그람 양성균인 *Bacillus subtilis*와 그람 음성균인 *Escherichia coli*에서 Protein secretion에 중요 역할을 하는 SecY에 대한 비교 연구

(A Comparative Study of the Major Component of the Protein Secretion Machinery, secY, in Gram Positive *Bacillus subtilis* and Gram Negative *Escherichia coli*.)

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INTRODUCTION

Although protein translocation in eukaryotes seems more complex than in prokaryotes because of posttranslational modifications of secreted proteins and diverse subcellular compartments, genetic and biochemical studies have shown several common features in protein translocation across the eukaryotic endoplasmic reticulum and the bacterial cytoplasmic membrane (Saier *et al.*, 1989). The common features include the structurally and functionally conserved signal sequences, which are interchangeable in the two different systems, an energy requirement as a form of ATP, the existence of a functionally similar machinery comprising cytoplasmic and membrane components, and the frequent coupling of protein synthesis to protein translocation (reviewed in Oliver, 1985; Saier *et al.*, 1989). Thus studies on the prokaryotic secretory machinery can serve as a model system to understand the mechanism of the protein translocation process, for which four basic models have been proposed: the signal hypothesis, the membrane trigger hypothesis, the direct transfer model, and the loop model or helical hairpin hypothesis (reviewed in Oliver, 1985).

The secretion system of the gram positive microorganism *Bacillus subtilis* has been a matter of interest because of its practical use. *B. subtilis* secretory proteins have longer signal peptides (40 vs 20 residues) than exported proteins of gram negative bacteria or higher eukaryotes (Sarras, 1986), and some soluble factors thought to be involved in the protein secretory process have been isolated (see Saier *et al.*, 1989 for a review). However, nothing is known about the secretory machinery in *B. subtilis* and most of our knowledge of the protein translocation machinery in prokaryotes comes from study of *E. coli*.

Recent genetic and biochemical analysis in *E. coli* have revealed several key components in the protein translocation machinery (reviewed in Oliver, 1985; Saier *et al.*, 1989). The *secY (prlA)* gene product has a central role in that process. The *prlA* (*prl* for protein localization) allele of *secY* (*sec* denotes secretion defective) was identified by selection for suppressors of export-defective LamB proteins with various mutations in the hydrophobic core of the signal sequence (Emr *et al.*, 1981). The *prlA* mutations can supress signal sequence mutations of *lamB*, *malE*, *phoA*, and *ompF*, suggesting that *secY* /PrlA directly interacts with the

signal peptide during secretion (Emr *et al.*, 1982; Shultz *et al.*, 1982), in a gene originally designated *secY* (Ito *et al.*, 1983; Shultz *et al.*, 1982). The complete nucleotide sequence was determined by Cerretti *et al.* (1983). The *secY* gene encodes an integral membrane protein of 49 kilodalton (Cerretti *et al.*, 1983; Ito, 1984; Akiyama and Ito, 1987). *secY* is essential for growth and the temperature-sensitive *secY24* mutation causes the accumulation of precursor forms of exported proteins under non-permissive temperature (Ito *et al.*, 1984; Shiba *et al.*, 1984). Bieker and Silhavy (1989) have presented evidence that the *secY* protein is the cellular component that is rate-limiting for protein export. Jamming of the protein export apparatus using LamB-LacZ hybrid protein with defective signal sequences and experiments using a dominant *secY* supressor *(prlA4)* also suggested that *secY* is the component of the translocation apparatus through which proteins cross the membrane (Bieker and Silhavy, 1989).

Recent biochemical analysis also have shown that *secY* is an essential component of the translocation machinery. Fandl and Tai(1987) showed that the temperature-sensitive *secY24* gene product interferes with protein translocation into inverted *E. coli* membrane vesicles *in vitro*. However, this translocation defect of *secY24* was supressed by adding purified SecA protein to the *in vitro* system (Fandl *et al.*, 1988), indicating that the SecA interacts, directly or indirectly, with *secY*. The gene for SecA has been sequenced, and temperature sensitvie *secA* mutation show that SecA is also essencial for translocation (Oliver and Beckwith, 1981; Schmidt *et al.*, 1988). The genetic evidence for the involvement of SecA in protein translocation *in vivo* have been supported by *in vitro* translocation experiments with inverted membrane vesicles (Cabelli *et al.*, 1988).

