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One of three transmembrane stretches is sufficient for the functioning of the SecE protein, a membrane component of the *E.coli* secretion machinery

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The *E.coli secE (prlG)* gene codes for an integral cytoplasmic membrane protein which is part of the cell's secretory machinery. A deletion of nearly the entire gene renders the cell dependent on the presence of a complementing *secE*$^+$ plasmid, indicating that the SecE protein is essential for growth. Deletions which remove carboxy-terminal sequences or substantial amounts near the amino-terminus of SecE can still complement the lethal deletion. This deletion analysis suggests that the essential domain of the SecE protein includes only a single one of its three hydrophobic membrane-spanning segments. Two of three dominant *prlG* signal sequence suppressors map to this segment. Consistent with the insensitivity of SecE to major structural changes, several cold-sensitive mutations cause lethality not because of any change in the protein, but because of a reduction in its level of expression. Our results suggest that higher levels of the protein are needed at the lower temperature. These findings are discussed in terms of the interactions between various components of the secretory machinery.

Key words: Escherichia coli/membrane proteins/secretion/structure-function relationship

Introduction

Genetic and biochemical studies have revealed a cellular machinery required for export of proteins from the cytoplasm of *Escherichia coli*. The genes that code for these components include *secA*, *secB*, *secD*, *secE/prlG*, *secF*, and *prlA/secY* (Emr *et al.*, 1981; Oliver and Beckwith, 1981; Kumamoto and Beckwith, 1983; Shiba *et al.*, 1984; Bankaitis and Bassford, 1985; Gardel *et al.*, 1987 and 1990; Riggs *et al.*, 1988). Mutations in these genes cause defects in the export of a wide variety of periplasmic and outer membrane proteins (Schatz and Beckwith, 1990). Of these six genes, four code for integral proteins of the cytoplasmic membrane (Ito, 1984; Akiyama and Ito, 1985; Schatz *et al.*, 1989; Gardel *et al.*, 1990). The *prlA/secY* gene product spans the membrane 10 times and is required for the functioning of *in vitro* translocation systems derived from *E.coli* membranes (Akiyama and Ito, 1987; Bacallao *et al.*, 1986; Fandl and Tai, 1987). The *secD* and *secF* genes are adjacent on the chromosome and, according to topological models based on alkaline phosphatase (AP) fusions, code for products with six membrane spanning stretches (MSS) and large periplasmic domains (Gardel *et al.*, 1990; K.Johnson and J.Beckwith, in preparation). The *secE/prlG* gene product is also integral to the cytoplasmic membrane (Schatz *et al.*, 1989).

Several lines of evidence suggest that SecE/PrlG and PrlA/SecY interact in the membrane. Both genes give rise not only to alleles that cause secretion defects, but also to suppressors of defective signal sequences, indicating that their products may both interact with signal sequences (Emr *et al.*, 1981; Shiba *et al.*, 1984; Riggs *et al.*, 1988; Stader *et al.*, 1989). The products of these suppressor alleles can be specifically jammed by hybrid proteins with defective signal sequences, leaving the wild-type protein (from a second copy of the gene) free to function (Bieker and Silhavy, 1989 and 1990). An extension of this analysis has shown the ability of a jammed PrlA/SecY complex to titrate SecE/PrlG, suggesting a direct physical association between the two proteins. Another indication for such an interaction comes from the observation of allele-specific synthetic lethality in one of nine combinations of *prlA* and *prlG* alleles (Bieker and Silhavy, 1990). A complex of membrane proteins containing both PrlA/SecY and SecE/PrlG has been detected biochemically and appears to have translocation activity *in vitro* (Brundage *et al.*, 1990). Additional data to support the existence of this complex comes from the observation that the PrlA/SecY protein can be substantially overproduced only in the presence of excess SecE/PrlG (P.C.Tai, personal communication; Matsuyama *et al.*, 1990).

The *secE/prlG* gene is the first gene in an operon; the second gene is *nusG*, which encodes a transcription factor (Downing *et al.*, 1990; Schatz *et al.*, 1989; Stader *et al.*, 1989). The sequence of SecE, a 13.6 kd protein, and the construction of AP (the product of the *phoA* gene) fusions to it suggest that it spans the *E.coli* inner membrane three times with its amino-terminus in the cytoplasm (Schatz *et al.*, 1989). In this paper, we continue our genetic analysis of *secE/prlG*. Sequencing of several cold-sensitive mutations in *secE* indicate that they cause lethality not because of an alteration in the structure of the protein, but rather because of a lowering of its level of expression. A large deletion of the gene is lethal to the cell, showing that SecE is essential for growth. Surprisingly, a small region of the protein, containing only one of the three transmembrane domains, is sufficient for cell survival. A deletion derivative of SecE, missing two of the transmembrane segments, is able to complement the large deletion. This same essential membrane spanning segment is also the location of two out of three *prlG* suppressor mutations. These results suggest that a single transmembrane domain plays a crucial role in the function of SecE/PrlG.

Table I. Strains used in this study

Strain	Genotype	Source or reference
MC4100	*lacΔU169 araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1*	Beckwith collection
KS272	*phoAΔPvuII ΔX74(lac) galE galK rpsL*	Kathy Strauch
MJC98	KS272 *pcnB80 zad1::Tn10*	Mike Carson
MJC224	KS272 *polAts12 zig::Tn10*	Mike Carson
LG3	MC4100 *lamB14D prlG1 zja::Tn10*	(Stader *et al.*, 1989)
SI1	MC4100 *lamB14D prlG2 zja::Tn10*	Steven Isakoff
SI2	MC4100 *lamB14D prlG3 zja::Tn10*	Steven Isakoff
PS8	MC4100 *secEcsE501 recA::cat*	(Schatz *et al.*, 1989)
PS141	MC4100 *argE::Tn10*	(Schatz *et al.*, 1989)
PS163	MC4100 *secEcs15 zijRK498::Tn5 secA::lacf181* (λ PR9)	this study
PS259	KS272 pJS65 *pcnB80 zad1::Tn10*	this study
PS273	KS272 pJS65 *secEΔ19−111 pcnB80 zad1::Tn10*	this study
PS274	KS272 pJS65 *secEΔ19−111 recA::cat pcnB80 zad1::Tn10*	this study
PS279	KS272 pJS65 *argE::Tn10 pcnB80 zad1::(Tn10 tet^s str^r)*	this study
PS284	KS272 pJS51 *secEΔ19−111 recA::cat pcnB80 zad1::Tn10*	this study
PS286	KS272 pJS82 *secEΔ19−111 recA::cat pcnB80 zad1::Tn10*	this study
PS291	KS272 pBRU *secEΔ19−111 pcnB80 zad1::(Tn10 tet^s str^r) recA::cat*	this study
KB35	MC4100 *lamB17D-lacZ42−1 prlG1 zja::Tn10*	(Bieker and Silhavy, 1990)
KB200	MC4100 *lamB17D zja::Tn10*	this study
KB249	MC4100 *lamB17D prlG1 zja::Tn10*	this study
KB182	MC4100 *lamB17D prlG2 zja::Tn10*	this study
KB201	MC4100 *lamB17D prlG3 zja::Tn10*	this study

