The EMBO Journal vol.13 no.3 pp.554 – 561, 1994

SecD and SecF facilitate protein export in *Escherichia coli*

Joseph A.Pogliano and Jon Beckwith

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue Boston, MA 02115, USA

Communicated by J.Beckwith

We show here that the rate of protein translocation in the bacterium *Escherichia coli* depends on the levels of the SecD and SecF proteins in the cell. Overexpression of SecD and SecF stimulates translocation in wild type cells and improves export of proteins with mutant signal sequences. Depletion of SecD and SecF from the cell greatly reduces but does not abolish protein translocation. A *secDF::kan* null mutant deleted for the genes encoding both proteins is cold-sensitive for growth and protein export, has a severe export defect at 37°C and is barely viable. The phenotypes of a *secD* null mutant and a *secF* null mutant are identical to the *secDF::kan* double null mutant. These results partially resolve the conflict between genetic studies and results from *in vitro* translocation systems which do not require SecD and SecF for activity, affirm the importance of these proteins to the export process, and suggest that SecD and SecF function together to stimulate protein export in a role fundamentally different from other Sec proteins. Our results provide additional support for the notion that an early step in protein export is cold-sensitive.

Key words: Escherichia coli/membrane proteins/secretion/SecD

Introduction

The ease of genetic manipulation of the organism *Escherichia coli* has led to the identification of six proteins that are involved in the translocation of proteins across the inner membrane. The soluble components SecA and SecB have been biochemically characterized in detail and many of the important functions they perform have been well studied (Kumamoto, 1991; Wickner *et al.*, 1991; Oliver, 1993). The membraneous components, on the other hand, are much less well understood even though they are thought to be part of the 'core' of the translocation complex (Bieker *et al.*, 1990; Mizushima *et al.*, 1992).

Two of the membrane proteins, SecD and SecF, were identified by cold-sensitive (Cs) mutations that cause the accumulation of precursors of exported proteins (Gardel *et al.*, 1987, 1990). The *secD* and *secF* genes, located at 9 min on the *E.coli* genetic map, form an operon along with a third gene of unknown function (Pogliano and Beckwith, 1994). An analysis of the topology of SecD and SecF shows that both have large periplasmic domains sandwiched between the first two of six transmembrane stretches (Pogliano and Beckwith, 1994). The SecD periplasmic domain is ~45 kDa, while that of SecF is ~11 kDa. Two other membrane components of the export system, SecE and SecY, are predicted to have almost all of their amino acid sequences integrated into the lipid bilayer, making SecD and SecF the only integral membrane Sec proteins with long stretches of hydrophilic sequences (Akiyama and Ito, 1987; Schatz *et al.*, 1989).

SecD and SecF differ from SecE and SecY in two other respects. First, the *in vitro* translocation systems developed to dissect the export pathway biochemically depend for activity on the SecE and SecY proteins but not the SecD or SecF proteins (Wickner *et al.*, 1991). These findings raise questions about the relative importance of SecD and SecF to the export process. However, recent experiments of Matsuyama *et al.* (1993) show that protein export in spheroplasts is inhibited when exposed to anti-SecD IgG, providing additional support for the involvement of SecD *in vivo*.

Second, many *prl* alleles of the *secE* and *secY* genes have been isolated. *prl* mutations restore export to proteins with defective signal peptides (Emr *et al.*, 1981). The ability to isolate both conditional mutations which block export and *prl* mutations which restore export in a gene provides strong evidence for the importance of that gene product to the export process. Despite extensive searches for *prl* mutants, no *prl* alleles have been found at the *secDF* locus (Schatz and Beckwith, 1990; Bieker-Brady and Silhavy, 1992). Thus, SecD and SecF do not satisfy two of the criteria that define a *sec* gene as being directly involved in the export process. Our goal is to determine the importance of SecD and SecF to the protein export process and to find out what functions they perform in the cell.

In vivo analysis of the export intermediates accumulated by the Cs *sec* mutants was recently carried out in an attempt to distinguish between possible models for what SecD and SecF do (Pogliano and Beckwith, 1993). In this study, it was shown that the Cs *secD* and *secF* mutants are phenotypically identical to the *secE* and *secY* Cs mutants, suggesting that SecD and SecF play an important role in protein export. Furthermore, the results led to the hypothesis that low temperature itself has an inhibitory effect on protein export. Therefore, the phenotypes of the Cs *sec* mutants at low temperatures are a complex manifestation of two separate effects: (i) the effect of the mutation on protein export and (ii) the effect of lowering the temperature on protein export.

In the absence of any biochemical assays for SecD and SecF functions and since the Cs mutants were not useful in analyzing the loss of function phenotypes, we have constructed and characterized a null mutant lacking both SecD and SecF. The null mutant is viable only at high temperatures and has a severe, cold-sensitive export defect. In addition, overexpressing SecD and SecF improves the export of proteins with defective signal sequences. Our results suggest SecD and SecF stimulate protein translocation and are essential for efficient protein export.

Results

Depletion of SecD and SecF reduces but does not eliminate protein translocation

The existence of *secD* and *secF* mutants that failed to grow at low temperatures suggested that the SecD and SecF proteins may be essential for cell viabililty. To examine the phenotype of a *secDF* null mutant, we constructed a strain in which the *secDF* operon is regulated by the arabinose promoter, P_{BAD}. Unlike the P_{tac} promoter which allows a considerable level of expression in the absence of inducer, growth on glucose reduces transcription from P_{BAD} to a very low level. The P_{BAD} promoter has been used to deplete cells of essential proteins present at only 40 molecules per cell (Guzman *et al.*, 1992).

