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Suppressor analysis suggests a multistep, cyclic mechanism for protein secretion in *Escherichia coli*

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The *sec/prl* gene products catalyze the translocation of precursor proteins from the cytoplasm of *Escherichia coli*. Recessive, conditionally lethal mutant alleles of these genes (*sec* mutations) cause a generalized defect in protein secretion; dominant suppressor mutant alleles (*prl* mutations) restore export of precursor proteins with altered signal sequences. In *prl* strains, a precursor protein with a defective signal sequence can be selectively targeted to the suppressor gene product. When a precursor LacZ hybrid protein is used, the targeted prl protein is inactivated by the large, toxic hybrid molecule, a result termed suppressor-directed inactivation (SDI). Using SDI, two different secretion-related complexes can be generated: a pretranslocation complex that contains a hybrid protein with an unprocessed signal sequence, and a translocation complex in which the hybrid protein is jammed in transmembrane orientation with the signal sequence cleaved. Additional Sec proteins that are contained within, and thus sequestered by, each of these complexes can be identified when their functional levels are lowered using the conditional lethal *sec* mutations. Results of this genetic analysis suggest a multistep pathway for protein secretion in which the translocation machinery assembles on demand.

Key words: protein secretion/*sec* mutants/signal sequence

Introduction

Genes encoding components of the protein secretion machinery in *Escherichia coli* have been identified using two fundamentally different genetic strategies. One approach exploits *lacZ* fusions to identify mutations in essential genes that confer generalized secretion defects. Genes identified in this manner have been termed the *sec* genes for the recessive, conditional lethal *sec*retion defects conferred. The second approach seeks dominant, extragenic suppressors of signal sequence mutations. Genes identified in this manner have been termed the *prl* (*pr*otein *l*ocalization) genes. The *prl* suppressors ameliorate the secretion defect conferred by a variety of signal sequence mutations in a number of different genes, and they are thought to act by broadening the specificity of the protein secretion machinery. Six *sec* genes have been recognized, *secA, secB, secD, secE, secF* and *secY*; three of these genes, *secA/prlD, secE/prlG* and *secY/prlA* were also identified using the suppressor approach. In the remaining three cases, *secB, secD* and *secF,* no corresponding *prl* alleles have been identified, perhaps because the function of these Sec proteins does not involve or depend upon the signal sequence. All of the *sec* genes have been cloned, the DNA sequences have been determined and the protein products have been identified (Bieker *et al.*, 1990; Schatz and Beckwith, 1990). The general properties of the Sec proteins are summarized in Table I.

The events that occur in the cytoplasm and involve the soluble components SecA and SecB are relatively well understood. SecB is a secretion-specific chaperonin that forms a stoichiometric complex with the precursor forms of secreted proteins, thus maintaining them in an export-competent conformation. SecB is essential only in rapidly growing cells, suggesting that its function is required only when demand on the secretion pathway is high (Randall *et al.*, 1987; Bassford, 1990; Kumamoto, 1990). SecA associates specifically with both precursor proteins and SecB, and targets them to the membrane component SecY and/or SecE (see below). When SecA is in contact with all of these essential components, it hydrolyzes ATP (Mizushima and Tokuda, 1990; Oliver *et al.*, 1990; Wickner *et al.*, 1991).

The four other *sec* genes, *secD, secE, secF* and *secY,* encode integral membrane proteins. It was generally assumed that at least some of these Sec proteins would function in the translocation of precursor proteins across the cytoplasmic membrane, a process analogous to protein translocation into the lumen of the endoplasmic reticulum in eukaryotic cells. Genetic analysis of the translocation reaction has been difficult because a defect in any of these components confers the same phenotype, i.e. precursor accumulation in the cytoplasm. The inability to detect different biochemical intermediates precludes the application of double mutants to establish functional order using traditional tests of epistasis.

