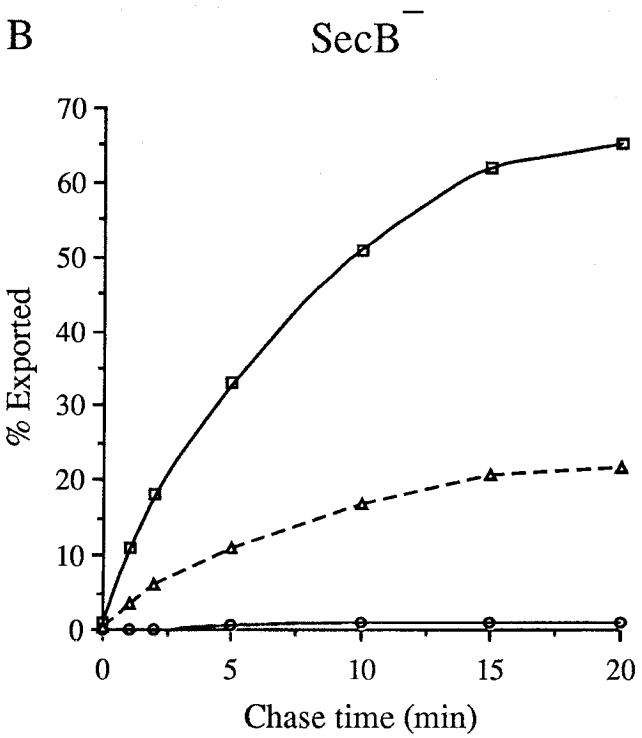

B
SecB−
% Exported
70
60
50
40
30
20
10
0
0
5
10
15
20
Chase time (min)

FIG. 5. **Posttranslational export of the wild-type LamB protein with or without the interfering region.** Glycerol grown cells of SE3001 F'*lac*iQ^{1}Z^{+}Y^{+}A^{+} (Δ*lam*B), containing either plasmid p*lam*Bwt or plamBΔIR, were induced with 20 μm IPTG 2 h prior to labeling. Carbonyl cyanide m-chlorophenylhydrazone was added 1 min prior to labeling, to introduce a translocation block. The samples were then pulse labeled for 1 min and chased by the addition of excess cold methionine. The translocation block was released 1 min later by the addition of β-mercaptoethanol. Control samples were pulse labeled for 1 min with no chase in the absence of carbonyl cyanide m-chlorophenylhydrazone. See Materials and Methods for complete experimental details.

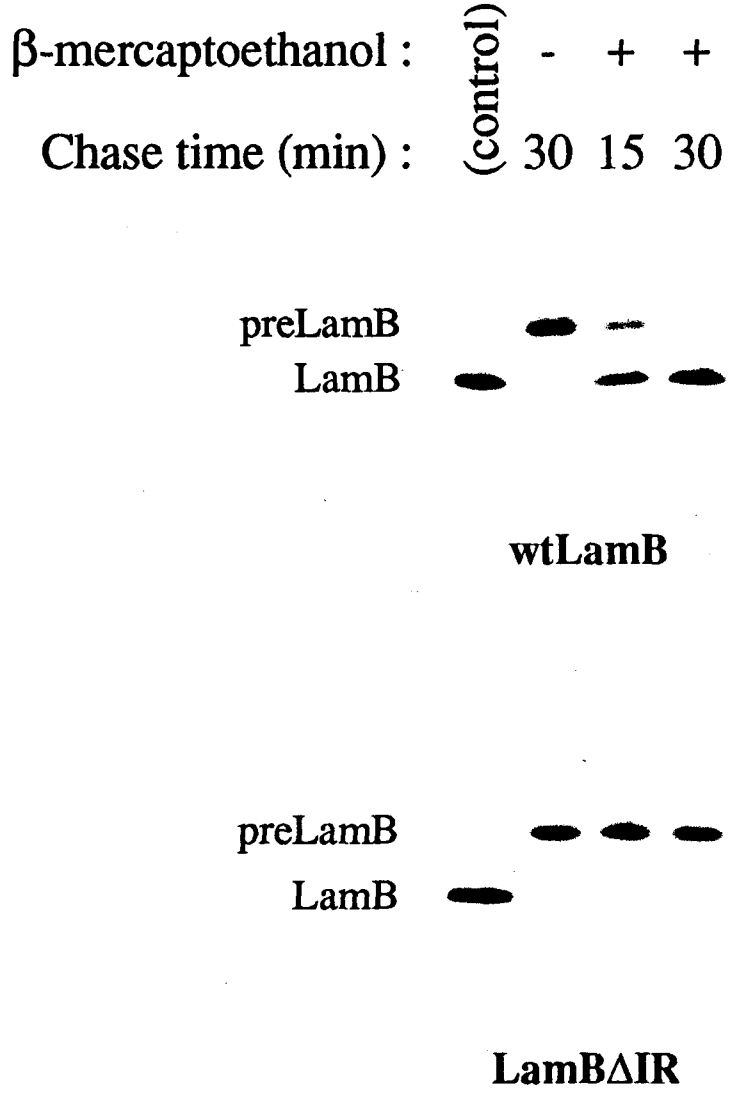
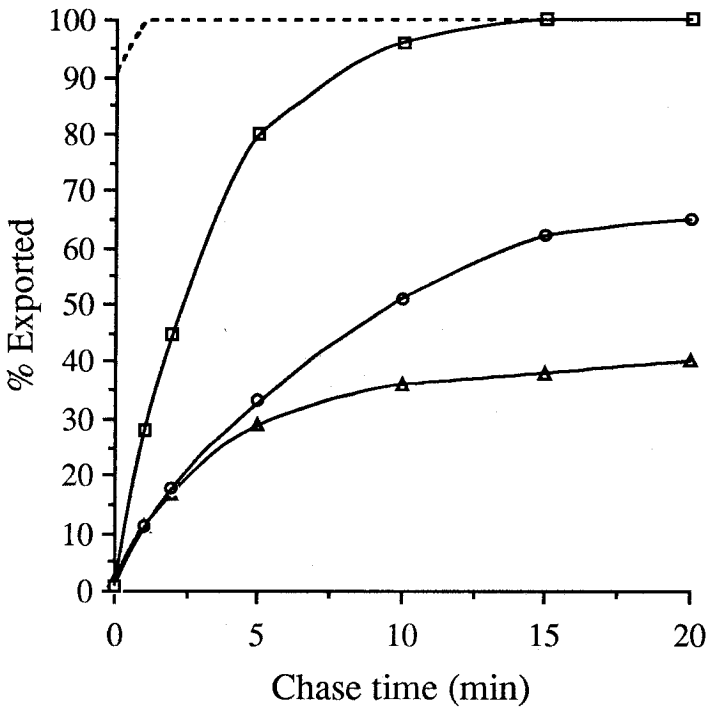

β-mercaptoethanol : - + +
Chase time (min) : (control) 30 15 30
preLamB
LamB
wtLamB
preLamB
LamB
LamBΔIR

FIG. 6. **Export kinetics of LamB, MBP, and OmpA in SecB$^+$ or SecB$^-$ backgrounds.** Maltose grown cells of either MC4100 (*lam*B$^+$, *mal*E$^+$, *omp*A$^+$, *sec*B$^+$) or MC4100 *sec*B::Tn5 (*lam*B$^+$, *mal*E$^+$, *omp*A$^+$, *sec*B$^-$) were pulse labeled for 30s and chased as indicated. LamB, MBP, and OmpA data points in a *sec*B null background are shown as open circles, triangles, and squares, respectively. Since the LamB, MBP, and OmpA data points were almost identical in a *sec*B$^+$ background, a single, averaged export profile for the three proteins in a *sec*B$^+$ background is indicated by a dashed line (the only variance in the data points were at the 0 min chase point, where the percent exported values were 86% for LamB, 97% for MBP, and 90% for OmpA).