The depletion strain JP91 contains a *secDF::kan* null mutation on the chromosome complemented by a plasmid, pGAP1, carrying the *secDF* operon under control of the P_{BAD} promoter. Growth experiments with this strain on Petri plates under various conditions indicated that SecD and SecF are essential at 30°C. While JP91 formed colonies on any media with arabinose, this strain did not form colonies on rich or minimal glucose medium at 30°C without arabinose. However, at 37°C the strain formed tiny colonies on rich medium and full size colonies on minimal glucose medium. Since these results suggested that SecD and SecF are not absolutely essential for cell viability at 37°C, we examined protein export in cells depleted of SecD and SecF by growing JP91 in minimal glucose medium at 37°C. While overproducing SecD and SecF has no observable deleterious effects on the cell, underproducing them during growth of the overnight cultures to be used for depletion reduces growth rate and provides a selection for suppressor mutations. To circumvent this problem, we used conditions in which SecD and SecF were present in excess at the beginning, anticipating that many generations of growth were likely to be required before the effects of depletion were observed.

An overnight culture of JP91 was grown in rich medium supplemented with arabinose at 37°C, pelleted, resuspended in M63 and diluted into M63 glucose supplemented with 18 amino acids at 37°C. OD_{600} readings were taken to monitor cell growth and pulse–chase [^{35}S]methionine labellings were performed to assay protein export. From the OD_{600} readings we calculated the doubling time at each time point, as described in Materials and methods (Figure 1). No growth defect was noticeable until ~13 h after the beginning of depletion (10 generations of growth), at which point the doubling time suddenly began increasing. Despite the fact that JP91 forms normal size colonies on glucose minimal medium, its doubling time in glucose minimal liquid medium is ~4 times higher than a wild type strain.

In the depletion experiment, pulse–chase labelling at 10 h (eight generations) showed only a slight decrease in the rate of export of OmpA and DegP (Figure 2) and no export defect was observed at two earlier time points (data not shown). However, labelling at 13.5 h (11 generations) showed a severe export defect. At the pulse point, for example, almost all of OmpA was in the precursor form. Precursor OmpA chased slowly and completely to mature OmpA with a half-life of ~3 min.

secDF::kan null mutants are viable

One trivial explanation for the finding that a *secDF* null mutant is able to grow at all at 37°C is that the plasmid pGAP1 allows a residual amount of expression of SecD and SecF even when glucose is present. If this were the case, we would not have truly tested the essentiality of the *secDF* genes at 37°C. Therefore, we isolated and characterized derivatives of JP91 that had lost the complementing plasmid. When JP91 is streaked onto NZ arabinose plates without ampicillin at 37°C, segregants that have lost the plasmid form minute colonies. These segregants, which restreak as tiny colonies, are not resistant to ampicillin at even very low

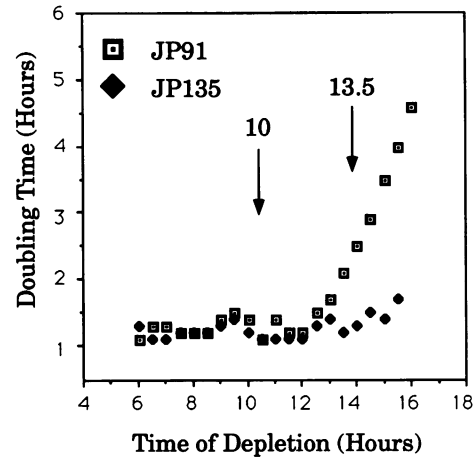

Fig. 1. Depleting cultures of SecD and SecF reduces growth rate and the rate of protein export. Strains were grown overnight in rich medium supplemented with arabinose and at time zero washed and diluted into glucose minimal media at 37°C. Logarithmic growth of the cultures was maintained by dilution into fresh medium as necessary. The approximate growth rate at each time point was calculated from the OD_{600} readings and expressed as doubling times, as described in Materials and methods, and plotted versus time of depletion. Both strains contain the *secDF::kan* knockout mutation on the chromosome and are isogenic except that in strain JP91 (square with dot) expression of the *secDF* operon is controlled by the P_{BAD} promoter, while in strain JP135 (filled diamond) the *secDF* operon is expressed constitutively from its own promoter. At 2.5, 4.5, 10 and 13.5 h of depletion samples were taken and the kinetics of export examined (Figure 2).

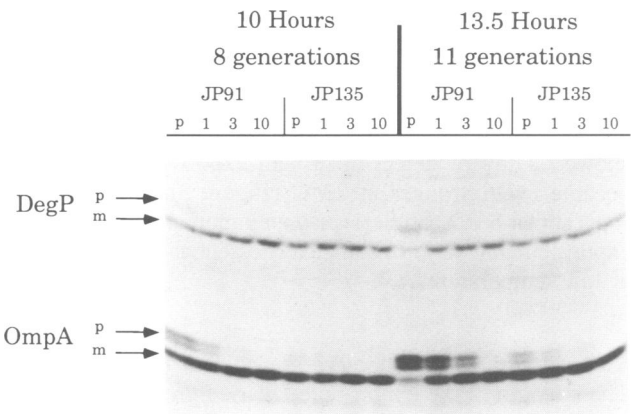

Fig. 2. Kinetics of protein export of cultures depleted of SecD and SecF. After 10 h (eight generations) and 13.5 h (11 generations) of growth in depleting conditions (indicated by arrows), samples of the cultures described in Figure 1 were pulse labelled for 30 s with [^{35}S]methionine and chased with cold methionine for 1, 3 and 10 min. The proteins DegP and OmpA were examined on 10% SDS–PAGE after immunoprecipitation. Precursor (p) and mature (m) proteins are indicated by arrows. No export defect was noticeable in pulse–chase experiments at earlier time points of depletion (2.5 and 4.5 h, data not shown).

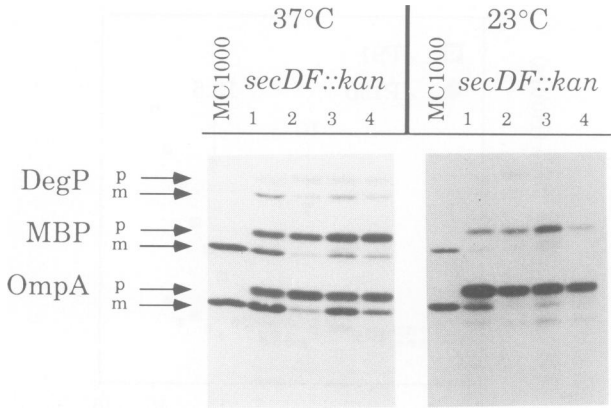

Fig. 3. Null mutants acquire suppressor mutations which improve protein export. *secDF:kan* null mutants are viable at 37°C and can be isolated from strains JP135 (*secDF::kan*/pCGSH1) and JP91 (*secDF::kan*/pGAP1) by screening for loss of the complementing plasmids. Four independent *secDF::kan* isolates (1, 2, 3 and 4) of JP135 that had lost the complementing plasmid and a wild type control strain MC1000 were grown in maltose minimal medium supplemented with 18 amino acids at 37°C. One-half of each culture was shifted to 23°C for 1 h. Each culture was pulse labelled for 30 s with [^{35}S]methionine. The proteins DegP, MBP and OmpA were examined on 10% SDS−PAGE after immunoprecipitation.