To gain insights into the nature of the secretion pathway and to probe the interactions between the various Sec proteins, we have developed two novel genetic approaches: suppressor-directed inactivation (SDI) and Sec titration (Bieker and Silhavy, 1989, 1990). SDI exploits the toxicity of secreted LacZ hydrid proteins in combination with dominant *prl* mutations to selectively inactivate one component of the secretion machinery. Sec titration extends this analysis to determine which Sec proteins are present in, and thus titrated by, the inactivated LacZ hydrid-Prl complex. Our data suggested that precursor proteins are targeted by SecA to SecE and that subsequent SecE-SecY interaction is required to form a functional complex that allows translocation.

Recent biochemical studies support the hypothesis that there is a SecE-SecY interaction and demonstrate directly that these two proteins function in translocation (Brundage *et al.*, 1990, 1992; Matsuyama *et al.*, 1990; Akimaru *et al.*, 1991). However, the nature of the interaction of SecA with the membrane components SecE and/or SecY remains

somewhat unclear. In addition, no functional role has yet been established for SecD or SecF. Indeed, the available biochemical data suggest that these two proteins are not required for protein translocation *in vitro*. We report here the specific application of SDI and Sec titration to address these important issues.

Background

High level synthesis of secreted LacZ (β-galactosidase) hybrid proteins is lethal. Accordingly, the *lamB−lacZ42-1* fusion confers an inducer-sensitive phenotype (Mal^s, Table II). Amino-terminal secretion signals in the LamB (maltoporin or λ receptor) portion of the hybrid protein direct the large carboxy-terminal fragment of LacZ to the cellular secretion machinery. LacZ sequences are not compatible with this machinery, probably owing to problems of protein folding (Lee *et al.*, 1989; Phillips and Silhavy, 1990) and this leads to lethal jamming. The secretion-specific nature of this jammed phenotype is demonstrated with signal sequence mutations. When the hybrid protein carries a defective signal sequence (*lamB17D−lacZ42-1*), it is not recognized by the secretion machinery and induction of hybrid protein synthesis results in benign accumulation of the hybrid molecule in the cytoplasm (Mal^r, Table II). When jamming does occur, the hybrid molecule is found in transmembrane orientation, apparently stuck in the translocation apparatus (for review, see Bieker *et al.*, 1990). We have shown previously that in this case cells die because SecY function is lost, and we conclude from this that SecY is a limiting component of the translocator (Figure 1 and Bieker and Silhavy, 1989).

Table I. Components of the *Escherichia coli* export machinery

Component			
Sec	Prl	Molecular mass (kDa)	Cellular location
SecA	PrlD	102	Peripheral membrane, cytoplasm
SecB	–	12	Cytoplasm
SecD	–	65	Integral inner membrane
SecE	PrlG	13.6	Integral inner membrane
SecF	–	35	Integral inner membrane
SecY	PrlA	49	Integral inner membrane

−indicates that no *prl* signal sequence suppressor alleles in the corresponding *sec* gene have been identified.

Suppressor-directed inactivation

SDI exploits the novel interaction between defective signal sequences and components of the secretion machinery altered by suppressor (*prl*) mutation. This interaction targets LacZ hybrid proteins bearing a signal sequence mutation (LamB17D−LacZ) to the Prl suppressor protein. If the interaction is stable, the Prl component will be sequestered by the toxic LacZ hybrid protein and inactivated, thus restoring the Mal^s phenotype. Although the *prl* suppressors are dominant, the Mal^s phenotype of SDI strains is recessive. When the wild-type (prl^+ or sec^+) allele is present *in trans*, the inactive Prl−LacZ hybrid protein complex forms, but function never becomes limiting since the wild-type Sec protein does not recognize the toxic LacZ molecule and remains free to perform its secretion-related business. SDI has been used in the analysis of two suppressors, *prlA* (*secY*) and *prlG* (*secE*) (Bieker and Silhavy, 1989, 1990). In *prlA* strains, the mutant LacZ hybrid protein jams in transmembrane fashion, producing an inactive complex (translocation complex) similar to that seen with wild-type fusions in wild-type strains. In *prlG* strains, the hybrid protein is stuck at a step that precedes signal sequence processing (pretranslocation complex, see Figure 1). Table 2 shows the Mal^s phenotype of the relevant strains quantified using a disk assay (see Materials and methods). Data presented here serve as controls for the experiments which follow.