The topology of the integral membrane protein *secY* has been deduced from the hydropathic character of its amino acid sequence, susceptibility to proteases, and *secY-phoA* (alkaline phosphatase) fusion data (Akiyama and Ito, 1987). Akiyama and Ito suggested that *secY* contains ten transmembrane segments and eleven hydrophilic domains, five exposed to the periplasm and six exposed to the cytoplasm, including the NH2- and COOH-termini regions. Watanable and Blobel (1989) also showed that the NH2- and COOH- temini of *secY* are exposed to the cytoplasm by using antibodies raised against the hydrophilic NH2- or COOH-terminal regions of *secY*. Watanabe and Blobel further proposed these NH2- and COOH- termini act as membrane-integrated signal sequence receptors.

A phylogenetic comparison of homologous protein can often supplement genetic and biochemical analysis by revelaing conserved structures that are critical for function (Waugh *et al.*, 1989). I therefore isolated a *secY* homologue from *B. subtilis*, a gram positive bacterium evolutionary distant from *E. coli*. The comparison and interplay between these two bacterial systems should contribute greatly to our understanding of the functions and interactions within systems evolved for protein translocation in both prokaryotic and eukaryotic organisms.

METHODS AND MATERIALS

Bacteria, phage, and plasmids

Plasmid pKY6, carrying *E. colli secY*, and the isogenic derevatives of *E. coli* strain MC4100, strains IQ85 (*secY24*) and IQ86 (*secY+*), were described by Shiba *et al.*, (1984). *E. coli* Y1090 was host for λgt11 (Young and Davis, 1983), which was grown as described by Davis *et al.* (1980). *E. coli* DH5α (Hanahan, 1985) was host for plasmid construction with pKK223-3 (Pharmacia-PL), pUC18, and pUC19 (Yanisch-Perron *et al.*, 1985). pST120 was made by moving the 1801 bp *Xmn*I-*Xmn*I fragment (Fig. 1) containing *B. subtilis secY* into the *Hind*III site of the expression vector pKK 223-3 (Pharmacia-PL). The *Xmn*I ends of the fragment were joined to *Hind*III linkers before ligation into pKK223-3. To make pST121, pST120 was cut at the unique *Eco*RV site within *secY* and inserted the 4.8 kb *Sma*I-*Stu*I fragment from pJF751 (Ferrari *et al.*, 1985), thus joining the thirteenth codon