100
90
80
70
60
50
40
30
20
10
0
% Exported
0
5
10
15
20
Chase time (min)

Chapter 4

Heat-shock Proteins Can Substitute for SecB Function During Protein Export in *Escherichia coli*

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Summary

In this study we have shown that 1) induction of the heat-shock response can substitute for SecB function in *Escherichia coli*, 2) SecB itself is not a heat-shock protein, and 3) a basal level of heat-shock proteins is required for cells to grow in the absence of a functional SecB protein. Overproduction of DnaK, or GroEL/ES, which were candidates for the heat-shock proteins that could substitute for SecB function, did not rescue the export defect caused when SecB was limiting or absent. In an attempt to identify the heat-shock protein(s) that could substitute for SecB function, unlinked suppressors of *sec*B were isolated and characterized. Interestingly, most of the suppressors mapped to the *rpo*H locus. Since *rpo*H encodes σ^{32}, the heat-shock transcription factor, it is likely that these suppressors affect the synthesis levels of heat-shock proteins which can substitute for SecB function. The remaining suppressors did not map to any known heat-shock or export genes. Collectively, our data suggest that these suppressors may represent unidentified heat-shock proteins or export factors which act in a manner similar to SecB in facilitating the export process in *E. coli*.

On the basis of several observations, it has become apparent that many exported proteins are not translocated across biological membranes in a simple vectorial manner in which translocation is tightly coupled to translation (Zimmerman and Meyer, 1986). Indeed in *Escherichia coli*, it has been demonstrated that most exported proteins are largely translated before the process of translocation is initiated (Randall and Hardy, 1984). In addition, it has been shown that the successful export of periplasmic maltose binding protein (MBP) depends on whether MBP can be translocated before folding into an export-incompetent form (Randall and Hardy, 1986). A simple prediction of these findings is that export factor(s) must be responsible for preventing the exported protein from folding into an nonexportable form. Workers in several labs have shown that SecB, a cytoplasmic component of the *E. coli* export machinery, functions as an antifolding agent which prevents certain exported proteins from folding into export-incompetent forms (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Liu *et al.*, 1989; chapter 3, this thesis).

Several heat-shock proteins have been shown to act in a variety of cellular processes to ensure that proteins are not misfolded before they assume their final functional form (Pelham, 1986). As a result, these heat-shock proteins have been termed chaperones (Ellis, 1987; Ellis and Hemmingsen, 1989; Ellis *et al.*, 1989). Since some heat-shock proteins function as antifolding agents, it is not unreasonable to postulate that heat-shock proteins could play a role in protein export. Indeed it has been convincingly demonstrated in yeast that the hsp70 class of heat-shock proteins are actively involved in the export process (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). More recent work has also implicated the participation of heat-shock proteins in *E. coli* export. Several labs have shown that the *gro*E locus, which encodes two heat-shock proteins, GroEL and GroES, may be involved in the export process in *E. coli*. GroEL has been shown to interact with exported proteins in a manner similar to that of SecB (Lecker *et al.*, 1989; Bochkareva *et al.*, 1988), and both GroEL and GroES are required for the efficient export of at least one secreted protein, β-lactamase (Kusukawa *et al.*, 1989). Additionally, Phillips and Silhavy (1990) have shown

that overproduction of either GroEL or DnaK facilitates the export of LamB-LacZ hybrid proteins in *E. coli*. In this study we present evidence which indicates that heat-shock proteins other than DnaK or GroEL/ES can substitute for SecB function and may act in a manner similar to SecB in facilitating the export process in *E. coli*.

Materials and Methods

Bacterial strains

The parental strains used in this study are listed in Table I. All subsequent strains were constructed either by matings or by transductions with P1 as described in Miller (1972).

Hfr recombinant mapping strains and strategy

Strains EA1001-EA1006 were constructed by transducing D7011, Hfr6, KL99, KL16, KL228, and KL14 with Tn10s from S10, CAG5054, CS819, AE1122, G2, and SY798, respectively. EA 1005 was selected as a *rec*A$^+$ transductant. The *sec*B suppressors that were not linked to *rpo*H were mapped using these strains. Each of the unlinked *sec*B suppressors was mated individually with EA1001-EA1006, and minimal glycerol Tet^r Str^r Kan^r recombinants were isolated. The recombinants were then subsequently scored for the ability to form single colonies on rich, tetracycline kanamycin plates.

Isolation of sec*B suppressors*

Single-colony isolates of CK1953 from a minimal glycerol plate were restreaked on a rich kanamycin plate and incubated at 37°C. One single-colony revertant was picked and

restreaked several times on rich plates to ensure that the *sec*B suppressor could indeed form single colonies on rich media.

Plasmids

pDS2, which encodes σ^{32} under *tac* control, is described in Grossman *et al.* (1987). pMOB45 *dna*K$^+$ (Tilly *et al.*, 1983), and pOF39 (Fayet *et al.*, 1986), are multicopy plasmids, which contain the *dna*K$^+$ and *gro*ES$^+$L$^+$ genes, respectively.

Radiolabeling, immunoprecipitation, polyacrylamide gel electrophoresis, and autoradiography

Pulse-chase experiments and subsequent immunoprecipitations were performed as described in chapter 2. The resulting immunoprecipitates were then resolved by SDS-PAGE and autoradiography as previously described, except that 10% resolving gels were utilized in Figures 1 and 4 (Altman *et al.*, 1985). Quantification of bands on autoradiograms was achieved using a LKB Ultrascan laser densitometer interfaced with an Apple II computer. Percent exported protein was determined as a ratio of mature protein/(mature + precursor protein).

Media

Minimal and rich plates used in this study were prepared as described by Miller (1972). Where appropriate kanamycin, ampicillin, tetracycline, and streptomycin were added at a final concentration of 40 μg/ml, 100 μg/ml, 20 μg/ml, and 125 μg/ml, respectively.

Reagents

Protein A-Sepharose CL-4B, IPTG, and all antibiotics were purchased from Sigma, while ^{35}S-methionine came from Amersham. Anti-LamB, MBP, and OmpA rabbit serum were the generous gifts of Tom Silhavy, Phil Bassford, and Paul Ray, respectively. The preparation of anti-SecB antisera has been previously described (Kumamoto *et al.*, 1989).

Results

Induction of the heat-shock response suppresses the export defect caused by limiting SecB

Successful export in *E. coli* depends on the ability of a protein to be translocated before it folds into an export-incompetent form. At least one component of the *E. coli* export machinery, SecB, acts as an antifolding agent to maintain the exported protein in an export-competent form. When SecB is limited due to the synthesis of export-defective MBP or LamB proteins, the export kinetics of SecB-dependent proteins is impaired and precursors accumulate (Collier *et al.*, 1988; chapter 2, this thesis). Since several heat-shock proteins have been shown to function as antifolding agents, we wanted to test whether induction of the heat-shock response might suppress the export defects caused by limiting SecB.