Results

secE is essential for growth

All of the existing conditional-lethal alleles of *secE* cause cold sensitivity. In order to determine whether *secE* is required for growth at all temperatures, we made a null allele in the gene. Because *secE* is the first gene in an operon containing the essential *nusG* gene (Downing *et al.*, 1990), a simple insertion mutation could perturb the expression of the downstream gene. To avoid such problems, we constructed an in-frame deletion in the 127 codon *secE* gene. The deletion, *secEΔ19−111*, removes the codons for amino acids 19−111, including those for the three predicted transmembrane domains (Schatz *et al.*, 1989), and should destroy the function of the gene completely.

In order to study the essentiality of *secE*, we recombined the *Δ19−111* deletion into the chromosome in the presence of a *secE*$^+$ plasmid, pJS65 (see Materials and methods). We verified the presence of the deletion in the chromosome using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988; see Materials and methods). Further confirmation of the presence of the deletion came from Western blots to detect SecE protein (see below).

If the deletion strain on its own were viable, then growth of strain PS274, carrying the deletion on the chromosome and *secE*$^+$ on the plasmid pJS65, should not be dependent on pJS65 (see Tables I and II for information on strains and plasmids). Plasmid pJS65 carries a fusion (*bla−phoA*) of the gene for AP to the *bla* gene that allows detection of the presence of the plasmid on AP indicator (XP) media. Loss of the plasmid is manifested by the presence of white colonies on such indicator media, while colonies that retain the plasmid form blue colonies. We found that a strain carrying the *secE* deletion on the chromosome was unable to lose the complementing plasmid, while an isogenic strain with an intact chromosomal *secE* locus lost the plasmid at high frequency (see Materials and methods).

We examined the deletion-carrying strain PS274 for its dependence on the complementing plasmid at a variety of temperatures on rich and minimal media. No segregation of the *secE*-carrying plasmid was observed at temperatures from 23°C−42°C on TYE (rich) and M63 (minimal) media. A *bla−phoA* fusion on pJS65 did cause sickness in the strain at 42°C leading to selection for deletion of the fusion gene, but not *secE*. When transformed with the *secE*$^+$ plasmids pJS51 and pBRU, strain PS274 lost pJS65 rapidly, while control transformants with the *secE*$^-$ vectors Bluescript and pBRU did not lose pJS65. The *secEΔ19−111* deletion apparently did not disrupt expression of the downstream *nusG* gene, because plasmids containing only *secE* (pJS65 and pJS51) complemented its lethal phenotype. We conclude that *secE* is essential for growth of *E.coli* under the conditions examined. The lethality caused by titration of SecE/PrlG with fusion proteins is consistent with this conclusion (Bieker and Silhavy, 1990).

Table II. Plasmids used in this study

Plasmid	Genes on plasmid	Source or reference
pJS51	*secE bla*	(Schatz *et al.*, 1989)
pBRU	*secE nusG bla tet*	(Downing *et al.*, 1990)
pJS65	*secE bla::TnphoA (kan)*	this study
pJS67	*secE-phoA-f40 bla*	this study
pJS70	*secE-phoA-f119 bla*	this study
pJS82	*secEΔ7−78 bla*	this study
pJS84	*secEΔ19−111 bla*	this study
pJS85	*secEΔ19−111 nusG bla*	this study
pJS86	*secEcsE501-phoA-f119 bla*	this study
pJS87	*secEcs13-phoA-f119 bla*	this study
pJS88	*secEcs15-phoA-f119 bla*	this study

Late TnphoA insertions in secE complement the deletion

A further series of transformation experiments served to define sequences necessary for *secE* function. We previously