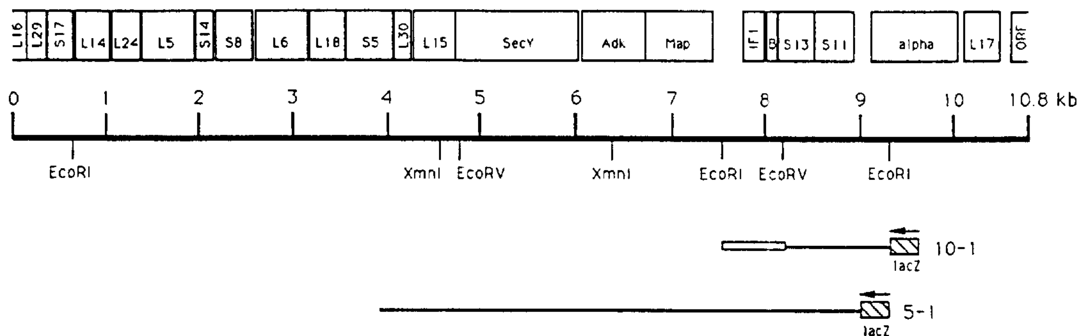

Fig. 1. Physical map of the *B. subtilis spc*-alpha region, derived from the DNA sequence. The sequence from 7.6 to 10.8 kb was reported previously (SUH *et al.*, 1986, Boylan *et al.*, 1989) and the remaining sequence will be published elsewhere (J.-W. Suh *et al.*, in preparation). Coding regions are represented by boxes labelled with the gene product and the direction of transcription is from left to right. The recombinant phages used in this study are shown beneath the map. λgt11 phage 10-1 was isolated previously (Boylan *et al.*, 1989) and the isolation of phage 5-1 is reported here. The *lacZ* gene (hatched boxes with arrows for the direction of transcription) indicates the orientation of the cloned *B. subtilis* inserts with respect to the right arm of λgt11 (Young and Davis, 1983). The insert of phage 5-1 is bounded by *Eco*RI linkers used during library construction whereas the insert of phage 10-1 is bounded by *Eco*RI sites from the *B. subtilis* genome. The 0.65 kb *Eco*RI-*Eco*RV probe used to isolate phage 5-1 is indicated by the open box on the left end of the phage 10-1 insert. This map shows only those *Eco*RV and *Xmn*I sites used to make the hybridization probe or plasmid constructions.

of *secY* to the eighth codon of *lacZ*. The in-frame fusion was confirmed by DNA sequencing.

DNA methods

Isolation of chromosomal, plasmid, and phage DNA, restriction endonuclease digestion, ligation, transformation, gel electrophoresis and Southern blotting were as previously described (Boylan *et al.*, 1989). Hybridization screening of the λgt11 libraries (Suh *et al.*, 1986) was done as described by Davis *et al.* (1980). For DNA sequencing by the dideoxynucleotide chaintermination method of Sanger *et al.* (1977), appropriate restriction fragments were first cloned into pUC18 and pUC19. I then made sets of nested deletions as previously described (Boylan *et al.*, 1989) and used sequenase (US Biochemicals Corp.) to label reactions primed on double-stranded templates with [α-^{35}S]-dATP (Amersham). Reaction conditions were those described by the manufacturer.

Computer analysis

Restriction analysis and translation of DNA sequence into amino acid sequence were done using the program of Pustell, version 4.1 (Pustell and Kafatos, 1984). The statistical significance of protein sequence comparisons was evaluated using the FASTP and RDF programs (Lipman and Pearson, 1985) on the National Biomedical Research Foundation VAX computer. Highly related sequences have an optimized alignment score greater than 100 and z value greater than 10v (Lipman and Pearson, 1985). Amino acid hydropathy plots were done by the method of Kyte and Doolittle (1982).

RESULTS

The predicted product of *B. subtilis secY* is a 4.72 kilodalton hydrophobic protein whose 431 predicted residue were 41% indentical to the 443 residue *E. coli* SecY protein. The alignment shown in figure 2 is highly significant by the criteria of Lipman and Pearson (1985), and the *B. subtilis* SecY homologue, like *E. coli secY*, has a charge distribution typical of transmembrane proteins (von Heijne, 1986). *E. coli secY* is an integral membrane protein thought to contain ten membrane-spanning segments and eleven hydrophilic domains (Alkiyama and Ito, 1987). From the align-