Regulation of heat-shock proteins is controlled by the transcription factor σ^{32} in *E. coli* (Grossman *et al.*, 1984; Landick *et al.*, 1984), and Grossman *et al.* (1987) have cloned *rpo*H, the gene that encodes σ^{32}, under control of the *tac* promoter. Because the heat-shock response is induced when σ^{32} is overproduced (Grossman *et al.*, 1987), we could easily test whether heat-shock proteins facilitated the export of precursors that accumulated as a result of SecB depletion. Figure 1 shows that when SecB is limited by the synthesis of an export-defective LamB protein, the export kinetics of MBP is drastically affected and MBP precursor accumulates. Surprisingly, however, when the heat-shock response is

concomitantly induced by overproducing σ^{32}, MBP precursor accumulation is completely suppressed (Figure 1). In a reciprocal experiment, the accumulation of LamB precursor that occurred due to the synthesis of an export-defective MBP protein was also suppressed by overproducing σ^{32} (data not shown).

SecB is not a heat-shock protein

The above results could be easily explained if SecB was a heat-shock protein. Induction of the heat-shock response would lead to higher levels of SecB synthesis and thereby overcome the limitation of SecB caused by the expression of export-defective LamB or MBP proteins. To test this possibility, we determined the amount of SecB protein in cells both before and after induction of the heat-shock response. As shown in Figure 2 and consistent with previous findings, induction of the heat-shock response has a pleiotropic effect on the synthesis levels of several cellular proteins. While the synthesis levels of heat-shock proteins are increased, the synthesis levels of nonheat-shock proteins are either unaffected, or are decreased (Lemaux *et al.*, 1978; Yamamori *et al.*, 1978). We find that induction of the heat-shock response causes the synthesis level of SecB protein to decrease. This result indicates that SecB is not a heat-shock protein.

Induction of the heat-shock response can substitute for SecB function

Taken together, our results indicate that one or more heat-shock proteins may be able to substitute for SecB function. To investigate this further, we took advantage of the conditional growth phenotype exhibited by *sec*B null mutants. While a *sec*B null strain grows like wild-type *E. coli* on minimal media, it is unable to form single colonies on rich media (Kumamoto and Beckwith, 1985). This allowed us to develop a plating assay in order to determine quantitatively whether induction of the heat-shock response could

suppress the growth defect exhibited by *sec*B null mutant strains. As shown in Figure 3, when 100 μl of a 10^{-5} dilution from a minimal overnight of *sec*B null mutant cells were plated on minimal media, approximately 5000 colonies were obtained. When the same number of cells were plated on rich media, no growth was seen. However, when 100 μl of a 10^{-5} dilution from a minimal overnight of *sec*B null mutant cells that were overproducing σ^{32}, were plated on either minimal or rich media, similar numbers of colonies were obtained (~2500). This result demonstrates that induction of the heat-shock response completely suppresses the conditional growth phenotype exhibited by a *sec*B null mutant and implies that heat-shock protein(s) are able to substitute functionally for SecB function.

To test directly whether induction of the heat-shock response improved the export defect caused by a *sec*B null mutation, we measured the export kinetics of three SecB-dependent proteins, LamB, MBP, and OmpA. As shown in Figure 4, the export kinetics of all three proteins were markedly improved in a *sec*B null background when the heat-shock response was induced. Interestingly, while the export kinetics of MBP is improved by only 50%, the export kinetics of LamB and OmpA is improved by 100%. It could be argued that the suppression of the *sec*B export defect, seen upon induction of the heat-shock response, is actually an artifact caused by the selective degradation of the cytoplasmic LamB, MBP, and OmpA precursors, by heat-shock proteases, such as Lon (Neidhardt and VanBogelen, 1987). This is not the case, however, because the total levels of these proteins does not change (±5%) when the heat-shock response is induced.

Overproduction of GroEL/ES or DnaK does not substitute for SecB function

We wanted to determine which heat-shock protein(s) in particular could substitute for SecB function. Three obvious candidates to consider were the GroEL, GroES, and DnaK proteins, because they had already been implicated in the export process in *E. coli*. Additionally, DnaK is homologous to the 70K class of heat-shock proteins that had been

shown to participate in the secretory process in yeast. Since the *gro*E and *dna*K genes had been cloned and multicopy plasmids were available, which directed overproduction of the GroEL/ES (Fayet *et al.*, 1986) or DnaK proteins (Tilly *et al.*, 1983), we could easily test whether these proteins could substitute for SecB function. Overproduction of either GroEL/ES or DnaK, however, did not noticeably suppress the lack of SecB protein caused by the synthesis of export-defective MBP or LamB proteins, or the presence of a *sec*B null mutation (data not shown).

secB::Tn5 null suppressors

Because induction of the heat-shock response substituted for SecB function and allowed a strain harboring the *sec*B null mutation to form single colonies on rich media, we reasoned that it might be possible to isolate extragenic suppressors of *sec*B by selecting for *sec*B revertants which could grow on rich media. To test this hypothesis, 25 independent suppressors of the *sec*B::Tn5 null mutation were isolated. We have designated these suppressors *sed*B-1—*sed*B-25, for suppressor of the export defect of SecB. Because a mutation that altered either the σ^{32} protein or its synthesis level could conceivably suppress the conditional phenotype elicited by the *sec*B null lesion, we first screened the suppressors to see if any of them mapped to the *rpo*H locus which codes for the σ^{32} protein. To accomplish this, the suppressors were transduced with a P1 lysate from a strain containing a Tn10 tightly linked to the wild-type *rpo*H gene. Multiple transductants of each suppressor mutant were then scored for the ability to form single colonies on rich media. If the suppressor was due to a mutation at the *rpo*H locus, then some of the transductants would lose the ability to form single colonies on rich media. As anticipated, 20 out of the 25 suppressors were linked to *rpo*H and likely contained a mutation that affected either the synthesis level or the activity of σ^{32}. The remaining five suppressors, however, were not

linked to *rpo*H and represented candidates for mutations that affected either a heat-shock protein or export factor which could compensate for the lack of SecB function.

We used a series of Hfr recombinant crosses to map the five suppressors to two different regions of the *E. coli* chromosome. As shown in Figure 5, Hfr recombinant crosses placed *sed*B-1, *sed*B-3, and *sed*B-14 to between 62-67 min, and *sed*B-7, and *sed*B-22 to between 41-48 min. Although no known heat-shock protein or export factor is known to be encoded by a gene that maps to the region between 41-48 minutes, a heat-shock protein is encoded by the *rpo*D gene, which maps to 66 minutes (Nakamura *et al.*, 1977; Harris *et al.*, 1977). To determine whether *sed*B-1, *sed*B-3, or *sed*B-14 represented mutations that affected *rpo*D, we transduced these three suppressors with a P1 lysate from a strain containing a Tn10 tightly linked to the wild-type *rpo*D gene. No transductants were obtained that had lost the ability to form single colonies on rich media, indicating that neither *sed*B-1, *sed*B-3, or *sed*B-14 mapped to the *rpo*D locus. Our mapping data indicate that these five suppressors may represent mutations that affect as yet unidentified heat-shock proteins or export factors which can substitute for SecB function.