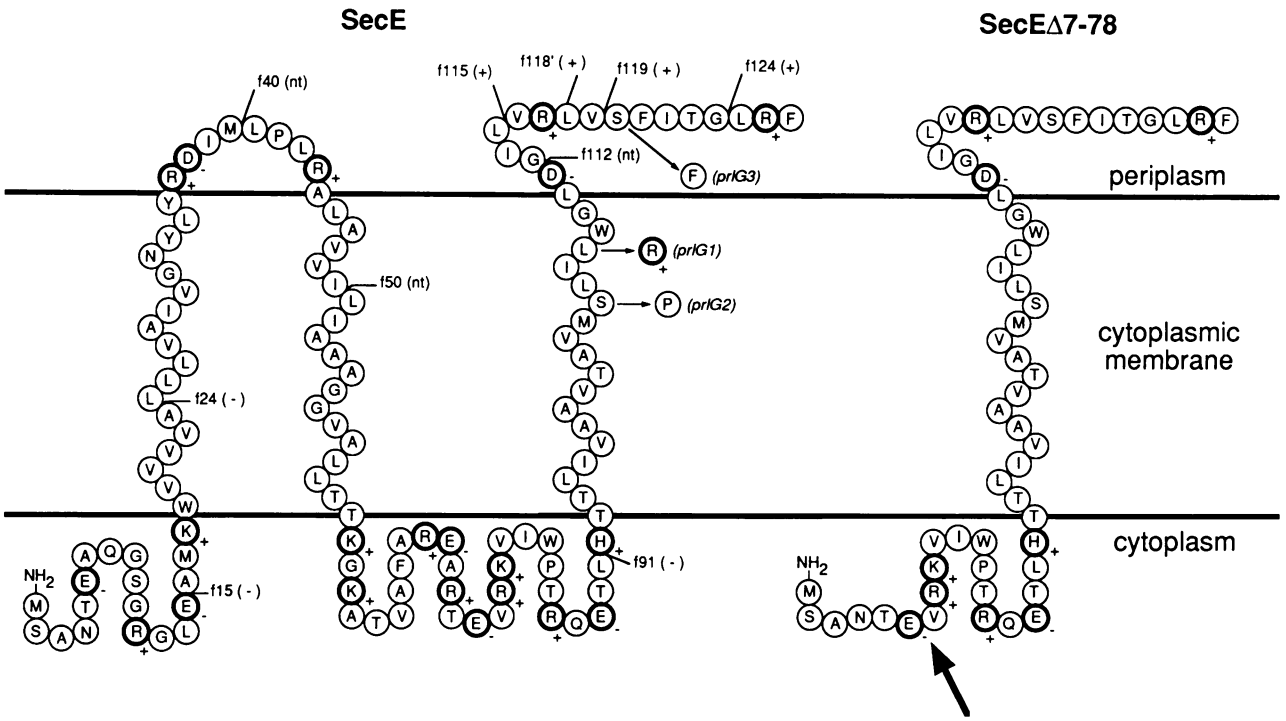

Fig. 1. Sequence and proposed topology of SecE (**left**) and SecEΔ7−78 (**right**) in the *E.coli* inner membrane. The SecE and the SecEΔ7−78 sequences are shown with their amino-termini at the left. The proposed membrane-spanning stretches, shown between the solid lines, are numbered 1−3 from left to right in SecE. Only membrane spanning stretch 3 remains in SecEΔ7−78. The large arrow indicates the site of the deletion in SecEΔ7−78. Dark circles represent charged amino acids, with the charges indicated to the lower right. The SecE−AP fusion joints are shown in SecE. Fusion plasmids failed to complement (−) or complemented (+) the chromosomal *secEΔ19−111* deletion. (nt) Fusion plasmids that yielded very slow-growing transformants of strain PS291. The amino acid changes caused by the *prlG* alleles are indicated with small arrows.

described the isolation of transposon Tn*phoA* insertions in the *secE* gene that disrupt the *secE* coding sequence at various points along its length (Schatz *et al.*, 1989) and generate AP fusions to the SecE protein. Each fusion is numbered according to the amino acid after which the fusion joint occurs along the 127 residue protein (Figure 1). We found previously that the Tn*phoA* insertions late in *secE* (f112, f115, f118′, f119, f124) retained their ability to complement the cold-sensitivity caused by *secEcsE501* (Schatz *et al.*, 1989).

To analyze the functionality of the *secE−phoA* fusions further, we tested plasmids carrying these fusions for their ability to complement the chromosomal *secE* deletion. None of the plasmids carrying fusions early in the *secE* gene, f15, f24 and f91 (all express low levels of AP), were able to replace the function of plasmid pJS65 in strain PS274 when tested as above on XP indicator medium. Most of the remaining plasmids carried fusions which expressed high levels of AP activity, so we could not use XP indicator plates to detect loss of the original pJS65 plasmid. Instead, the plasmids were introduced into strain PS291 which harbors the plasmid, pBRU (*secE*$^+$, tetracycline-resistance, ampicillin-resistance) and *secEΔ19−111* on the chromosome. The ability of the fusion plasmids to express sufficient *secE* activity to maintain cell viability was analyzed by testing for loss of the tetracycline resistance associated with pBRU. Three of the fusion plasmids, f40, f50 and f112, yielded very slow-growing transformants of PS291. Presumably, these SecE−PhoA fusion proteins are deleterious to the recipient cells even though there is an intact *secE* gene. They were not studied further. However, the plasmids carrying the latest insertions (f115, f118′, f119, and f124) yielded healthy transformants of PS291 that rapidly

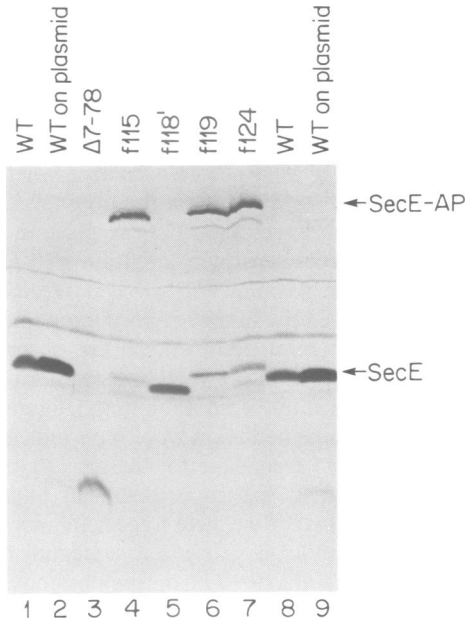

Fig. 2. Western blot of *secE* mutant strains. Protein extracts from several strains were electrophoresed on a SDS−PAGE gel, transferred to nitrocellulose and detected with an anti-SecE antibody. Lanes 1 and 8, MJC98 (wild-type); lanes 2 and 9, PS274 (*secEΔ19−111*, *secE*$^+$ on plasmid); lane 3, PS286 (*secEΔ19−111*, *secEΔ7−78* on plasmid); lanes 4−7 contained extracts from strains with *secEΔ19−111* on the chromosome and f115, f118′, f119 and f124, respectively, on plasmids.

lost the *secE*$^+$ plasmid pBRU. A Western blot (Figure 2) confirmed the loss of intact SecE in the resulting strains,

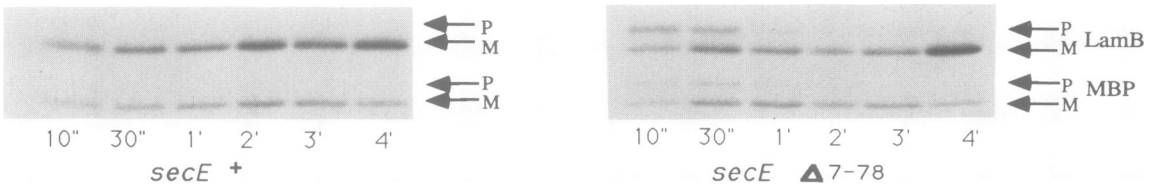

Fig. 3. Kinetics of LamB and MBP signal sequence processing in *secE* deletion strains. *p* designates precursor, *m* designates the processed mature form. The rate of processing is an indication of the efficiency of secretion since the processing takes place after translocation of the precursor across the inner membrane. ^{35}S-methionine pulse-labeling and LamB and MBP co-immunoprecipitation were performed as described in Materials and methods. **Left**: PS284 (*secEΔ19−111* pJS51 with *secE*$^+$). **Right**: PS286 (*secEΔ19−111* pJS82 with *secEΔ7−78*).

and the presence of the fusion gene products. Strains containing the three in-frame fusions (f115, f119, and f124) had SecE-reactive proteins the size of the predicted fusion proteins as well as presumed proteolytic degradation products with gel mobility close to that of intact SecE. It remains unclear which of these species are functional. The f118′ fusion results in a precise deletion of the last nine amino acids of SecE due to the introduction of a nonsense codon. Consistent with this, the f118′ strain produced a protein with slightly greater mobility than that of intact SecE. Thus, plasmids missing as many as 12 of the normal amino acids from the carboxy-terminus of SecE can complement both a *secEcs* mutation and the chromosomal deletion. These 12 amino acids of SecE are not essential for cell viability.