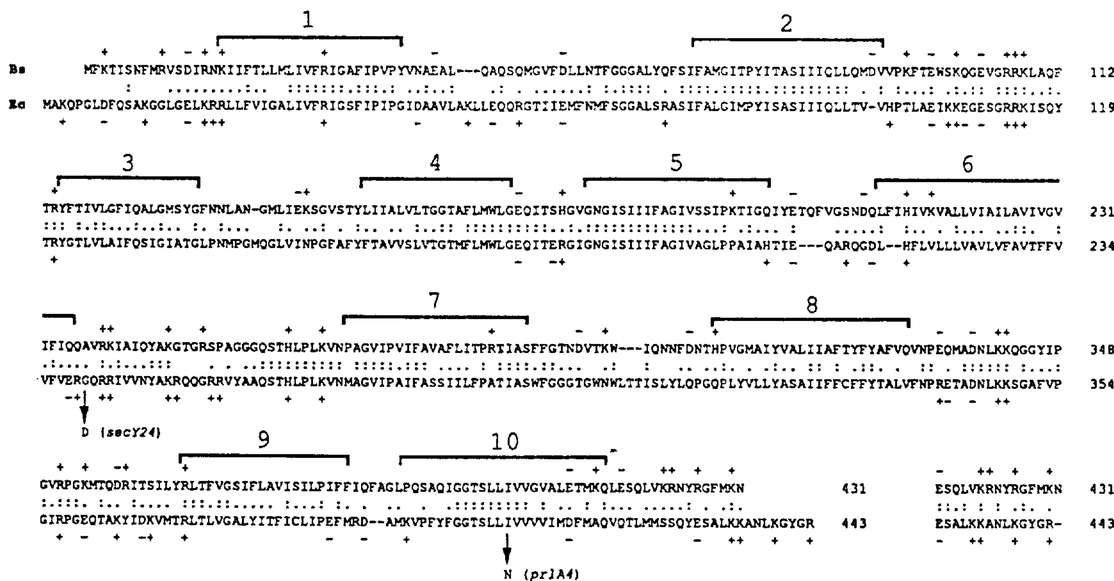

Fig. 2. Alignment of the predicted amino acid sequence of *B. subtilis* and *E. coli* SecY proteins determined by the FASTP program of Lipman and Person (1985). The primary sequences are given by the single letter code of Dayhoff *et al.* (1978). Identical residues are shown by a colon and conserved replacements (Dayhoff *et al.*, 1978) by a single dot. The *E. coli* SecY sequence is from Cerretti *et al.* (1983). Charged residues are designated above and below the lines. The statistical significance of the alignment comparison, given as optimized alignment score and z volue, are 903 and 36.6, respectively. By the criteria of Lipman and Pearson (1985), optimized scores over 100 and z values over 10 are highly significant. Brackets above the alignment delineate the ten membrane-spanning segments of *E. coli* SecY predicted by Akiyama and Ito (1987) and the corresponding regions from *B. subtilis* SecY. Following the FASTP alignment is an alternate alignment of the C-terminal 15 residues that increses primary sequence identity and charge similarity by introducing a gap between L416 and E417 of *B. subtilis* SecY. Also shown are the locations of the *E. coli secY24* (G-240-D) mutation, determined by Shiba *et al.* (1984), and the *secY/prlA* mutation *prlA4* (I408-N), determined by Sako and Iino (1988).

ment (Fig. 2), the distribution of those amino acid residues which are identical in the *secY* proteins from the two evolutionarily divergent bacteria showed that some regions are remarkably conserved whereas other regions are not. The regions which are highly conserved during evolution are likely important for *secY* configuration and function in the process of protein translocation (see Waught *et al.*, 1989).

As shown in Figure 3, the hydropathy profile of *B. subtilis secY* is essentially the same as *E. coli secY*. Although there is a minor difference in hydrophilic domain eight, which is thought to be exposed to the *E. coli* periplasm (Akiyama and Ito, 1987), it is clear that *B. subtilis secY* also contained ten potential membrane-spanning segments (Fig. 2 and 3). This remarkably similar hydropathy profile suggests that *B. subtilis secY* protein likely has a similar configuration in the cytoplasmic membrane, and this configuration might be necessary for the function of this protein. Then, which of the domains predicted by the topology study of Akiyama and Ito (1987) are important for *secY* function ? As shown in Figure 4, the calculated primary sequence identity between *B. subtilis* and *E. coli secY* suggests regions important for function. The periplasmic domains were much less conserved than the 41% average of the entire sequence. I interpret this to indicate that these regions are not important for function, but the lack of consevation could also reflect structural differences outisde the cytoplasmic membrane