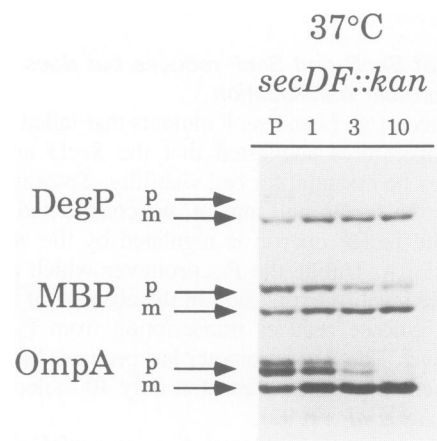

Fig. 4. Kinetics of protein export in *secDF::kan* null mutants at 37°C. A *secDF::kan* null mutant was isolated and prepared as described in Materials and methods. Cultures were pulse labelled for 30 s with [^{35}S]methionine and chased with cold methionine for 1, 3 and 10 min. The proteins DegP, MBP and OmpA were examined on 10% SDS−PAGE after immunoprecipitation.

concentrations (5 μg/ml), and behave identically to JP91 grown under depleting conditions. These null mutants are cold-sensitive for growth, but grow like wild type on minimal glucose medium at 37°C. Since the null mutant grows more slowly than wild type in liquid medium, the possibility of revertants arising that take over the population is of great concern. Therefore, we picked four isolated null mutants and performed pulse labelling experiments at 37°C and 23°C to examine the export defect. All four of the null mutants showed a severe defect in protein export at 37°C which became even stronger at 23°C (Figure 3). These results suggest that SecD and SecF play an important role in protein export, but that they are completely essential for growth only at low temperatures.

Null mutants readily acquire suppressor mutations

The strength of the export defect varied greatly among the null mutants pulse labelled in Figure 3, indicating that some of them had already begun to accumulate suppressor mutations. For example, at 37°C ~80% of OmpA was in the precursor form after a 30 s pulse in isolate 2, while only 40% was precursor in isolate 1. The difference between these two isolates was even more dramatic at 23°C (Figure 3). While the null mutants form colonies that are similar in size to the wild type on minimal plates at 37°C, they grow poorly in all liquid media tested (both rich and minimal). Suppressor mutations arise at a very high frequency in overnight liquid cultures, probably due to a strong selection. Since the depletion strain in the presence of glucose appears to mimic the null mutant, we suspect that the depletion strain may be more useful than the null mutant for biochemical studies. In subsequent experiments using the null mutant, we have taken extreme care to avoid accumulating suppressor mutations, including keeping the number of generations of growth in liquid media to a minimum, performing experiments with multiple independent isolates and analyzing liquid cultures for the presence of suppressor mutants.

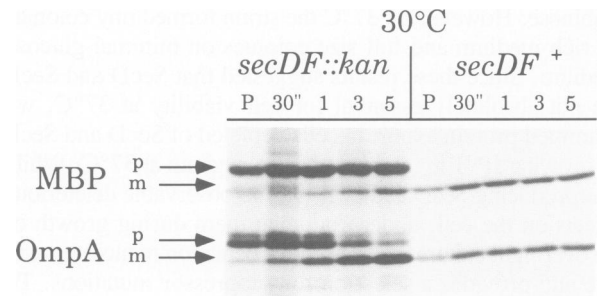

Fig. 5. Kinetics of export in *secDF::kan* null mutants at 30°C. A *secDF::kan* null strain containing either a control plasmid or a *secDF*$^+$ plasmid was grown at the permissive temperature of 37°C in maltose minimal medium and then shifted to 30°C for 1 h. Cultures were pulse labelled with [^{35}S]methionine for 30 s and chased with cold methionine for 0.5, 1, 3 and 5 min. The proteins MBP and OmpA were examined on 10% SDS−PAGE after immunoprecipitation.

The magnitude of the translocation defect is protein dependent

We sought to examine more closely the rate at which proteins are exported in the null mutant by performing pulse−chase experiments at 37°C and 30°C. At 37°C an extreme reduction in the rate of export was observed for all three proteins examined (Figure 4). As seen in the depletion experiment, preOmpA chased almost quantitatively to the mature form by 10 min and ~50% of maltose binding protein (MBP) was processed by 10 min. At 30°C, very little MBP was exported while almost all of OmpA was eventually exported (Figure 5). Thus, some proteins, such as OmpA, can be slowly and completely exported even at the nonpermissive temperature of 30°C, while other proteins are extremely susceptible to the inhibitory effects of low temperatures.

Phenotypes of the individual null mutants

We examined the phenotypes of a *secD* null mutant and a *secF* null mutant individually. We introduced plasmids expressing only the *secD* gene or only the *secF* gene into a strain containing the *secDF::kan* null mutation. The growth

phenotypes of a *secD* null mutant and of a *secF* null mutant were identical to that of the *secDF::kan* null mutant (data not shown).

Overexpression of SecD and SecF results in a Prl phenotype

Proteins with defective signal sequences are secreted more efficiently in strains with *prl* mutations (Emr *et al.*, 1981). Of the genes known to be required for protein export, *prl* mutations have been found in *secY*(*prlA*), *secE*(*prlG*) and *secA* (*p1prlD*). Numerous searches for *prl* mutants had not yielded any mutations mapping to the *secD* locus. However, we found that overexpression of the *secDF* operon on a multicopy plasmid suppresses the export defect of proteins with signal sequence mutations as judged by both phenotypic analysis and pulse−chase labelling experiments.