A large deletion in secE supports cell growth

During the oligonucleotide mutagenesis to create the *secEΔ19−111* deletion, another in-frame deletion arose, apparently as the result of recombination between two eight base pair direct repeats within *secE*. The resulting deletion removes the codons for amino acids 7−78 and is called *secEΔ7−78*. The missing sequences include the first two hydrophobic transmembrane domains as well as about half of each of the cytoplasmic domains (see Figure 1). Expecting that this deletion would destroy *secE* function, we used the plasmid containing it as a negative control when testing complementation of *secEcsE501* by the original *Δ19−111* plasmid. To our surprise, the *Δ7−78* plasmid, pJS82, complemented the cold sensitivity of strain PS8 almost as well as the wild-type plasmid pJS51. When introduced into PS274, pJS82 allowed the loss of pJS65, implying that the *secEΔ7−78* allele could complement the chromosomal deletion. The absence of intact SecE in the resulting strain, PS286, was confirmed by Western blotting (Figure 2). The strains used for complementation were *recA* indicating that recombination is not responsible for the growth seen. The surprising conclusion is that more than half of the SecE molecule (including two of the three transmembrane regions) can be removed without destroying its essential function. Strains containing *secEΔ19−111* on the chromosome and *secEΔ7−78* on a plasmid were, however, cold-sensitive at 23°C and 30°C. Since the *secEΔ7−78* allele does complement the cold sensitivity caused by *secEcsE501*, it appears that without some intact *secE* gene product it cannot support cell growth in the cold.

To test further the ability of the *secEΔ7−78* allele to fulfil the function of *secE* in the strain carrying *secE*Δ19−111 on the chromosome, we measured growth rates and the efficiency of secretion. At 37°C, PS286 (with *secE*Δ7−78 on the plasmid) had a doubling time of 49 min in rich (LB) medium and 125 min in minimal (M63 glycerol) medium compared to 46 min in rich medium and 109 min in minimal

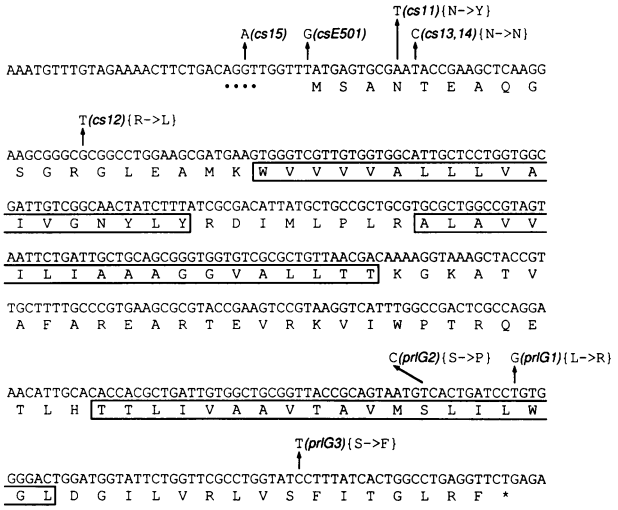

Fig. 4. Sequences of *secE/prlG* mutations. The DNA and corresponding amino acid sequences of the *secE* gene are shown, comprising bases 206−625 as reported previously (Downing *et al.*, 1990). The base changes, allele numbers and amino acid changes are indicated above the sequence. The proposed transmembrane domains of SecE are enclosed in boxes. The consensus ribosome binding site is indicated by dots below the DNA sequence.

medium for PS284 (with wild-type *secE* on the complementing plasmid). For comparison, an isogenic strain with no deletion in the chromosome and no plasmid grew somewhat more quickly than PS284 (37 min doubling time in rich medium and 97 min in minimal medium). While the deletion plasmid did not restore a completely normal growth rate, it did allow nearly normal levels of secretion (Figure 3). Thus, the truncated protein substitutes rather effectively for an intact SecE protein.

Sequences and consequences of secE/prlG alleles

Two types of alleles have been identified in the *secE/prlG* gene. The *secE* alleles confer cold-sensitive lethality and secretion defects (Riggs *et al.*, 1988; Schatz *et al.*, 1989) while the *prlG* alleles are suppressors of signal sequence mutations (Stader *et al.*, 1989). In order to increase our understanding of structure−function relationships in the protein, we have sequenced most of the known mutations. The previously described *secE* alleles include *secEcsE501*, a relatively strong cold-sensitive lethal (Riggs *et al.*, 1988), along with *secEcs11, 12, 13,* and *14,* weak alleles that confer cold sensitivity only in the presence of the tetracycline resistance protein (Schatz *et al.*, 1989). We have isolated an additional cold-sensitive allele, called *secEcs15*, which is the strongest of the group. This allele arose from a localized mutagenesis procedure essentially identical to that described previously (Schatz *et al.*, 1989), except that a

Table III. Expression of *secE-phoA*-f119 from mutant genes

	37°C		23°C		
mutation	*AP/β*-lactamase	mut/WT	AP/β-lactamase	mut/WT	Phenotype
wild-type	12.3[a]	100%[b]	26.2[a]	100%[b]	wild-type
secEcs13	6.9	56%	13.1	50%	weak cs
secEcsE501	5.8	47%	9.6	37%	cs
secEcs15	5.2	42%	7.7	29%	strong cs

[a]ratio of AP activity to β-lactamase activity, arbitrary units. The assays were done on duplicate cultures of MJC98 transformants. In no case did the error exceed 8%.
[b]mutant ratio divided by wild-type ratio

linked Tn5 insertion (encoding kanamycin resistance) was used to eliminate the reliance on the previously used tetracycline resistant Tn10 element. Three of seven previously identified *prlG* suppressor alleles *(prlG1, 2,* and *3)* were also sequenced.

To isolate molecular clones of each mutation, we amplified the *secE/prlG* region of the chromosome using PCR and cloned it in place of the wild-type gene in plasmid pJS51 (see Materials and methods). Sequencing of the resulting clones identified the mutations shown in Figure 4. The changes reported were observed in clones derived from two independent amplification reactions. In three cases, additional base changes occurred in individual clones, presumably due to the high error rate of the *Thermus aquaticus* DNA polymerase (Tindall and Kunkel, 1988). In all of these cases, additional clones from the same reaction did not contain the error.

secE alleles

We were surprised to find that the two strong cold-sensitive *secE* alleles, *secEcsE501* and *secEcs15*, affected sequences 5′ to the AUG initiation codon (Figure 4). Both are changes expected to lower the translation level of the *secE* message. The *secEcsE501* allele is an alteration in the base before the initiator codon from a T to a G; G is an unfavorable base at this position according to consensus rules for translation initiation sites (Stormo *et al.*, 1982). The *secEcs15* allele is an alteration in the consensus ribosome binding site just upstream of the initiation codon. Thus, these alleles must cause cold sensitivity not because of any alteration in the protein structure, but because of lower levels of *secE* expression. The conditional lethal phenotype of the mutants could be due either to cold-sensitive translation of the mutant mRNA and/or to a greater need for SecE at lower temperatures.