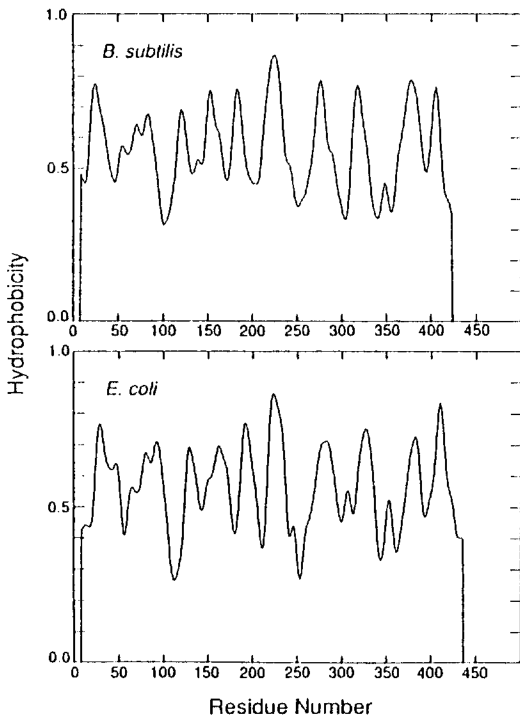

Fig. 3. Comparison of hydrophobic profiles of *B. subtilis* and *E. coli* SecY proteins. Kyte-Doolittle plots (window=17) shows remarkably similar hydrophobic profiles of *B. subtilis* (top) and *E. coli* (bottom) SecY, each containing ten potential membrane spanning domains. Using protease accesibility studies and *phoA* fusion analysis of *E. coli SecY*, Akiyama and Ito (1987) proposed that hydrophillic domains 1, 3, 5, 7, 9, and 11 are exposed to the cytoplasm whereas domains 2, 4, 6, 8, and 10 are exposed to the periplasm (see Fig. 4).

between the gram positive and negative organisms. In contrast, four of the six cytoplasmic regions representing the NH2- and COOH- terminal regions of SecY very dissimilar based on the FASTP alignment (Fig. 4). However, the alternate alignment of the COOH- terminal shown in Figure 2 yields greater consrevation in the amino acid sequences and a similar charge distribution in the segment of 14 residues. The most remarkable conservation was in the proposed menbrane-spanning segments. The first, second, fourth, fifth, seventh, and tenth membrane-spanning segments were particulary conserved, showing between 50

Table 1. secY complementation

STRAIN	32℃ vec	32℃ Ec	32℃ Bs	43℃ vec	43℃ Ec	43℃ Bs
E. coli IQ85 *secY24*	+ + +	+ − +	+	+	+ − +	-
E. coli IQ86 *secY*+	− + −	+ - +	− +	+ + +	+ + +	-

E. coli strains contained the pKK223-3 expression vector alone (vec), the pKY6 plasmid carrying *E. coli secY* (Ec), or pST120 carrying *B. subtilis secY* (Bc.) Growth was determined on polypeptone plates (Ito *et al.*, 1983) at the permissive and nonpermisive temperature for *E. coli secY24* mutant.
+ + + =good growth, + =weak growth, - =*no growth*

and 73% identity with the corresponding *E. coli* segment.

Because of the remarkable similarities in the hydropathic profile and in the conserved regions, I tested whether the *B. subtilis secY* product could complement the *E. coli secY24* temperature sensitive mutant (Shiba *et al.*, 1984). The 1.8 Kb *Xmn*I *Xmn*I fragment (Fig. 1) contained the 3′ end of the preceding L15 gene, encoding the 51 COOH-terminal residues of L15, the entire coding region of *secY*, and the 5′ end of the succeeding adenylate kinase gene (encoding the 98 NH2- terminal residues of Adk). This 1.8 Kb *Xmn*I-*Xmn*I fragment was ligated with *Hind*III linkers and cloned into the unique *Hind*III site of the expression vector pKK223-3, placing *B. subtilis secY* under the control of the promoter. This plasmid was called pST120.

The expression of *B. subtilis secY* in *E. coli* was confirmed by Susan Thomas in our lab, who made a gene fusion between the thirteenth codon of *secY* and the eighth codon of *lacZ*, using the *Eco*RV site (Fig. 1) in *secY*. This fusion plasmid pST121 showed high expression (6000-8000 Miller units) in logarithmic growth in *E. coli* IQ85.