MBP, encoded by the *malE* gene, is a periplasmic protein required for transport of maltose into the cell and is required for growth on maltose as the sole carbon source. Some mutations in the *malE* gene, such as *malE18-1*, alter the signal sequence of the precursor protein and prevent most of the precursor form of MBP18-1 from being exported (Ryan and Bassford, 1985). Bacteria producing MBP18-1 still grow on maltose at 37°C, since only a small amount of MBP needs to be exported for sufficient transport of maltose into the cell to allow growth. Export of MBP18-1 is further inhibited at lower temperatures (23°C) and such strains do not grow on maltose at this temperature (Pogliano and Beckwith, 1993). A multicopy plasmid carrying the wild type *secDF* locus (pCGSH1) restores efficient growth to this strain at low temperatures, while control plasmids (pBR322, pCGp/h, pF110, pD45) containing mutations in the *secD* or *secF* genes do not. SecD and SecF overproduction increased export of a variety of signal sequence mutant proteins. The effect was seen with signal sequence mutants of alkaline phosphatase (*phoA21*, *phoA73*, *phoA82* and *phoA93*) (Michaelis *et al.*, 1986), MBP (*malE10-1*, *malE14-1*, *malE16-1* and *malE18-1*) (Bedouelle *et al.*, 1980) and LamB (*lamBs78*) (Emr and Silhavy, 1983) according to several plate tests: growth on minimal maltose or minimal maltodextrin plates, and color on Nz XP or maltose tetrazolium plates (data not shown). Both SecD and SecF were required for suppression. The first gene in the *secD* operon, *yajC*, was not necessary for signal sequence suppression (data not shown).

To quantitate the effectiveness of suppression and to demonstrate that the phenotypes we observed were due to increased signal sequence processing, we performed a pulse−chase [^{35}S]methionine labelling experiment on a *malE16-1* mutant carrying either the *secDF* overproducing plasmid or the vector plasmid (Figure 6). The fraction of MBP16-1 that is processed to the mature form is higher at all time points examined in the strain overproducing SecD and SecF. Quantitation of the effect showed that export of MBP16-1 was stimulated by at least 2-fold. Therefore, overexpression of SecD and SecF can improve export of proteins with defective signal sequences.

Overexpression of SecD and SecF can improve the export efficiency of a wild type strain

How does *secDF* overexpression restore export to proteins with defective signal sequences? The mechanisms by which *prl* alleles of the *secA*, *secE* and *secY* genes restore export

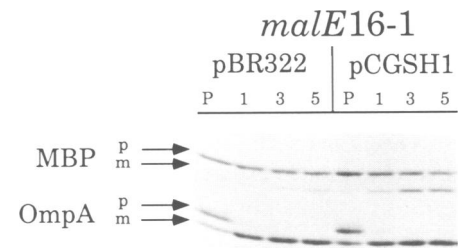

Fig. 6. Overexpression of the *secDF* operon suppresses the export defect of MBP16-1. A *malE16-1* mutant containing either a control plasmid (pBR322) or a *secDF*$^+$ overproducing plasmid (pCGSH1) was grown in maltose minimal medium at 30°C, pulse labelled for 30 s with [^{35}S]methionine and chased with cold methionine for 1, 3 and 5 and min. The proteins MBP and OmpA were examined on 10% SDS−PAGE after immuoprecipitation. A substantial increase in the amount of MBP16-1 that is processed to the mature form is seen in the *secDF* overproducing strain relative to the control strain.

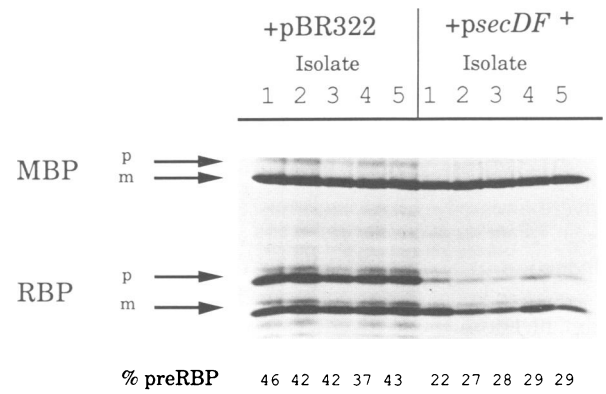

Fig. 7. Overexpression of the *secDF* operon improves export in wild type cells. MC4100 was transformed with either the *secDF*$^+$ overproducing plasmid (pCGSH1) or a control plasmid (pBR322). Five independent isolates from each transformation were grown in maltose minimal medium and given a pulse of [^{35}S]methionine for 15 s at 30°C. Samples were stopped on ice and the proteins MBP and RBP were examined by 10% SDS−PAGE after immunoprecipitation. The precursor (p) and mature (m) forms of the proteins are indicated by arrows and were quantitated using a PhosphoImager. The fraction of RBP present as precursor in each lane (% preRBP) is shown under the corresponding lane.

are unknown. However, we reasoned that the Prl phenotype from multicopy expression of SecD and SecF may be easier to interpret. Since we know that the immediate effect is to produce more SecD and SecF, it seemed possible that the net result could be an overall improvement in the rate at which the protein export machinery functions. By increasing the efficiency at which all proteins are exported, those with defective export signals may be exported more efficiently also. We tested directly whether *secDF* overexpression improved export of wild type proteins.

A wild type strain was transformed with either pBR322 or the *secDF* overexpression plasmid. Five independent isolates from each transformation were grown in minimal maltose medium at 30°C and pulse labelled with [^{35}S]methionine for exactly 15 s. The secreted proteins MBP and ribose binding protein (RBP) were immunoprecipitated and analyzed by SDS−PAGE. The fraction of protein present as precursor on the pulse provides an accurate measurement for comparison if numerous samples are

analyzed. Very little MBP is present as precursor (Figure 7), while a significant fraction of RBP is unprocessed after a 15 s pulse. A substantial increase in the fraction of RBP that is processed at the pulse is observed in the strain that overexpresses SecD and SecF. Thus, overexpression of SecD and SecF improves protein export even in a wild type strain.