All of the weak cold-sensitive alleles are changes early in the protein coding portion of the gene (Figure 4). The *secEcs13* and *secEcs14* mutations, although isolated independently, are due to identical changes in the third base of the fourth codon, substituting one asparagine codon for another. Again, the phenotype caused by the mutation is not due to an alteration in protein structure, but must be due to an effect on the expression level. Since the new codon is not rare, the mutation probably exerts its effect by altering the mRNA structure near the initiation codon, a region of *E.coli* mRNAs known to have nonrandom structure (Stormo *et al.*, 1982). Alternatively, the mutation could affect mRNA stability. The other two weak alleles are base changes early in the gene that do lead to amino acid substitutions. It remains unclear, however, whether the phenotype of each mutation

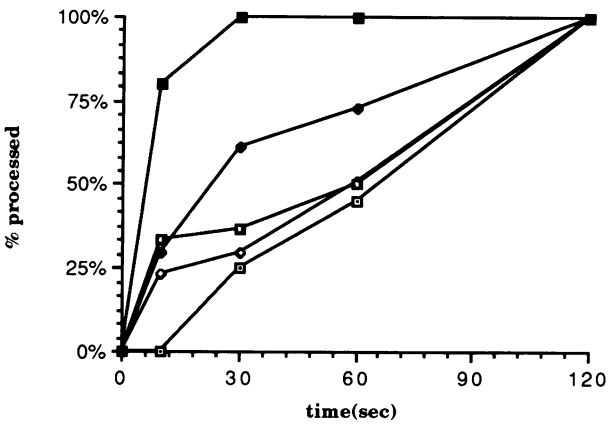

Fig. 5. Processing of the LamB17D signal sequence. Strains were grown at 37°C and pulse-labeled with ^{35}S methionine followed by immunoprecipitation with LamB antiserum. Samples were displayed on a SDS–polyacrylamide gel and the percent of LamB processed to the mature form was quantified by densitometry (Stader *et al.*, 1986). ⊡ KB200 *(prlG⁺, lamB17D);* ◆ KB249 *(prlG1, lamB17D);* □ KB182 *(prlG2, lamB17D);* ◇ KB201 *(prlG3, lamB17D);* ■ MC4100 *(prlG⁺, lamB⁺).*

is due to the base change in the mRNA or to the amino acid change in the protein.

To determine the degree to which the *secEcsE501*, *cs15*, and *cs13* alleles affect expression of *secE*, we cloned the mutations in front of a *secE–phoA* gene fusion (f119) encoding an active AP. Both the mutant derivatives and the parent wild-type fusion were assayed for the levels of AP activity. These levels should mirror the transcription and translation of the *secE* gene to which the *phoA* is fused. To correct for any fluctuation in copy number of the plasmids, the AP assays were normalized to the levels of β-lactamase activity from a *bla* gene on the same plasmid (Table III). The data are also shown in terms of the ratio of normalized AP levels from *secE* mutant fusions compared to expression from the *secE*–wild-type f119 fusion. At both temperatures, the activities correlate with the allele strength. For example, the *secEcs15* allele, which causes the tightest cold-sensitive phenotype, also expressed the lowest level of AP. At the permissive temperature (37°C), the three mutant plasmids expressed 42%–56% of the wild-type level and at the non-permissive temperature (23°C), the level relative to wild-type dropped ~10% further, for each mutant. Thus, a decrease in expression of *secE* to ~40% of wild-type levels is sufficient to cause a cold-sensitive phenotype.

A comparison of the normalized AP activities for the wild-type *secE–phoA* fusion reveals a two-fold increase in *secE* expression at 23°C relative to 37°C. Assuming the *bla* gene on the plasmids is not regulated by temperature, it appears that cells increase expression of *secE* at lower temperature. We also assume that the fusion genes on the plasmids are expressed similarly to the chromosomal *secE* gene. The data also show that the mutant *secE* genes, while expressed at lower levels than wild-type at both temperatures, still exhibit an increase at low temperature. In spite of the elevated levels of SecE relative to 37°C, these mutations cause cold sensitivity. Therefore, our results suggest that there is a greater need for SecE at lower temperature. The regulatory effect which leads to higher expression at 23°C may reflect this need.

The fact that the mutant *secE* genes are expressed at an even lower level relative to wild-type at 23°C than at 37°C

(~10–30%) raises the possibility that there is also a lower translation efficiency of the mutant mRNAs in the cold. In the absence of knowledge of the basis of the temperature-dependent regulatory mechanism and the effects of the mutations on this regulation, we cannot speculate further on this phenomenon. However, this lowered relative expression may contribute to the cold-sensitive phenotype.

prlG alleles

The LamB protein is required for growth on maltodextrins (Dex) and it serves as the receptor for bacteriophage λ. Seven *prlG* alleles of *secE* were identified as suppressors of the signal sequence mutation *lamB14D* (an Asp for Val substitution at position 14 of the signal sequence) by selection for Dex$^+$ and one of these, *prlG1*, has been characterized in some detail (Stader *et al.*, 1989). Using phenotypic tests that monitor the LamB phenotypes it was shown that *prlG1* has an ameliorating effect in most *lamB* signal sequence mutants. However, in no case is pronounced suppression observed. To provide biochemical evidence for suppression, pulse–chase experiments were performed using a series of *lamB* signal sequence mutants. Such experiments follow the time-dependent conversion of the mutant precursor to the mature form of LamB. Since processing occurs at the periplasmic face of the inner membrane, this method provides an indication of the rate of LamB translocation from the cytoplasm. An increase in the rate of processing could be detected with only two *lamB* signal sequence mutations, *lamB17D* (Asp for Gly at position 17) and *lamB17R* (Arg for Gly at position 17). These signal sequence mutations confer only modest export defects; they are quite leaky. In contrast, several *prlA* suppressors such as *prlA4* restore the phenotypes of many *lamB* signal sequence mutants to wild-type, and they cause pronounced increases in the rates of processing in pulse–chase experiments (Stader *et al.*, 1986). Accordingly, we concluded that *prlG1* was a weak suppressor of signal sequence mutations.