Growth of a secB null strain is dependent on the expression of a basal level of heat-shock proteins

Unlike other export components encoded by the *sec*A and *prl*A/*sec*Y genes, the *sec*B gene is nonessential (Bankaitis *et al.*, 1986). Because induction of the heat-shock response could substitute for SecB function, we wondered if the basal level of heat-shock proteins always present in cells was required for the viability of a *sec*B::Tn5 null strain. The availability of a *rpo*H amber mutation that could be suppressed by a temperature sensitive *sup*C amber suppressor allowed us to test this hypothesis, since even at permissive temperatures the levels of heat-shock proteins in such a temperature sensitive *rpo*H background are lower than that of wild-type (Neidhardt and VanBogelen, 1981; Yamamori

and Yura, 1982). We rationalized that a *sec*B::Tn5 null-*rpo*H amber-temperature sensitive *sup*C triple mutant might actually be inviable, if a basal level of heat-shock proteins was necessary for growth in the absence of a functional SecB protein. For this reason the triple mutant strain was constructed on minimal glycerol media at 16°C, since the *sec*B null mutant grows like wild-type on minimal glycerol, and it had been shown that a *rpo*H null mutation is viable at 16°C (Zhou *et al.*, 1988). As shown in Figure 6, isogenic strains that harbor the temperature sensitive *sup*C mutation and either the *sec*B::Tn5 null or *rpo*H amber mutations are viable on minimal media at 16°C, 23°C, or 30°C. When all three mutations are combined however, the resulting triple mutant is viable only at 16°C. At 23°C the triple mutant grows very poorly and at 30°C the triple mutant is inviable. The synthetic lethality seen when a heat-shock protein defect is combined with a SecB protein defect indicates that the basal level of heat-shock proteins does indeed compensate for the absence of SecB protein in a *sec*B::Tn5 null strain.

Discussion

Exported proteins in *E. coli* must be maintained in an export-competent, unfolded form if they are to be successfully translocated. This is accomplished by export factors, which function as antifolding agents. The best characterized of these antifolding factors is the SecB protein, a 17K cytoplasmic component of the *E. coli* export machinery (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989; Watanabe and Blobel, 1989). SecB is an unusual component of the *E. coli* export apparatus, as the cells' requirement for SecB appears to be different from that of other export components, such as SecA or Pr1A/SecY. First, while the *sec*A and *pr1*A/*sec*Y genes are essential, the *sec*B gene is not (Bankaitis *et al.*, 1986). Although a *sec*B null strain exhibits a severe growth defect on rich media it grows like a *sec*B$^+$ strain on minimal media (Kumamoto and Beckwith, 1985). Second, SecB is necessary only for the efficient export of a subset of the proteins that are dependent on

SecA and Pr1A/SecY. To date, SecB has been shown to be necessary for the efficient export of MBP, LamB, OmpA, and OmpF proteins, but is not required for the export of ribose binding protein (RBP), alkaline phosphatase, β-lactamase, lipoprotein, or M13 coat protein (Kumamoto and Beckwith, 1983, 1985; Collier *et al.*, 1988).

It has been shown that the synthesis of export-defective MBP or LamB protein interferes with the normal export process by limiting the availability of SecB protein (Collier *et al.*, 1988; chapter 2, this thesis). This interference imposes an export block on other SecB-dependent proteins, and precursors of these proteins accumulate inside the cell (Bankaitis and Bassford, 1984). We report here that induction of the heat-shock response completely alleviates the interference caused when SecB is limited by the synthesis of export-defective MBP or LamB proteins, but that SecB itself is not a heat-shock protein. A simple prediction of these results is that induction of the heat-shock response substitutes for SecB function. We find that this is indeed the case. Induction of the heat-shock response completely alleviates the conditional growth phenotype caused by a *sec*B::Tn5 null mutation and markedly improves the export rates of MBP, LamB, and OmpA proteins in a *sec*B null background. On the basis of these observations, we reasoned that the basal level of heat-shock proteins, which are expressed by cells under all growth conditions, may be what permits *sec*B null mutants to survive on minimal media. We found that consistent with this idea a reduction in the basal level of heat-shock proteins was lethal to *sec*B null cells but not to *sec*B$^+$ cells. Because induction of the heat-shock response could substitute for SecB function and a basal level of heat-shock proteins was necessary for cells to survive in the absence of SecB protein, it seemed likely that heat-shock protein(s) might also be involved in the export process and function in a manner similar to SecB.

Two obvious candidates for the heat-shock protein(s) that could substitute for SecB function were the GroEL and GroES proteins, which are encoded by the *gro*E locus, as both of these proteins appear to participate in the *E. coli* export process. GroEL has been shown to act in a manner similar to SecB in maintaining certain exported proteins in

an export-competent form (Lecker *et al.*, 1989; Bochkareva *et al.*, 1988), and when overproduced, GroEL facilitates the export of LamB-LacZ hybrid proteins (Phillips and Silhavy, 1990). GroEL and GroES, however, appear to be required for the export of an even smaller subset of proteins than SecB. To date GroEL and GroES have been shown to be necessary only for the efficient export of β-lactamase and are not necessary for the export of MBP, OmpA, OmpF, alkaline phosphatase, or lipoprotein (Kusukawa *et al.*, 1989). Another candidate to consider was the DnaK protein, because it shares significant homology with the 70K heat-shock proteins that have been shown to facilitate the translocation of certain yeast proteins across the endoplasmic reticulum membrane (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). Also, when overproduced, DnaK facilitates the export of LamB-LacZ hybrid proteins (Phillips and Silhavy, 1990). We found that overproduction of DnaK or GroEL/ES, however, could not substitute for SecB function, either when SecB was limited due to the synthesis of export-defective MBP or LamB proteins, or when SecB was absent due to the presence of a *sec*B::Tn5 null mutation.

In an attempt to identify the heat-shock protein(s) that could substitute for SecB function, extragenic suppressors of a *sec*B null mutation were isolated and characterized. Not unexpectedly, most of these suppressors mapped to the *rpo*H locus, which encodes σ^{32} (Grossman *et al.*, 1984; Landick *et al.*, 1984). Since σ^{32} regulates the heat-shock response, it is likely that these suppressors affect either the synthesis level or the activity of the σ^{32} protein. A few of the suppressors were not linked to *rpo*H, however, and represented mutations that might affect heat-shock protein(s) or other export factor(s), which could substitute for SecB function. Subsequent mapping experiments revealed that none of these suppressors mapped to loci which currently are known to encode either a heat-shock protein or an export factor.

Collectively, the data from studies on the *sec*B and the *gro*E loci suggest that there may be antifolding agents yet to be identified, which facilitate the export of proteins that do not utilize SecB or GroEL/ES. It is possible that the suppressors isolated in this study

represent other antifolding factors that normally facilitate the export of SecB-GroEL/ES-independent proteins, which when mutated or overproduced can substitute for SecB function. Cloning and characterizing the products of these suppressor loci should enable us to discriminate between these possibilities, and may uncover additional components of the *E. coli* export apparatus.