Discussion

SecD and SecF facilitate export

SecD and SecF were originally identified by genetic selections for mutants defective in protein export. The phenotypes of *secD* and *secF* Cs mutants are identical to *secE* and *secY* Cs mutants of equivalent strength. However, attempts to show a dependence on the SecD and SecF proteins in *in vitro* protein translocation systems have thus far failed. These results have raised questions about the importance of these two proteins in the export process. Several lines of evidence support a role for the two proteins in facilitating protein translocation.

First, overproduction of SecD and SecF stimulates protein translocation in a wild type cell and increases export of a variety of proteins with signal sequence defects. The strength of the suppression of signal sequence mutants (~2-fold) is greater than that of the strongest *prlG* (*secE*) suppressor mutation, *prlG1*, and comparable with that seen when SecA is overexpressed (Fikes and Bassford, 1989). The Prl phenotype conferred by *secDF* overexpression cannot be due to induction of SecA, as synthesis of the SecA protein is not increased under these conditions (K.J.Pogliano, personal communication).

Second, a strain deleted for both the *secD* and *secF* genes is severely defective in protein export, does not grow at low temperatures and is barely viable at 37°C. The rate of export is decreased at least 10-fold in the null strain when cultured at 37°C. The null mutant grows poorly at 37°C in liquid medium, whether rich or minimal, and forms tiny colonies on rich plates. The null strain grows best on minimal plates, forming colonies approximately the same size as the wild type.

Third, the only mutants to appear in a general selection for secretion mutants were in the *secA*, *secE*, *secD* and *secF* genes. In fact, the strongest Cs mutations affecting export are alleles of *secD* and *secF* (Pogliano and Beckwith, 1993).

Finally, Matsuyama *et al.* (1993) reported the inhibition of protein export from spheroplasts when anti-SecD IgG was included in the assay system. Taken together, the available evidence suggests that SecD and SecF are important components of the export pathway.

Why then do in vitro systems not require SecD and SecF?

Why do *in vitro* translocation systems not require SecD and SecF for activity? We suggest two explanations that may help resolve the apparent conflict. First, translocation *in vitro* is inefficient compared with translocation *in vivo* (Akimaru *et al.*, 1991). Our results show that SecD and SecF are not completely essential for the translocation reaction to take place *in vivo*, in accord with *in vitro* studies; however, translocation rates are greatly affected by the levels of these two proteins *in vivo*. If SecD and SecF provide only a stimulatory function, translocation may take place at a low rate in their absence. *In vitro*, the cause of inefficiency may be unrelated to the functions of SecD and SecF. For example, a rate limiting step that requires as yet unidentified components or conditions that more closely mimic the physiology of protein export may prevent efficient export, such that the absence of SecD and SecF has little or no effect.

Second, *in vitro* systems are not designed to assay for terminal steps in translocation, such as the folding or release of newly translocated proteins. The assay for *in vitro* translocation involves measuring resistance of translocated proteins to proteases added externally to inverted membrane vesicles. If *secD* and *secF* mutants were specifically defective in a terminal export step, then the effect of their absence from the *in vitro* system might not be detected. *In vivo*, the absence of SecD and SecF might lead to a log jam effect in which mature proteins back up at the site of translocation. Such an effect would explain why these mutants accumulate precursor proteins in the cytoplasm even though they were altered in a late step of protein export. *In vitro*, newly translocated proteins may not accumulate to high enough levels to manifest a log jam effect. It may be that as the *in vitro* systems continue to develop, the presence of these proteins will prove to have a measurable effect (Nishiyama *et al.*, 1993).

Possible functions of SecD and SecF

SecA and SecE are essential for growth of *E.coli* and are essential for protein translocation *in vivo* and *in vitro*. (A *secY* null mutant has not been characterized.) In contrast, SecD and SecF are not absolutely essential for growth or for protein translocation *in vivo*. Furthermore, we note that suppressor mutations easily arise in the *secDF::kan* null mutant, while it has not been possible to obtain mutations that eliminate the requirement for SecE (C.Murphy, personal communication). These observations and other properties of SecD and SecF suggest that they play a fundamentally different role in protein export than other Sec proteins.

The topological arrangement of SecD and SecF in the inner membrane provides the basis for speculation that they are involved in a late step in the export process, such as folding or release of newly translocated proteins, recycling the export complex after each round of translocation, or presenting newly translocated proteins to leader peptidase. Based on *sec* titration experiments, Bieker-Brady and Silhavy (1992) suggested that SecD and SecF function at a late step in the secretion pathway. Matsuyama *et al.* (1993) proposed that SecD is involved in a release step based on the appearance of an intermediate form of MBP (processed but unfolded) when export in spheroplasts is inhibited with anti-SecD IgG. However, a similar intermediate accumulates *in vivo* in *secA*, *secE*, *secY*, *secD* and *secF* mutants (Geller, 1990; Ueguchi and Ito, 1990; Pogliano and Beckwith, 1993). Presently, it is not clear whether the intermediate species observed in *sec* mutants are translocation, folding or release intermediates.

While the phenotypes reported here argue strongly that SecD and SecF are required *in vivo* for efficient protein export, indirect mechanisms are not entirely excluded. In the absence of SecD and SecF, membrane structure could be drastically perturbed, upsetting the function of many cellular processes that take place in the cytoplasmic membrane. While hypotheses invoking indirect mechanisms are formally possible, the accumulated evidence suggests that such interpretations are less likely.

Do SecD and SecF form a complex?

We present genetic evidence that supports the previous suggestion that SecD and SecF may function together, possibly in a complex (Gardel *et al.*, 1990). First, we could detect an improvement in the export of proteins with signal sequence mutations only when SecD and SecF were overexpressed together. Second, a strain deleted for both the *secD* and *secF* genes is phenotypically identical to strains deleted for only one of the genes. While the phenotypes of the individual null mutants and the double null mutants are identical, they differ greatly from null mutants of *secA*, *secB* and *secE*. Third, SecD and SecF are produced in equal amounts in the cell (Pogliano and Beckwith, 1994). The expression levels of these two proteins appear to be ~10-fold less than that of SecE or SecY. Further genetic and biochemical studies are needed to verify the hypothesis that SecD and SecF form a complex in the innner membrane.