The six remaining *prlG* alleles were characterized according to the phenotypic tests described above. Each of these exhibited varying degrees of suppression and allele numbers were assigned according to the strength of suppression observed, *prlG1* > *prlG2* > *prlG3* etc. The alleles *prlG4–6* are very poor suppressors. Pulse–chase experiments with *prlG2* and *prlG3* yielded data similar to that obtained with *prlG1* (Stader *et al.*, 1989); no effect on the rate of processing was observed except with leaky signal sequence mutations. Results obtained with the leaky signal sequence mutation *lamB17D* are shown in Figure 5. Although differences are small, these experiments are consistent with phenotypic tests, *prlG1* is a stronger suppressor than *prlG2* and *prlG3*.

Recently we have described an additional phenotypic test for monitoring the degree of suppression caused by a particular suppressor of signal sequence mutations (Bieker and Silhavy, 1989). This test employs a *lamB–lacZ* fusion strain. Owing to the incompatibility of LacZ sequences with the cellular export machinery, these strains exhibit a characteristic maltose-sensitive (Mal^s) phenotype. Signal sequence mutations in the *lamB* portion of the hybrid gene such as *lamB17D* confer maltose-resistance (Mal^r) because they prevent efficient entry of the hybrid protein into the export pathway. Strong suppressors of signal sequence mutations such as *prlA4* restore a Mal^s phenotype to such strains while weak *prlA* suppressors do not. Despite the fact that *prlG1* behaves as a weak suppressor in LamB phenotypic tests and pulse–chase experiments, it restores Mal^s to a degree comparable to that seen with *prlA4* (Bieker and Silhavy, 1990). Restoration of Mal^s is also observed with strains carrying *prlG2*. In contrast, *prlG3* has no effect on the phenotype of the *lamB17D–lacZ* fusion strain; derivatives containing this suppressor remain Mal^r. Based on these results we suggest that *prlG1* and *prlG2* are more potent suppressors than *prlG3*.

The behavior of *prlG1* and *prlG2* is exceptional. On the basis of LamB phenotypic tests and pulse–chase experiments, they are weak suppressors. Yet, with the *lamB17D–lacZ* fusion strain suppression is quite dramatic. A possible explanation for this exception is provided by pulse–chase experiments that monitor processing of the LamB17D–LacZ hybrid protein. These studies suggest that PrlG1 acts at an early step in the secretion pathway, a step preceding signal sequence processing. Efficient recognition of mutant signal sequences at this step can restore Mal^s. However, subsequent steps in the pathway may still be defective owing to the mutant signal sequence and thus complete translocation would not be substantially increased.

All of the *prlG* alleles are missense mutations late in the coding sequence of the *secE* gene (Figures 1 and 4). Because these alleles are dominant and because changes in the expression of *secE/prlG* do not cause a PrlG phenotype, we can safely assume it is the amino acid substitution in the protein which is responsible for the suppressor phenotype. Both *prlG1* and *prlG2* lead to alterations in the third transmembrane stretch of the protein that should have significant effects on its structure. *prlG1*, the strongest suppressor, changes leucine to arginine, thus introducing a positive charge into the hydrophobic environment of the membrane. *prlG2* changes serine to proline, which may have some effect on the presumed α-helical structure of the transmembrane domain. *prlG3*, the weakest suppressor whose sequence was determined, leads to a serine to phenylalanine substitution in the nonessential, carboxy-terminal, periplasmic domain of the protein.

secE is not regulated according to the secretion needs of the cell

The *E.coli secA* gene is induced by a variety of conditions that block secretion, including the presence of fusion proteins that block the export apparatus and the presence of various *sec* mutations (Oliver and Beckwith, 1982; Riggs *et al.*, 1988; Rollo and Oliver, 1988). In order to determine whether *secE* also is regulated according to the secretion needs of the cell, we examined the expression of two of the *secE–phoA* fusions. Fusions f119 and f40 were recombined onto a λ-*secE*$^+$ transducing phage in order to measure the regulation in lysogenic cells carrying a single copy of the fusions. The transducing phage carries regions upstream of the *secE* gene, including about half of the *tufB* gene, making it likely that all *secE* regulatory sites are present. Downing *et al.*, have shown that transcription of *secE* begins in a region between *tufB* and *secE* (Downing *et al.*, 1990).

We made derivatives of the lysogenic strains which carried cold-sensitive alleles of *secD*, *secE*, and *secY*, as well as temperature-sensitive alleles of *secA* and *secY*. The temperature-sensitive strains were exposed to the non-permissive temperature (42°C) for 2 h and the cold-sensitive

strains were exposed to 23°C for 3 h. Measurements of fusion gene expression in terms of AP activity (data not shown) showed no induction of *secE* in the mutants shifted to the nonpermissive temperature. We also examined the temperature-sensitive mutants by pulse-labeling followed by immunoprecipitation of the fusion protein (data not shown), which also showed a lack of induction of *secE*. We conclude that, unlike *secA*, *secE* is not regulated by the secretion needs of the cell.

We can estimate the levels of expression of the *secE* gene from the amounts of AP activity produced from *secE–phoA* fusions. The *secE–phoA* fusions carried in single copy on a λ prophage make ~50 units (Brickman and Beckwith, 1975) of AP. Based on calculations of the relationship between units of activity and numbers of molecules of AP per cell (M.J.Carson, J.Barondess and J.Beckwith, submitted), we estimate that there are 250–500 copies of the SecE protein in each cell. This calculation may be somewhat of an underestimate if the λ phage carrying the fusions is inserted at the λ attachment site. If it is, it would be present in lower copy number than the wild-type *secE* gene which is located close to the origin of chromosomal replication.

Discussion

We have shown that the *secE* gene is essential for growth of *E.coli*. A strain carrying a large deletion of *secE (Δ19–111)* on the chromosome is dependent on the presence of a complementing *secE*$^+$ plasmid. Loss of the plasmid is apparently lethal to the cell. More recently, we have shown directly that a strain without functional *secE* is defective in secretion. In a strain carrying the *secEΔ19–111* deletion, a temperature-sensitive amber suppressor on the chromosome and a *secE* amber mutation on a plasmid, a strong secretion defect is observed upon shift to high temperature (C.Murphy, E.Brickman, P.J.Schatz and J.Beckwith, unpublished data).