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Table I. **Bacterial strains**

Name	Genotype	Reference or Source
MC4100	F⁻ *ara*D139 Δ*lac*U169 *rps*L150 *thi f1b*B5301 *deo*C7 *pts*F25 *rel*A1	Casadaban, 1976
SE2087	MC4100 *lam*BS87	Emr and Silhavy, 1980
SE9100	MC4100 *rps*L⁺ *rps*D F'*lac*iQ^{1}Z⁺Y⁺A⁺	Altman *et al.*, 1990a
CK1953	MC4100 *sec*B::Tn5	Kumamoto and Beckwith, 1985
SC122	F⁻ *lac*^{am} *trp*^{am} *pho*^{am} *sup*C^{ts} *rps*L *mal*^{am}	Cooper and Ruettinger, 1975
K165	SC122 *htp*R^{am}	Cooper and Ruettinger, 1975
D7011	HfrC *trp*R *thi*	Miller, 1972
Hfr6	Hfr *met*B1 *rel*-1 *mut*-2 *mtl*-8 *mal*-20 λ^{r} λ⁻	Low, 1973
KL99	Hfr *thi*-1 *rel*-1 *lac*-42 λ⁻	Low, 1973
KL16	Hfr *thi*-1 *rel*-1 λ⁻	Low, 1973
KL228	Hfr AB313 *thi*-1 *leu*-6 *sup*-54 *lac*Y1 or *lacZ*4 *gal*-6 λ⁻	Low, 1973

KL14	Hfr *thi*-1 *rel*-1 λ^-	Low, 1973
S10	AP2246 Amp^r *lac*UV5 *rel*A(Su) *rps*E *lys*A29 Δ*cya*-854 *arg*E::Tn10 *ilv*::Tn5 F'*lacZ*^{am}	D. Oliver
CAG5054	KL96 Hfr *thi*-1 *rel*-1 λ^- *trp*::Tn10	C. Gross
CS819	KL16 *omp*C::Tn5 *nal*A::Tn10	C. Schnaitman
AE1122	Hfr *met*B *arg*G6 *rsp*L104 *thy*A *zeb*1::Tn10	D. Oliver
G2	Hfr(KL16) *srl*300::Tn10 *rec*A56 *ilv*318 *thr*300 *thi*1 *rel*A *rps*E300 *rps*L	G. Weinstock
SY798	F^- *zje*::Tn10 *hfl*1 Δ*lac*M445 *lac*I *his arg*E *rps*L *mtl xyl* Δ(*rec*A-*srl*) *pco*lE1, *rec*A^+, *srl*$^+$	D. Oliver
CAG18638	MG1655 *zhg*-3086::Tn10 Tn10 >50% linked to *rpo*H^+	C. Gross
CAG1126	C600 *zgg*::Tn10 Tn10 >50% linked to *rpo*D^+	C. Gross

Fig. 1. Effect that overproducing σ^{32} has on the interference caused by an export-defective LamB protein. Maltose grown cells of MC4100 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ (*lam*B$^+$) and SE2087 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ (*lam*BS87) with or without the plasmid pDS2 (σ^{32} under *tac* control) were fully induced with 1 mM IPTG 15 min prior to pulse labeling with ^{35}S met for 30 s. The cells were then TCA precipitated, immunoprecipitated using MBP antisera, and subjected to SDS-PAGE and autoradiography. Because the synthesis levels of MBP were reduced twofold when the heat-shock response was induced, immunoprecipitation of SE2087 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ pDS2 was performed with twice as many labeled cells as were used for the other two strains. A similar reduction in the synthesis levels of MBP was also seen when MC4100 F'*lac*iQ^{1}Z$^+$Y$^+$A$^+$ cells that contained the plasmid pDS2 (σ^{32} under *tac* control) were fully induced with 1 mm IPTG (data not shown).

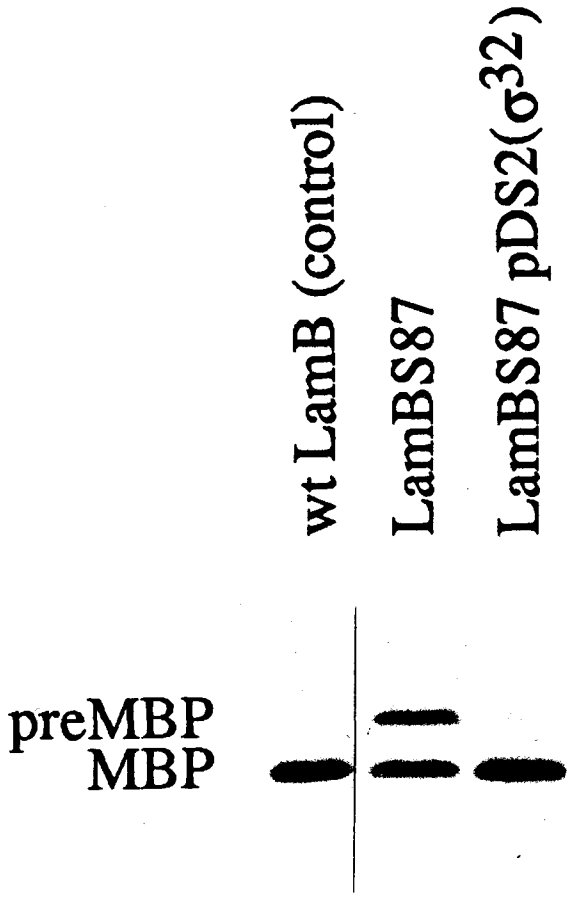

wt LamB (control)
LamBS87
LamBS87 pDS2(σ32)
preMBP
MBP

Fig. 2. Synthesis levels of SecB protein with or without induction of the heat-shock response. Cells of MC4100 F'*lac*iQ^{1}Z^{+}Y^{+}A^{+} (*sec*B^{+}) with or without the plasmid pDS2 (σ^{32} under *tac* control) were grown up in minimal glycerol media. 15 min prior to pulse labeling with ^{35}S met for 30 s the cells containing the plasmid pDS2 were fully induced with 1 mM IPTG. The cultures were then TCA precipitated, immunoprecipitated using SecB antisera, and subjected to SDS-PAGE and autoradiography (Part B). As a control an aliquot of each sample was removed prior to the addition of anti-SecB rabbit serum and analyzed by SDS-PAGE and autoradiography (Part A). Molecular-weight standards (in kilodaltons) are shown to the left of the uninduced whole cell extract in Part A. As expected, overproduction of σ^{32} caused a marked increase in the synthesis levels of known heat-shock proteins, which are indicated in the figure.