As shown in Table 1, the *E. coli* strain IQ85 (*secY24*) and IQ86 (*secY*+) carrying the pKK223-3 expression vector alone grew normally, like the original strains without the plasmid, and the plasmid pKY6 carrying *E. coli secY* (Shiba *et al.*, 1984) did complement the *secY24* defect at non-permissive temperature 43℃, as expected. However, pST 120 carrying *B. subtilis secY* not only failed to

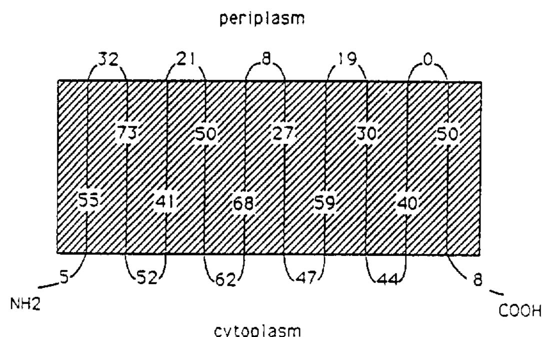

Fig. 4. *B. subtilis* and *E. coli* SecY have highly conserved cytoplasmic and membrane-spanning segments. The configuration of SecY in the *E. coli* membrane (hatched) is shown as proposed by Akiyama and Ito (1987). The values given for each segment indicate the % amino acid identity shared with the corresponding segments predicted for *B. subtilis* SecY (Fig. 2 and 3), calculated relative to the longer sequence for each segment and counting every gap as a mismach. In general, the domains thought to be exposed to the cytoplasm and the membrane spanning domains were strikingly conserved, whereas the domains exposed to the periplasm showed little similarity.

complement the *secY24* defect as the non-permissive temperature, but also inhibited growth of the *secY24* mutant and wild type in both permissive and non-permissive conditions. The growth inhibition was more severe with minimal growth medium than with rich media. The growth inhibition phenomenon with pST120 disappeared with the *lacZ* cartridge was inserted in *secY* to make pST 121, indicating the expression of *B. subtilis secY* in *E. coli* caused the growth inhibition, not just over-expression of protein with the expression vector.

DISCUSSION

The secretion system of *B. subtilis* has been intensively studied because of its prctical use in industry (Debabov, 1982; Mezes and Lampen, 1982). However, there is almost no data on the secretory machinery of *B. subtilis. B. subtilis* secretory proteins have longer signal peptides of around 40 residues rather than about 20 as in *E. coli* (Sarras, 1986). Some soluble factors involved in the protein secretory process of *B. subtilis* have been isolated (Saier *et al.*, 1989), but none of the genes for the secretory machinery have yet been identified. Therefore, most of the study on the mechanism and machinery involved in protein translocation across the prokaryotic cytoplasmic membrane have been done in the *E. coli* system.

In *E. coli*, the *secY (prlA)* gene product has a central role in secretion, as a part of the protein translocation machinery through which secretory proteins cross the cytoplasmic membrane. I found the gene for *B. subtilis secY* as the corresponding position of *E. coli secY* in the *spc* operon. The primary sequence alignment (Fig. 2) showed highly significant similarity, and the fact that some regions are highly conserved suggest that these conserved regions play an important structural or functional role for the protein.

Genetic studies on *E. coli secY* showed that its gene product is essential for growth and protein export (Shiba *et al.*, 1984). Biochemical and genetic studies have shown that *E. coli secY* is an integral membrane protein which has ten membrane-spanning domains and eleven hydrophillic domains, five exposed to the periplasm and six exposed to the cytoplasm, including the NH2- and COOH-terminal regions (Akiyama and Ito, 1987). *In vitro* biochemical studies showed that the *secY* product was essential for translocation and interacted directly or indirectly with cytoplasmic secretion factor SecA (Fandl *et al.*, 1988).