Direct support for the Cs hypothesis

Pogliano and Beckwith (1993) have previously proposed that mutations that reduce the rate of protein export render the cell sensitive to the inhibitory effects of low temperatures. Accordingly, the Cs mutations in the SecD and SecF genes were predicted not to encode thermolabile proteins. Instead, they were predicted to produce either mutant proteins with reduced activity at all temperatures or wild type proteins but at lower levels. While none of the mutations in the *secD* locus have been sequenced, the results presented here have confirmed this prediction.

Null mutants lacking either one or both of the *secD* and *secF* genes have a cold-sensitive export defect. Certainly, such a defect cannot be due to a thermolabile SecD or SecF protein. In addition, strains producing both wild type SecD and SecF proteins at reduced levels have a cold-sensitive export defect (Pogliano and Beckwith, 1994). Taken together, these data support the idea that cold sensitivity is intrinsic to the protein export pathway. When *secE* Cs mutations were sequenced, the strongest alleles reduced the level of SecE ~2-fold but did not alter its structure (Schatz *et al.*, 1991). Thus, there are now three cases where Cs export can be attributed to a reduction in the levels of a component of the export pathway. We are pursuing genetic studies to further understand how low temperatures affect the protein export pathway.

Materials and methods

Media, reagents and growth conditions

NZ medium containing 0.5% yeast extract (Difco), 1.0% NZamine (Difco), 1.5% agar and 0.8% NaCl was used as rich medium. M63 was used as

Table I. Bacterial strains and plasmids

	Genotype	Reference
Strain		
MC1000	*araD139* Δ*(ara-leu)7679* Δ*(lac)X74 rpsL150 thi*	Beckwith collection
MC4100	*araD139 relA1 thi rpsL150 flbB5301* Δ*(lac)U139 deoC7 ptsF25*	Beckwith collection
MM4	MC4100 *malE*18-1	Beckwith collection
DHB4	*araD139* Δ*(ara-leu)7679* Δ*(lac)X74 rpsL150 thi* Δ*malF3* Δ*phoA(PvuII) phoR*/F′*lacI*^{Q} *pro lac*	Beckwith collection
KJ255	MC1000 Δ(*phoA-proC*) *recD1903*::miniTn*10*/F′KJ2 *secDF*$^{+}$ *lac*$^{+}$	Kit Johnson Pogliano
JP15	*relA1 thi rpsL150 flbB5301* Δ*(lac)U139 deoC7 ptsF25 secA-lacZ-f181* (λPR9*secA*$^{+}$) *recA::cat secD1 zaj::*Tn*10* Ara^{r}/pOXgen	This study
JP37	MC1000 *pcnBuvc41-2 zad1*::Tn*10*/F′KJ2 *secDF*$^{+}$ *lac*$^{+}$	This study
JP63	MC4100 *malE16-1 leu*::Tn*10 ara*Δ714/pBR322	This study
JP64	MC4100 *malE16-1 leu*::Tn*10 ara*Δ714/pCGSH1	This study
JP73	Δ*(lac)X74 rpsL150 thi secDF15::kan pcnBuvc41-2 zad1*::Tn*10 leu*$^{+}$ *ara*Δ714 *recA::cat*/F′KJ2 *secDF*$^{+}$ *lac*$^{+}$	This study
JP91	Δ*(lac)X74 rpsL150 thi secDF15::kan pcnBuvc41-2 zad1*::Tn*10 leu*$^{+}$ *ara*Δ714 *recA::cat*/pGAP1	This study
JP135	Δ*(lac)X74 rpsL150 thi secDF15::kan pcnBuvc41-2 zad1*::Tn*10 leu*$^{+}$ *ara*Δ714 *recA::cat*/pCGSH1	This study
JP281	JP73/pCGSH1	This study
JP282	JP73/pGAP1	This study
JP283	JP73/pGAP10	This study
JP284	JP73/pBAD18	This study
JP285	JP73/pCGEE	This study
JP286	JP73/pCGNH15	This study
Plasmid		
pCGSH1	*secDF*$^{+}$	Gardel *et al.* (1990)
pGAP1	P_{BAD} *secDF*$^{+}$	This study
pGAP3	*secDF15::kan*	This study
pGAP10	P_{BAD} *secF*$^{+}$	This study
pCGEE	pBR322 vector	Gardel *et al.* (1990)
pCGNH15	*secD*$^{+}$	Gardel *et al.* (1990)
pCGp/h	*secD*$^{-}$ *secF*$^{+}$	Gardel *et al.* (1990)
pD45	*secD45::*Tn*phoA*	Gardel *et al.* (1990)
pF110	*secD*$^{+}$ *secF110::*Tn*phoA*	Gardel *et al.* (1990)
pJS51	*secE*$^{+}$	Schatz *et al.* (1991)
pBAD18	P_{BAD} vector	Luzmaria Guzman Verduzco

minimal medium (Miller, 1972). Carbon sources were added at 0.2%. When necessary, amino acid supplements were added as described (Maloy, 1990). Tetrazolium and MacConkey indicator plates were prepared as described (Miller, 1972). Antibiotics were added at the following concentrations: ampicillin 200 μg/ml for *pcnB*$^+$ strains and 20 μg/ml for *pcnB*$^-$ strains, tetracycline 20 μg/ml, kanamycin 60 μg/ml, chloramphenicol 10 μg/ml and gentamycin 10 μg/ml. An indicator of alkaline phosphatase activity, 5-bromo-4-chloro-3-indolylphosphate (XP) (Bachem Co.), was added at a final concentration of 40 μg/ml. All other buffers, reagents and standard molecular biology techniques were prepared and performed as described (Maloy, 1990; Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA ligase and Klenow were purchased from New England Biolabs (Beverly, MA).