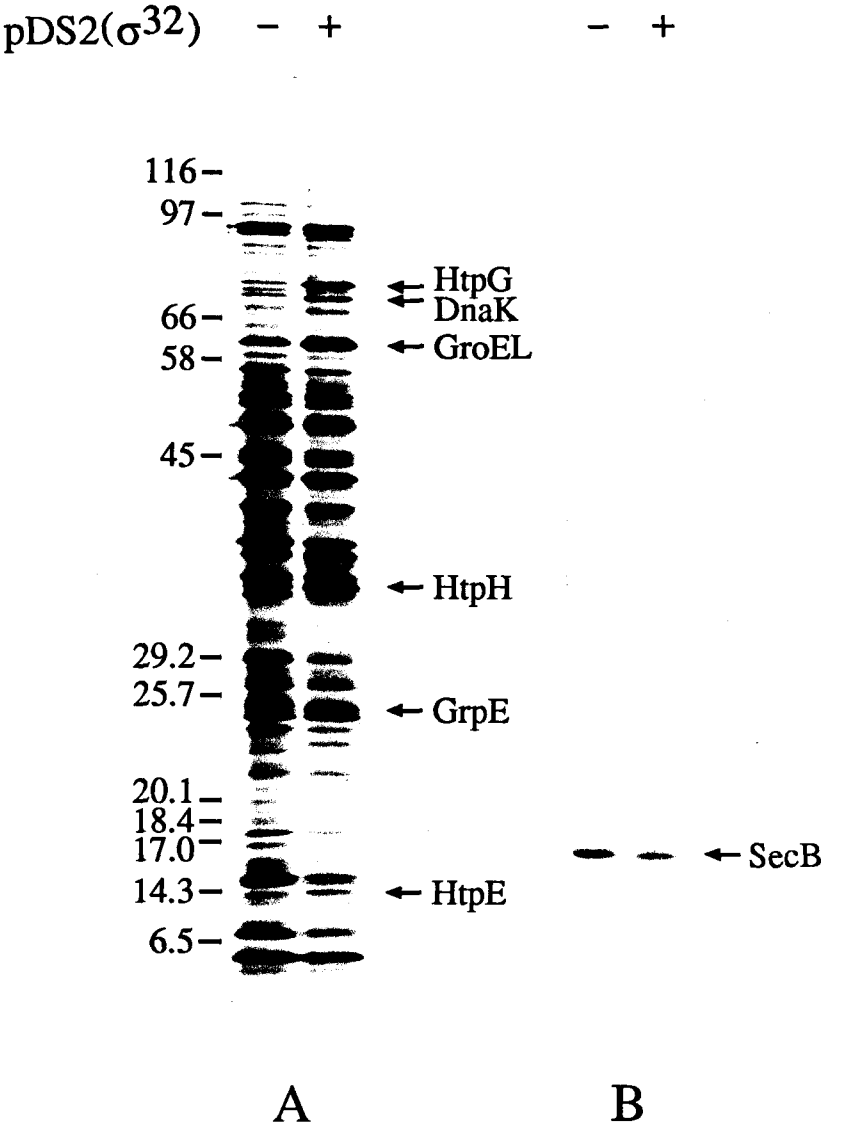

pDS2(σ32) − + − +
116
97
66
58
45
29.2
25.7
20.1
18.4
17.0
14.3
6.5
HtpG
DnaK
GroEL
HtpH
GrpE
HtpE
SecB
A
B

Fig. 3. Growth properties of a *sec*B null strain with or without the plasmid pDS2, which encodes σ^{32} on minimal glycerol versus rich media. 100 µl of a 10^{-5} dilution from minimal glycerol overnights of CK1953 F'*lac*i$Q^1Z^+Y^+A^+$ (*sec*B^-) with or without the plasmid pDS2 (σ^{32} under *tac* control) were plated on both a minimal glycerol and a rich plate and incubated at 37°C. For quantification purposes 100 µl of a 10^{-6} dilution were also plated (data not shown).

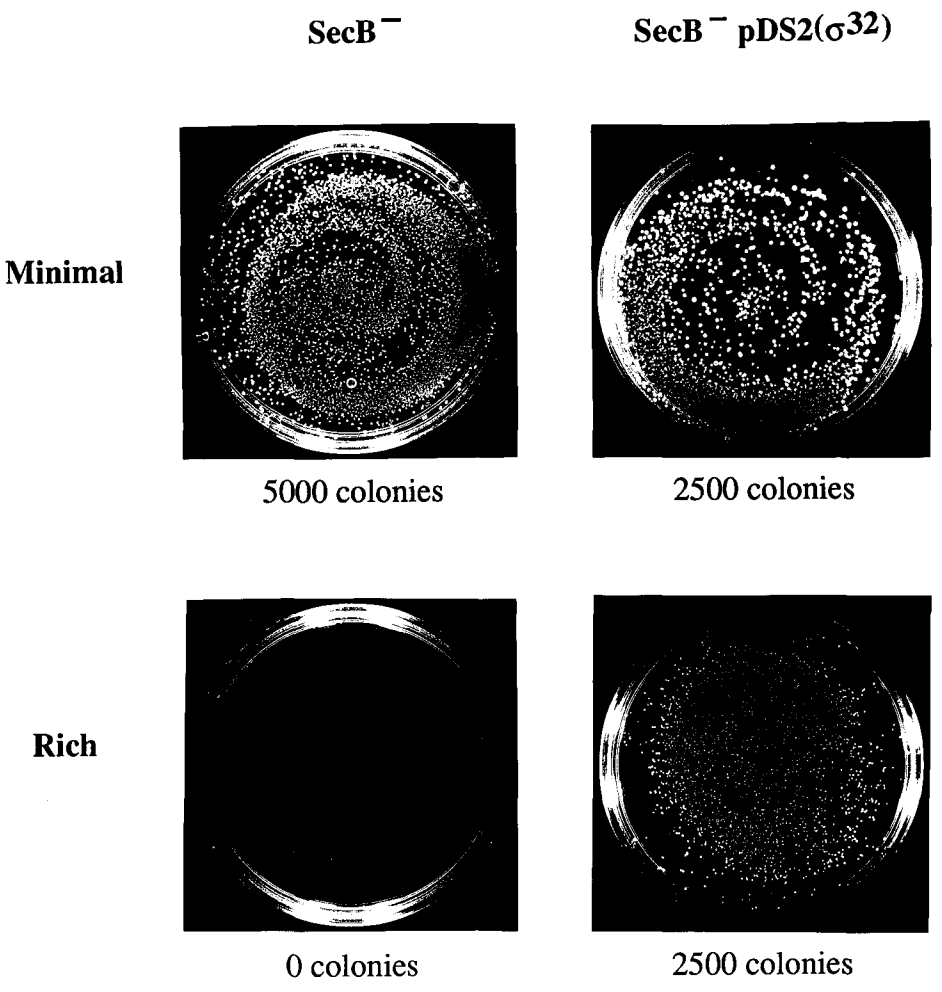

SecB⁻
SecB⁻ pDS2(σ32)
Minimal
5000 colonies
2500 colonies
Rich
0 colonies
2500 colonies

Fig. 4. Effect that overproducing σ^{32} has on the export kinetics of LamB, MBP, and OmpA proteins in a *sec*B null strain. Maltose grown cells of CK1953 F'*lac*i$Q^1Z^+Y^+A^+$ (*sec*B^-) with or without plasmid pDS2 (σ^{32} under *tac* control) were pulse labeled for 30 s, with ^{35}S met and chased as indicated. The cells were then TCA precipitated, immunoprecipitated using either LamB, MBP, or OmpA antisera, and subjected to SDS-PAGE and autoradiography. To quantify the effect that induction of the heat-shock response had on the export rates of LamB, MBP, and OmpA in a *sec*B$^-$ background, chase times were chosen such that a 50% export block was observed in the control samples. The synthesis levels of LamB, MBP, and OmpA protein were within 5% of each other in CK1953F'*lac*i $Q^1Z^+Y^+A^+$ cells whether the plasmid pDS2 (σ^{32} under *tac* control) was present or not.