Using this deletion strain, we have investigated the portions of *secE* that are required for its essential function. Late transposon Tn*phoA* insertions in the gene complement the deletion, indicating that the last 12 amino acids of the protein are not essential. More surprising is the ability of an extensive deletion of the gene to complement the chromosomal knockout. This deletion, *secEΔ7–78*, codes for a product missing two of the three proposed transmembrane stretches of the protein and about half of each of the two cytoplasmic domains (Schatz *et al.*, 1989). When present on a plasmid, *secEΔ7–78* suffices for growth and restores secretion competence to a strain with a chromosomal *secEΔ19–111* allele although the strain is cold sensitive and has reduced growth rate at other temperatures. We assume that the transmembrane stretch remaining in the secEΔ7–78 protein has the same topology in the membrane as wild-type SecE (Figure 1).

While a portion of the hydrophilic cytoplasmic domains of SecE remain in SecEΔ7–78, the only obviously intact domain common to it and the complementing *phoA* fusions is the third membrane spanning stretch (MSS). These results raise the possibility that this MSS alone is sufficient for the function of SecE. It is tempting to speculate that this MSS functions via an interaction with one or more MSS of another *sec* gene product. The obvious candidate is *prlA/secY*, given the evidence (see Introduction) that SecE and SecY interact. Support for this possibility comes from the observation that one of nine combinations of *prlG* and *prlA* alleles is lethal to *E.coli*, *prlG1* with *prlA4* (Bieker and Silhavy, 1990). Both of these mutations cause changes in transmembrane stretches, *prlG1* in the third MSS of SecE/PrlG and *prlA4* causes changes in both the seventh and tenth MSS of PrlA/SecY (Sako and Iino, 1988). The change in MSS 10 of PrlA4 is the suppressor (Stader *et al.*, 1986) but it is unknown which change causes the lethality in combination with *prlG1*. The proximity in the membrane of these amino acid substitutions might cause a lethal alteration in the activity of the proposed complex. The simplest (but not the only) interpretation of these data is that MSS three of SecE/PrlG directly contacts MSS seven and/or ten of PrlA/SecY when they form a complex in the membrane. This proposal is testable via mutation and suppressor analysis of this very small region.

The central role of MSS three of SecE is supported by our finding that two of three *prlG* alleles cause changes in this region. These mutations allow increased efficiency of export of proteins with defective signal sequences. Both of these alleles cause changes in the protein that should cause significant structural perturbations. One substitutes a charged lysine and the other introduces a proline into what we presume is an alpha helix crossing the hydrophobic environment of the membrane. Apparently, these changes broaden the specificity of the export apparatus so that proteins with defective signal sequences exit the cell more efficiently. We have recently obtained by oligo-directed mutagenesis a missense mutation in MSS three which renders the SecE protein non-functional (C.Murphy and J.Beckwith, unpublished data).

In our previous searches for *secE*-defective mutants, we did not obtain ones in which a missense mutation appears to have altered protein function. Both of the strong cold-sensitive alleles cause changes in the region upstream of the initiation codon. *secEcs15* is an alteration in the ribosome binding site and *secEcsE501* is a change in the base before the start codon. These mutations affect the phenotype not because of any alteration in the *secE* gene product, but because of a lowering in its level of expression. Most of the weaker alleles probably were detected for a similar reason. Our results show that there is a greater need for SecE at lower temperatures. The failure to find strong missense cold-sensitive alleles among this set is probably due to the relative resistance of the protein to major structural changes. Since more than half of the protein can be completely lost without killing the cell, very few of the possible missense mutations would have a sufficient effect on the activity of the protein to cause conditional lethality. A relatively small decrease in the level of the whole protein, however, does cause severe problems for the cell.

The *secEΔ7–78* allele presents something of a paradox. It complements the cold sensitivity caused by *secEcsE501*, but alone it cannot support cell growth in the cold. One possible interpretation of this result is that more than one SecE molecule is present in each membrane protein complex. According to this interpretation, complexes with both SecE and SecEΔ7–78 proteins or complexes with just SecE would be cold resistant, while complexes containing only SecEΔ7–78 would be cold sensitive. When SecE was present in limiting amounts in the *secEcsE501* mutant, the diploid cell would be able to survive in the cold using the

partially functional *secEΔ7−78* gene product in mixed complexes. Alternatively, the SecEΔ7−78 protein may be proteolytically unstable, and the lowered amounts at 23°C are not sufficient to complement the complete deletion. In any case, the strain with only *secEΔ7−78* is an excellent candidate for reversion analysis, which may be used to identify proteins that can interact with SecEΔ7−78 to restore cold resistance.

From the expression levels of *secE−phoA* fusions, we estimate that there are ~250−500 molecules of SecE protein per cell. Measurements of the level of SecA protein give a similar figure (Oliver and Beckwith, 1982). However, the *secA* gene can be derepressed 10−20-fold when secretion is blocked by various means (Rollo and Oliver, 1988), while *secE* cannot. These findings suggest that SecA may not form a stoichiometric complex with membrane components of the secretory machinery. Alternatively, high levels of SecA may help maintain precursors in a secretion-competent form when the cell's secretion mechanism is inhibited.

It remains to be determined whether the complex of Sec membrane proteins forms a pore in the inner membrane through which exported proteins pass, or if it assists the export process, by some other mechanism. In any case, once all components of this complex have been identified, it should be possible to engineer alterations in both the specificity of the pore and its capacity by making modifications in the structure and amounts of these components. Such alterations could have practical benefits for the production of secreted proteins in *E.coli* and would also enhance our understanding of the mechanism by which proteins pass through lipid bilayers.

Materials and methods

Media and bacterial strains

Media for bacterial and phage growth have been described (Riggs *et al.*, 1988). The *E.coli* strains used are shown in Table I.

Plasmid constructions

The plasmids used are summarized in Table II. The plasmids pBRU and pJS51 have been described (Downing *et al.*, 1990; Schatz *et al.*, 1989). Plasmids pJS82 and pJS84 were made by mutagenesis of pJS51 with the oligonucleotide GCGGCCTGGAAGCGATGAAG'GATGGTATTCT-GGTTCGCCT (provided by Annick Jacq) and their structure was confirmed by sequencing. The *secEΔ19−111* deletion was transferred from pJS84 to pBRU by subcloning to make pJS85.

Plasmids pJS70 and pJS67 were made by deleting the transposase and Kan^r functions from the original pJS51::Tn*phoA* plasmids by cleavage with *Xho*I and reclosure with ligase. The plasmids pJS86, pJS87, and pJS88 were made by subcloning *Pst*I to *Kpn*I fragments containing the *secE* mutations in place of the wild-type *secE* region of pJS70.