To determine which regions of *secY* might be important for its translocation and functions, I compared *E. coli* and *B. subtilis secY* by the hydropathy profiles and by the conservation of primary sequence and charged residues in each of the proposed membrane-spanning and hydrophillic segments. The fact that the hydropathy profile of *B. subtilis* SecY was essentially the same as that of *E. coli* SecY indicated that the membrane configuration of *B. subtilis* SecY might be very similar to that of *E. coli* suggested by Akiyama and Ito (1987). Furthermore, the remarkable conservation

of the first, second, fourth, fifth, seventh, and tenth membrane-spanning segments suggests that these regions are important for *secY* function, perhaps to form a protein translocation tunnel through which the secretory proteins pass across the cytoplamic membrane. The different structures outside the cytoplasmic membrane in gram positive and gram negative organisms might be responsible for the decreased conservation of the periplasmic domains. However, the remarkable conservation of the four central cytoplasmic regions-not including the NH2- and COOH- terminal portion-suggests that these might be important for interaction directly or idirectly with cytoplasmic secretory factors. The SecA protein, the signal peptide, and signal peptide recognition proteins are likely cadidates (Saier *et al.*, 1989).

Watanabe and Blobel (1989) found that antibodies raised against the hydrophillic NH2- and COOH- terminal region blocked the translocation process. They therefore proposed that the NH2- and COOH- termini of *E. coli secY* are important for function as a membrane-integrated signal sequence receptor. However, even though interactions between other translocation factors and the COOH- terminal region of *secY* cannot be ruled out based in the alternate alignnment of the COOH- terminal region (Fig. 2), the FASTP alignment (Fig. 1), showing the lack of conservation in the NH2- and COOH- terminal for signal peptide interactions.

Together with my results of the similarity in primary structure in each region, the location of the two available mutations in *E. coli secY* support the suggested importance of the conserved regions. The *prlA4* mutation alters Ile 408 to Asn within the highly conserved tenth membrane-spanning segment, which I proposed is important for forming a protein translocation channel. Since this mutation supresses mutations in the hydrophobic core of the signal peptide (Stader *et al.*, 1986). I assume that the signal peptide interacts directly or indirectly with the tenth membrane spanning segment of *secY*. The temperature senitive *secY24* mutation alters Gly 240 to Asp (Shiba *et al.*, 1984) in a region of the fourth cytoplasmic segment which is not highly conserved, but this location also lies very near conserved third membrane-spanning segment. Therefore, the phenotype of the *secY24* mutation (and *prlA4*) might be caused by changed SecY confirmation rather than the specific interactions of the mutation with other components of the secretory machinery.

There are several possible explanations for the fact that *B. subtilis SecY* not only failed to complement the *E. coli secY24* under restrictive conditions but also inhibited growth of the mutant and wild type under permissive conditions. Given the remarkable conservation of primary sequence and disapperance of the growth inhibition when the *lacZ* cartridge was inserted into *secY*, two interesting possibilities are that the growth inhibition might be caused either by titrating an *E. coli* secretion factor by overexpressed *B. subtilis secY*, or because the *E. coli* cytoplasmic membrane is dearranged by incorporation of overexpressed *B. subtilis secY*. If it could be shown that the *B. subtilis secY* had the expected configuration in the *E. coli* membrane by such methods as *phoA* fusion, then domain swapping between *B. subtilis* and *E. coli secY* might show the functions of each domain as well as the regions of *B. subtilis* SecY responsible for inhibiting growth of wild type *E. coli*.

The high conservation of *secY* in the gram-positive *B. subtilis* and gram-negative *E. coli* suggests that a similar mechanism of protein translocation across the membrane exists in both organism. The available *B. subtilis secY* gene suggests the use of the elegant methods of genetic selections developed in *E. coli* (reviewed in Riggs *et al.*, 1988; and in Schatz *et al.*, 1989) to isolate other *B. subtilis* genes which code for essential components in the protein secretion apparatus. Furthermore, the comparison and interplay between these two evolutionarily divergent prokaryotic systems can serve as a model system to understand the functions and interactions of the various components evolved for protein translocation in prokaryotes as well as in eukaryotes.

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