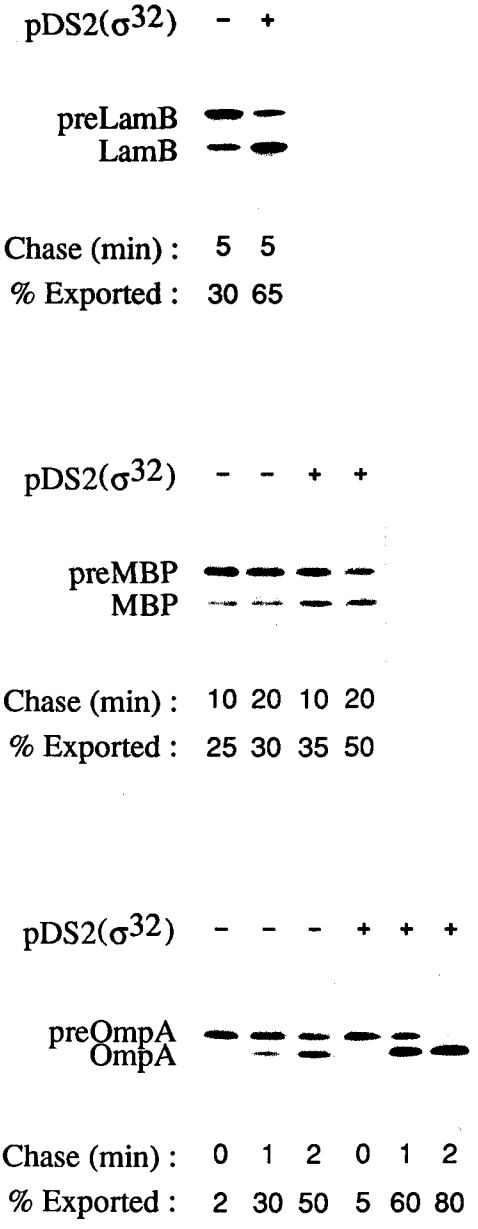

pDS2(σ32) - +
preLamB
LamB
Chase (min) : 5 5
% Exported : 30 65
pDS2(σ32) - - + +
preMBP
MBP
Chase (min) : 10 20 10 20
% Exported : 25 30 35 50
pDS2(σ32) - - - + + +
preOmpA
OmpA
Chase (min) : 0 1 2 0 1 2
% Exported : 2 30 50 5 60 80

Fig. 5. Hfr recombinant mapping of the *sec*B suppressors not linked to *rpo*H. *sed*B-1, *sed*B-3, *sed*B-7, *sed*B-14, and *sed*B-22 were mated with Hfr strains EA1001-EA1006 and recombinants were scored for their ability to form single colonies on rich media. For each Hfr the origin and direction of transfer are indicated by a line with an arrow at the start, while the end point of transfer is shown as a bar at the end of the line. A "+" indicates that the recombinants are still able to form single colonies on rich media, while a "-" denotes that recombinants can be obtained that have lost the ability to form single colonies on rich media. See Materials and Methods for additional information.

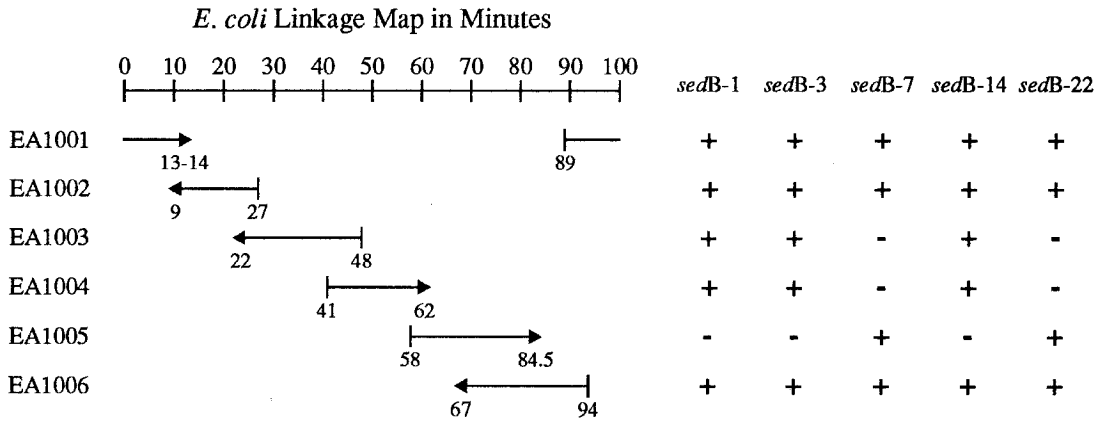

E. coli Linkage Map in Minutes
0 10 20 30 40 50 60 70 80 90 100
*sed*B-1 *sed*B-3 *sed*B-7 *sed*B-14 *sed*B-22
EA1001 13-14 89 + + + + +
EA1002 9 27 + + + + +
EA1003 22 48 + + - + -
EA1004 41 62 + + - + -
EA1005 58 84.5 - - + - +
EA1006 67 94 + + + + +

Fig. 6. Effect that a temperature sensitive σ^{32} mutation has on the growth properties of a *sec*B null strain. Single colonies from minimal glycerol tryptophan plates of either SC122 *sec*B::Tn5 (*sup*C^{ts} *sec*B^{-}), K165 (*sup*C^{ts} *rpo*H^{am}), or K165 *sec*B::Tn5 (*sup*C^{ts} *rpo*H^{am} *sec*B^{-}), were restreaked on minimal glycerol tryptophan plates and incubated at either 16°C, 23°C, or 30°C. Sets of plates at the three different temperatures were incubated until distinct single colonies were visible from the control strain K165. "+++" indicates that a strain grows normally and is able to form single colonies, while "+/-" denotes that a strain grows extremely poorly and is unable to form single colonies. An inviable strain is indicated by a "-". Although *sec*B null strains grow like *sec*B^{+} strains on minimal media at 37°C, *sec*B null strains are mucoidy at 30°C, 23°C, or 16°C. This phenotype is denoted by a "*" in the figure.