Labellings

Pulse–chase [^{35}S]methionine labellings were performed as described (Pogliano and Beckwith, 1993). Briefly, log phase cells were added to a prewarmed tube of [^{35}S]methionine at 40 μCi/ml. After 20 s, 0.1 vol of 0.5% methionine was added and samples were taken immediately and after various chase times and stopped on ice. Samples were immunoprecipitated with antibodies against RBP, MBP, DegP and OmpA and examined by 10% SDS–PAGE. Antiserum against RBP was prepared by Jeff Garwin and MBP/DegP antiserum was prepared by Kathy Strauch. OmpA antiserum was a generous gift from Carol Kumamoto. Precursor and mature forms of MBP, RBP, DegP and OmpA were quantitated using a Molecular Dynamics PhosphoImager.

Construction of a secDF::kan null mutation

All bacterial strains are derivatives of *E.coli* K12 (Table I). The *secD* and *secF* genes comprise an operon with one other gene of unknown function, *yajC* (Pogliano and Beckwith, 1994). A null mutant was constructed by first replacing the *secD* and *secF* genes of the *yajC-secD-secF* operon on a multicopy plasmid with a kanamycin resistance gene and then recombining the *secDF::kan* mutation onto the chromosome by linear transformation. The plasmid pCGSH1 carries the *yajC-secDF* operon and flanking DNA, including 0.75 kb of DNA encoding the unrelated *tgt* gene immediately 5′ to the *yajC-secDF* promoter and 0.6 kb of DNA downstream (Gardel *et al.*, 1990). This plasmid was digested with *Bsu*36I and *Bst*XI and the overhanging ends were filled using the Klenow fragment of DNA polymerase. The kanamycin resistance gene from plasmid pUC4K (Pharmacia) was ligated with the pCGSH1 *Bsu*36I–*Bst*XI fragment after pUC4K was digested with *Pst*I and made blunt-ended using Klenow. Following ligation with T4 DNA ligase and selection for kanamycin resistance, the desired plasmids were checked by restriction mapping. One of the plasmid products was pGAP3. This plasmid carries '*tgt, yajC-kan*'-*secF*. All of the *secD* gene and all but 108 nucleotides of the *secF* gene were replaced with the kanamycin resistance gene while the *yajC* gene was left intact. pGAP3 was linearized by digestion with several restriction enzymes that cut only pBR vector sequences and then electroporated into a *recD* strain, KJ255, selecting Kan^r (Russel *et al.*, 1989). When *recD* mutants are transformed with linear DNA which cannot replicate, most of the transformants are recombinants. KJ255 is diploid for the *secD* locus due to the presence of the temperature-sensitive F′*lac*$^+$ *secDF*$^+$ episome, F′KJ2 (Pogliano and Beckwith, 1994). In strain KJ255, the *secDF::kan* mutation can recombine onto the chromosome or the F′*lac*$^+$ *secDF*$^+$ episome. Kanamycin-resistant recombinants containing the deletion on the chromosome do not readily lose the complementing F′KJ2 episome at 42°C and can be detected by their inability to form white (Lac$^-$) colonies on MacConkey lactose medium at 42°C. The *secDF::kan* null mutation was transduced into a *pcnB* derivative of MC1000, JP37, complemented by F′KJ2. This strain was transduced to *leu*$^+$ *araΔ714* and *recA* to construct strain JP73, and then *secDF*$^+$ plasmids were transformed into this strain and the temperature-sensitive F′*lac*$^+$ *secDF*$^+$ episome cured by growth at 42°C. JP91 carries the plasmid pGAP1, which produces SecD and SecF only when arabinose is present. JP135 is isogenic to JP91 except that it carries the original *secDF*$^+$ plasmid pCGSH1 which expresses the *secDF* operon constitutively from its own promoter.

Construction of pGAP1 and pGAP10

A plasmid which allows tight control of expression of the *secDF* operon was constructed by cloning the *secDF* operon under control of the P_{BAD}. The plasmid pBAD18 is a pBR322 derivative which carries all of the P_{BAD} control region, including *araC*, and has a multiple cloning site immediately 3′ to the P_{BAD} promoter (Guzman *et al.*, 1992). A fragment of DNA from pCGSH1 containing all of the *yajC-secDF* operon except transcriptional start elements was subcloned into pBAD18 such that expression of *secDF* was arabinose-dependent. A 3.9 kb *Ssp*I fragment of pCGSH1 was ligated with a fragment of pBAD18 that had been digested with *Sma*I and dephosphorylated with calf intestinal phosphatase. Following transformation and selection for Amp^r, colonies were pooled and diluted into fresh media and the M13 single-stranded phage f1R408 was used to infect the pools. pBAD18 contains an M13 origin and is packaged by the phage while the pCGSH1 plasmid does not. This step separates products resulting from religation of the pCGSH1 vector from the desired pBAD18 recombinants. Phage were harvested and a *secD1* strain, JP15, was transfected followed by selection for Amp^r. Plasmids which complemented the *secD1* mutation in an arabinose-dependent manner were restriction mapped. One of these, pGAP1, was chosen for further study.

A derivative of pGAP1 was constructed which only expresses SecF. pGAP1 contains only two *Kpn*I sites, one in the multiple cloning site of the vector sequences 5′ to the translational start signals of the *yajC-secDF* genes and one very near the end of the *secD* gene. pGAP1 DNA was digested with *Kpn*I, religated and transformed into DHB4. DNA from the transformants was isolated and restriction mapped. One plasmid with the desired restriction map was designated pGAP10 and further characterized. pGAP10 fully complemented the Cs phenotype of a *secF62* mutant when 0.2% arabinose was provided.