Sec titration

SDI indicates that the limiting component in the pretranslocation and translocation complexes is PrlG/SecE and PrlA/SecY, respectively. Additional Sec proteins could be present in either of these complexes provided they are present in functional excess. To test this possibility, we have developed the Sec titration assay. Sec titration takes advantage of the conditional lethal *sec* alleles. By growing *sec* mutant strains at semi-permissive temperatures, the functional level of a given Sec protein can be lowered to the point at which it becomes limiting for protein secretion. One can then ask if induction of an SDI complex titrates the remaining Sec protein, causing lethality (Bieker and Silhavy, 1990). This strategy is illustrated in Figure 2.

The SDI strains carry the *lamB17D−lacZ42-1* gene fusion and are heterozygous for the suppressor allele and its wild-type counterpart (*prlA4/secY*$^+$, Figure 1C). These diploids are Mal^r because nothing is limiting for protein secretion when the jammed complex is formed. If a particular *sec* allele is introduced into these strains, they will remain Mal^r at the

Table II. Suppressor-directed inactivation

Strain		Chromosome	Plasmid	Diameter of sensitivity[a]		
				30°C	35°C	37°C
Controls	pop3186	*lamB−lacZ*		26	20	14.5
	SE1073	*lamB17D−lacZ*		0	0	0
Pretranslocation complex	KB215	*prlG1, lamB17D−lacZ*		19	2	0
	KB382	*prlG*$^+$, *lamB17D−lacZ*	*prlG1*	0	0	0
	KB375	*prlG1 lamB17D−lacZ*	*prlG*$^+$	0	0	0
Translocation complex	BKR73A4	*prlA4 lamB17D−lacZ*		22	16	14
	KB942	*prlA*$^+$ *lamB17D−lacZ*	*prlA4*	0	0	0
	KB941	*prlA*$^+$ *lamB17D−lacZ*	*prlA*$^+$	0	0	0

[a]Numbers indicate diameter of sensitivity (mm) to 0.01 ml 10% maltose after the subtraction of disk diameter (7 mm) at the indicated temperature.

permissive temperature regardless of the nature of the interaction of the Sec protein with the SDI complex. At semi-permissive temperatures, when functional levels of the Sec protein are limiting, two possible outcomes follow the induction of hybrid protein synthesis. If the Sec component being tested is not stably associated with the SDI complex, the strain will remain Mal^r. Although the Sec protein is limiting for protein secretion under these conditions, its ability to function at lowered levels is not further compromised by the formation of the SDI complex (Figure 2B). Conversely, if the Sec protein is a stable component of the SDI complex, maltose induction at the semi-permissive temperature will be lethal (Mal^s); the remaining Sec protein present in the cell will be titrated, and thus inactivated, by incorporation into the SDI complex (Figure 2D). As noted above, we have used this method previously to show that SecE (PrlG) is present not only in the pretranslocation complex, but also in the translocation complex (Bieker and Silhavy, 1990).

Results

Strains used for Sec titration at the pretranslocation complex carry the *lamB17D−lacZ42-1* gene fusion, a $secE^+$ chromosomal gene and a low copy number plasmid bearing either the *prlGl* suppressor allele or the corresponding wild-