Phage strains

λ-PS6 and λ-PS8 are cI^+ derivatives of λ YU112 (*cI857 int2 secE::kan*, provided by Susan Sullivan) carrying the *secE−phoA* fusions f119 and f40 respectively. To construct them, plasmids pJS70 and pJS67 were first introduced into strain MJC98. Lysates of λ AJ2 (a cI^+ derivative of λ YU112 made by Annick Jacq) were grown on these strains and examined on XP plates for the presence of phage that had acquired the fusion gene and lost the *kan* insertion by recombination. The presence of the fusion gene was confirmed by Western blotting of phage lysogens. Although the phages from which λ PS6 and PS8 were derived carried an int^- mutation, subsequent crosses may have replaced it with an int^+ gene. We have not tested this possibility and, therefore, cannot infer whether lysogens are at the attachment site or integrate by homology.

Construction of the chromosomal deletion of secE

Deletion *secEΔ19−111* was recombined onto the chromosome using plasmid pJS85, a derivative of pBRU containing the deletion. Strain MJC224 carries the *polAts12* allele, which renders temperature-sensitive the replication of plasmids with a ColE1-derived origin (Gutterson and Koshland, 1983). In order to force recombination with the chromosome, MJC224 was transformed with pJS85 at 30°C followed by selection for ampicillin resistance at 42°C. The plasmid was maintained at 42°C through integration into the chromosome by homologous recombination with the *secE* locus, the only region with homology to the plasmid. This integration event produced a duplication of the *secE* operon, with one copy of intact *secE* and one deleted copy.

The site of plasmid integration was confirmed by showing linkage, in a P1 transduction, between the ampicillin resistance determinant on the plasmid and an *argE::Tn10* mutation linked to the *secE* locus. The recipient in this cross was a $polA^+$ strain (PS141), allowing the reactivation of the plasmid origin. After several rounds of purification on high concentrations of ampicillin (200 μg/ml), all of 24 independent transductants examined contained free plasmid, presumably because of the selective disadvantage of having an active plasmid replication origin in the chromosome and the selective advantage of a higher copy number ampicillin resistance *(bla)* gene. Ten of these strains contained plasmids with an intact copy of *secE*. Assuming plasmid excision occurred by a reciprocal recombination event, the deletion of *secE* should have remained in the chromosome in these strains. In subsequent crosses, the deletion was transduced linked to $argE^+$.

An isogenic pair of strains was constructed carrying a $secE^+$ plasmid and either a wild-type *secE* gene or the presumed deletion on the chromosome. In order to easily detect bacteria which had lost the plasmid, we used a $secE^+$ plasmid, pJS65, with a transposon Tn*phoA* insertion in the *bla* gene, derived from plasmid pJS51. The maintenance of pJS65 in a strain could be easily observed by detection of the AP activity produced from the *bla−phoA* fusion using the indicator XP. The strains also contain the *pcnB80* allele, which causes a marked decrease in plasmid copy number and stability (Lopilato *et al.*, 1986). In the parent $secE^+$ strain, PS279, without selection for the kanamycin resistance marker, the plasmid was lost from the strain at very high frequency. On a streak on XP indicator media, 10−30% of the colonies of PS279 were white indicating absence of the plasmid. Derivatives of PS279 which carried the *secEΔ19−111* deletion on the chromosome failed to exhibit any segregation of the plasmid on XP plates. Presumably, those bacteria which had lost the plasmid could not survive and did not form colonies.

The deletion of *secE* was also introduced into the chromosome by a simpler two-step procedure. Integrated pJS85 was transduced from the MJC224 background into strain PS259 (*pcnB80* pJS65), followed by screening for loss of the ampicillin resistance associated with pJS85. In one of the resulting strains (PS273), the pJS65 plasmid appeared unable to segregate, presumably because of the presence of the deletion in the chromosome. To prevent recombination regenerating the wild-type chromosomal allele, a *recA* null mutation was introduced into PS273 to make strain PS274.

Sequencing and PCR

DNA sequence analysis of plasmid DNA was performed using Sequenase (US Biochemicals) as described previously (Schatz *et al.*, 1989). The primers used were secE#1 [GTTGAGGGCGTATAATCCG: corresponding to bases 137−156 in (Downing *et al.*, 1990)] and secE#2 (GCCGTAGTAATTC-TGATTGC: corresponding to bases 378−397). PCR (Saiki *et al.*, 1988) was carried out with the GeneAmp kit (Perkin Elmer Cetus) using primers secE#1 and secE#3 (CACGGATTTCAACCACTTC: the reverse complement of bases 766−784). To clone *secE* mutations, amplified DNA was digested with *Mlu*I and *Kpn*I and ligated in place of the wild-type gene on pJS51. These primers were also used to demonstrate the presence of the *secEΔ19−111* allele in the chromosome. Because the sequence corresponding to primer secE#3 is not present in plasmid pJS51 and its derivatives, only the chromosomal DNA was amplified. Compared to control strains, the amplified fragment was 269 bp smaller in deletion carrying strain PS274 (data not shown).

Assays

AP activity was measured by determining the rate of *p*-nitrophenyl phosphate hydrolysis in permeabilized cells normalized to the A_{600} of the cell suspension (Michaelis *et al.*, 1983). β-lactamase activity was measured by following the decrease in A_{240} due to hydrolysis of penicillin G by a cell lysate (Ross and O'Callaghan, 1975).

Anti-SecE antibodies, Western blots and labeling

A peptide corresponding to amino acids 64−92 of the SecE sequence was generously prepared by Dr Viren Sarin (Abbott Labs). The peptide was coupled to Keyhole limpet hemocyanin via an amino-terminal cysteine using the coupling reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Liu *et al.*, 1979). Two rabbits (#891 and 892) were injected with 1.5 mg each of the conjugate homogenized in Freund's complete adjuvant and boosted

several times with conjugate in Freund's incomplete adjuvant. The rabbits were bled once per month and the serum was stored in frozen aliquots. The anti-SecE antibodies were purified on a column made from the peptide coupled to activated thiol-Sepharose 4B (Pharmacia) according to the directions supplied by the manufacturer. The crude serum was loaded onto the column in 0.1 M Tris pH 7.5, 1 mM EDTA, 0.5 M NaCl, washed with the same buffer and eluted with 0.2 M HCl adjusted to pH 2.2 with 2 M glycine and rapidly neutralized with 1 M K_2HPO_4. Western blots were done with the Protoblot Western blot AP system (Promega) essentially according to the instructions provided by the manufacturer. Purified anti-SecE 891-bleed 4 was used at a 1:400 dilution. Pulse-labeling, immunoprecipitations and quantification were as described (Stader *et al.*, 1986) except that cultures were grown and labeled at 37°C

All other methods were as previously described (Schatz *et al.*, 1989).

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