Depletion of SecD and SecF and calculation of growth rates

The strain JP91 expresses the *secDF* operon only when arabinose is provided. Using this strain and an isogenic strain, JP135, which contains a plasmid with the wild type *secDF* locus as a positive control, we examined the effect of depleting cells of SecD and SecF. JP91 and JP135 were grown overnight in NZ broth containing 0.002% arabinose at 37°C. One milliliter of culture was pelleted in a microfuge to remove arabinose, resuspended in 1 ml M63 and diluted 1/50 into M63 0.2% glucose supplemented with 18 amino acids. Cultures were shaken in a 37°C water bath and OD_{600} readings taken to monitor cell growth. When cell densities reached an OD_{600} of 0.6, cultures were diluted 1/12 into the same prewarmed medium to maintain exponential growth of the culture. To calculate the approximate rate of doubling at each time point, we used a moving window of three OD_{600} readings of log phase culture, plotted the $\ln(OD_{600})$ versus time and calculated the slope of the best-fit line using linear regression analysis. The doubling time, g, is related to the slope, k, by the equation: $g = \ln 2/k$. We plotted the doubling time versus time of depletion in Figure 1. At four different points of depletion (2.5, 4.5, 10 and 13.5 h) samples were taken for pulse–chase experiments to assay the secretion defect.

Complementation of the secDF::kan mutation

We exploited the ability of *secDF::kan* null mutants to grow at high temperatures to construct strains for complementation analysis (see Results). In order to test the phenotypes of a *secD* null mutant and *secF* null mutant individually, we constructed *secDF:kan* null mutants containing plasmids expressing either SecD or SecF. Strain JP73 (*secDF::kan pcnB recA*/ F′KJ2) was transformed to Amp^r at 30°C with the relevant plasmids listed in Table I. Three isolates from each resulting strain (JP281, JP282, JP283, JP284, JP285 and JP286) were streaked on NZ Amp X-gal plates at 42°C. Strains with plasmids that complement the *secDF::kan* null mutation form large, white Lac$^-$ (F$^-$) segregants at 42°C on X-gal plates. Strains with plasmids that do not complement still yield Lac$^-$ segregants on NZ Amp X-gal plates at 42°C, since the *secDF::kan* mutant is viable. However, these Lac$^-$ segregants form very small colonies on rich medium and are cold-sensitive for growth. The phenotypes of Lac$^-$ (F$^-$) derivatives of these strains (which contain only the Amp^r plasmid and not the F′KJ2 episome) were tested on NZ Amp 0.2% arabinose plates at 37°C and 30°C. Similar results were obtained if ampicillin was not included in the media.

Acknowlegements

We are grateful to Beckwith lab members for thoughtful advice and critical reading of the manuscript, especially Kit Johnson Pogliano, Luzmaria Guzman Verduzco, Alan Derman and Chris Murphy. Theresa Luna provided excellent technical assistance. J.B. is an American Cancer Society Research Professor. This work was suppported by grants from the National Institutes of Health and the American Cancer Society to J.B.

References

Akimaru,J., Matsuyama,S., Tokuda,H. and Mizushima,S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 6545–6549.

Akiyama,Y. and Ito,K. (1987) *EMBO J.*, **6**, 3465–3470.

Bedouelle,H., Bassford,P.J., Fowler,A.V., Zabin,I., Beckwith,J. and Hofnung,M. (1980) *Nature*, **285**, 18–21.

Bieker,K.L., Phillips,G.J. and Silhavy,T.J. (1990) *J. Bioenerg. Biomembr.*, **22**, 291–310.
Bieker-Brady,K. and Silhavy,T.J. (1992) *EMBO J.*, **11**, 3165–3174.
Emr,S.D. and Silhavy,T.J. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 4599–4603.
Emr,S.D., Hanley-Way,S. and Silhavy,T.J. (1981) *Cell*, **23**, 79–88.
Fikes,J.D. and Bassford,P.J. (1989) *J. Bacteriol.*, **171**, 402–409.
Gardel,C., Benson,S., Hunt,J., Michaelis,S. and Beckwith,J. (1987) *J. Bacteriol.*, **169**, 1286–1290.
Gardel,C., Johnson,K., Jacq,A. and Beckwith,J. (1990) *EMBO J.*, **9**, 3209–3216.
Geller,B.L. (1990) *J. Bacteriol.*, **172**, 4870–4876.
Guzman,L.M., Barondess,J.J. and Beckwith,J. (1992) *J. Bacteriol.*, **174**, 7716–7728.
Kumamoto,C.A. (1991) *Mol. Microbiol.*, **5**, 19–22.
Maloy,S.R. (1990) *Experimental Techniques in Bacterial Genetics*. Jones and Bartlett, Boston, MA.
Matsuyama,S., Fujita,Y. and Mizushima,S. (1993) *EMBO J.*, **12**, 265–270.
Michaelis,M., Hunt,J.F. and Beckwith,J. (1986) *J. Bacteriol.*, **167**, 160–167.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mizushima,S., Tokuda,H. and Matsuyama,S. (1992) In Neupert,W. and Lill,R. (eds), *Membrane Biogenesis and Protein Targeting*. Elsevier, Amsterdam, pp. 21–32.
Nishiyama,K., Mizushima,S. and Tokuda,H. (1993) *EMBO J.*, **12**, 3409–3415.
Oliver,D.B. (1993) *Mol. Microbiol.*, **7**, 159–165.
Pogliano,K.J. and Beckwith,J. (1993) *Genetics*, **133**, 763–773.
Pogliano,K.J. and Beckwith,J. (1994) *J. Bacteriol.*, in press.
Russel,C.B., Thaler,D.S. and Dahlquist,F.W. (1989) *J. Bacteriol.*, **171**, 2609–2613.
Ryan,J.P. and Bassford,P.J.,Jr (1985) *J. Biol. Chem.*, **260**, 14832–14837.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning, A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schatz,P.J. and Beckwith,J. (1990) *Annu. Rev. Genet.*, **24**, 215–248.
Schatz,P.J., Riggs,P.D., Jacq,A.J., Fath,M.J. and Beckwith,J. (1989) *Genes Dev.*, **3**, 1035–1044.
Schatz,P.J., Bieker,K.L., Ottemann,K.M., Silhavy,T.J. and Beckwith,J. (1991) *EMBO J.*, **10**, 1749–1757.
Ueguchi,C. and Ito,K. (1990) *J. Bacteriol.*, **172**, 5643–5649.
Wickner,W., Driessen,A.J.M. and Hartl,F.U. (1991) *Annu. Rev. Biochem.*, **60**, 101–124.

Received on August 20, 1993; revised on October 25, 1993