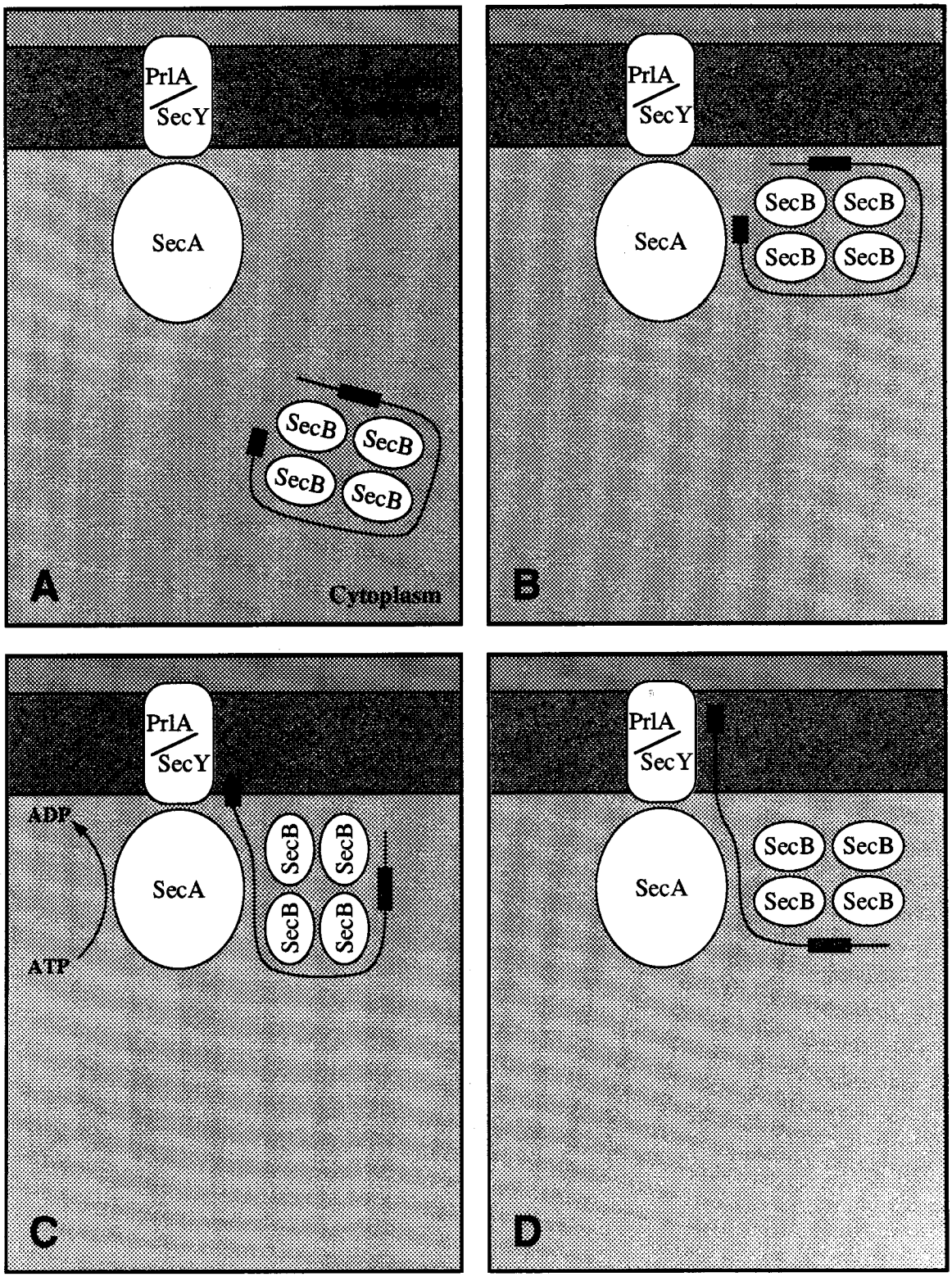
	rpoH^{am} *supC*^{ts}	*sec*B::Tn5 *supC*^{ts}	*sec*B::Tn5 *rpoH*^{am} *supC*^{ts}
16°C	+++	+++*	+++*
23°C	+++	+++*	+/−
30°C	+++	+++*	−

30°C

Appendix A

MODEL FOR THE EXPORT OF SecB-DEPENDENT PROTEINS

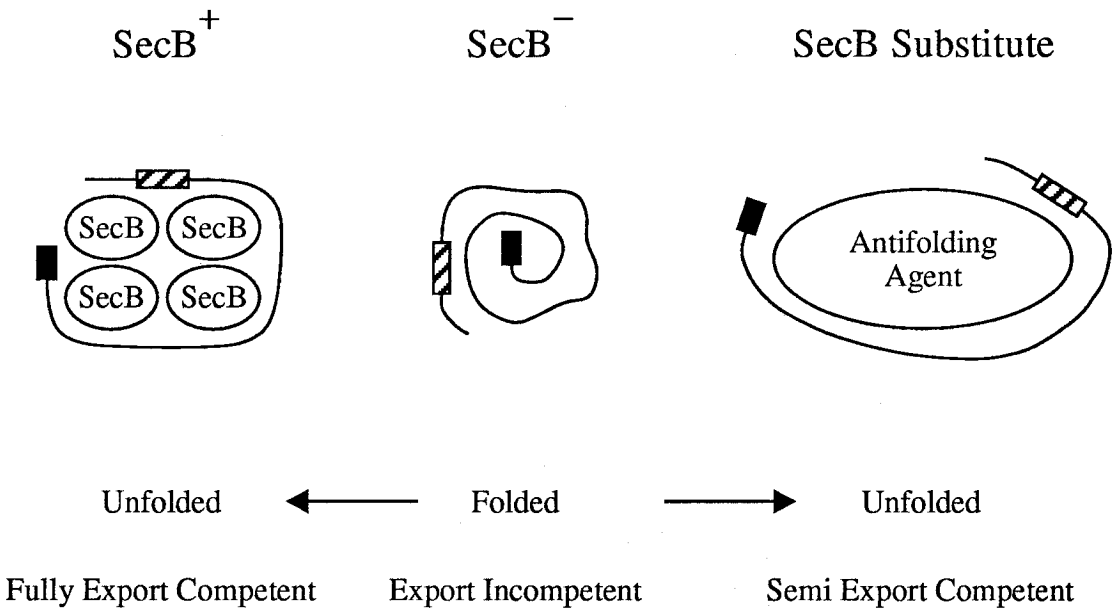
The data presented in Chapters 2 and 3 of this thesis have allowed us to develop a model for how SecB facilitates the export of SecB-dependent proteins. The exported protein is indicated by a solid line, while the signal sequence and interfering region are denoted by a solid and striped box, respectively. In panels A and B the exported protein is recognized by SecB and presented to the rest of the cellular export machinery. In panels C and D the signal sequence is released from SecB and translocated across the membrane by the export machinery. During this process, SecB's association with the interfering region keeps the protein from folding up into an export-incompetent form.


PrlA
SecY
SecA
SecB
SecB
SecB
SecB
A
Cytoplasm
PrlA
SecY
SecA
SecB
SecB
SecB
SecB
B
PrlA
SecY
ADP
ATP
SecA
SecB
SecB
SecB
SecB
C
PrlA
SecY
SecA
SecB
SecB
SecB
SecB
D

Appendix B

OTHER CHAPERONES CAN SUBSTITUTE FUNCTIONALLY FOR THE SecB PROTEIN

The data presented in Chapter 4 of this thesis indicates that other chaperones are able to substitute for SecB during the export process. This three-part figure suggests a mechanism by which this might occur. The exported protein is indicated by a solid line, while the signal sequence and interfering region are denoted by a solid and striped box, respectively. In light of the data presented in Chapters 2 and 3 of this thesis, it is clear that the interaction of SecB with the exported protein is modulated by discrete regions within the protein. It will be interesting to see if this is also the case for the antifolding factor(s) that can substitute for SecB function.


SecB+
SecB−
SecB Substitute
SecB
SecB
SecB
SecB
Antifolding
Agent
Unfolded
Folded
Unfolded
Fully Export Competent
Export Incompetent
Semi Export Competent