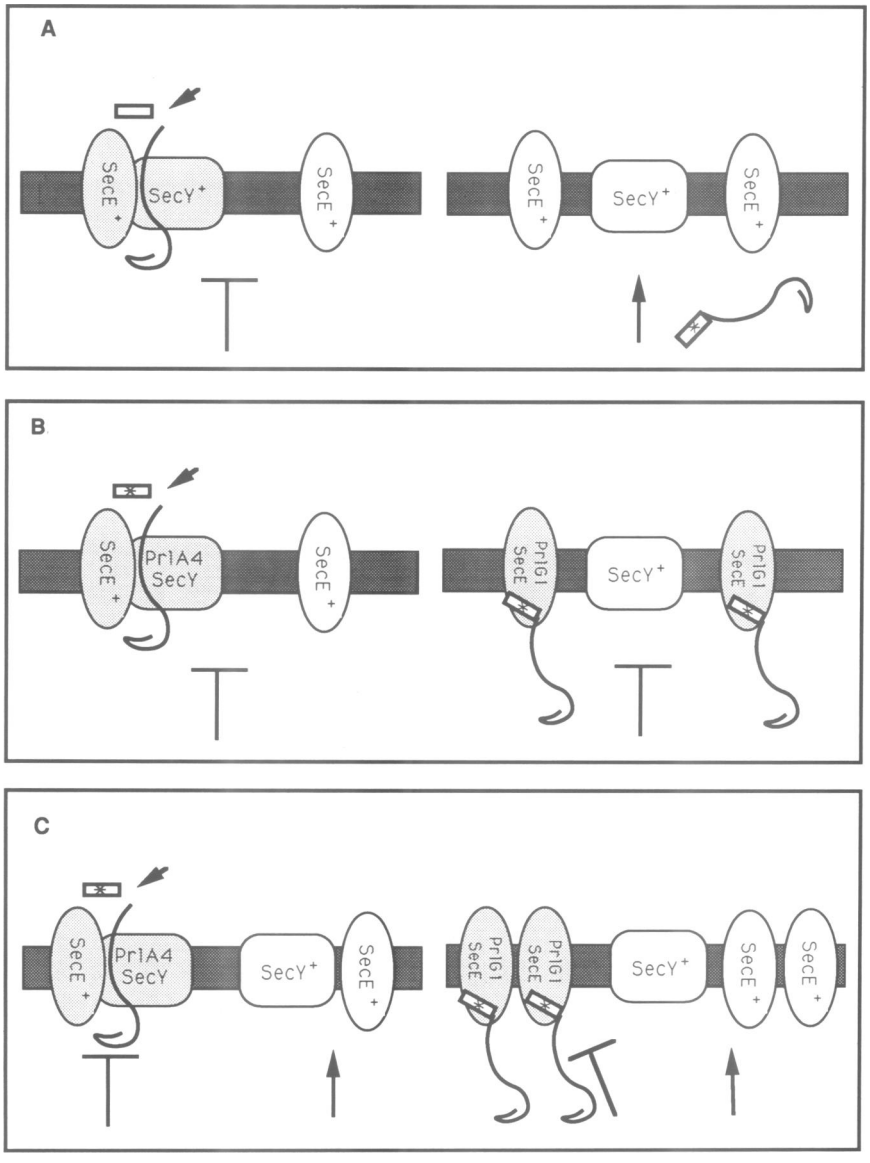

Fig. 1. Suppressor directed inactivation (SDI). (A) (Left) Transmembrane jamming of a LamB−LacZ hybrid protein with a wild-type signal sequence. SecY and SecE and sequestered in the jammed complex and export is blocked. (Right) Cytoplasmic localization of a LamB−LacZ hybrid protein with a defective signal sequence. Export is not blocked. (B) (Left) SDI at PrlA4 (SecY): the *prlA4 (secY)* suppressor allele restores recognition and transmembrane jamming of the LamB−LacZ hybrid protein with a defective signal sequence. PrlA4 (SecY) and SecE are sequestered and rendered non-functional, and export is blocked. (Right) SDI at PrlG1(SecE): the *prlG1 (secE)* suppressor allele restores recognition of the hybrid protein with a defective signal sequence and the PrlG1(SecE) protein is sequestered and rendered non-functional. Export is blocked. (C) (Left) SDI at PrlA4(SecY) in the presence of $SecY^+$: the SDI complex is formed, but translocation proceeds via $SecY^+$ and excess SecE. (Right) SDI at PrlG1 (SecE) in the presence of $SecE^+$: the SDI complex forms at PrlG1 (SecE), but export proceeds via $SecE^+$. See text for additional details. Symbols: inner membrane is indicated by dark shaded rectangle with the cytoplasmic face below and the periplasmic space above; sequestered components are indicated by light shadings; ▭ : signal sequence, *: defect in signal sequence; ⌒ : mature portion of hybrid protein; small arrow: cleavage by signal peptidase; large arrow: available pathway for export; ⊣ : blocked export pathway.

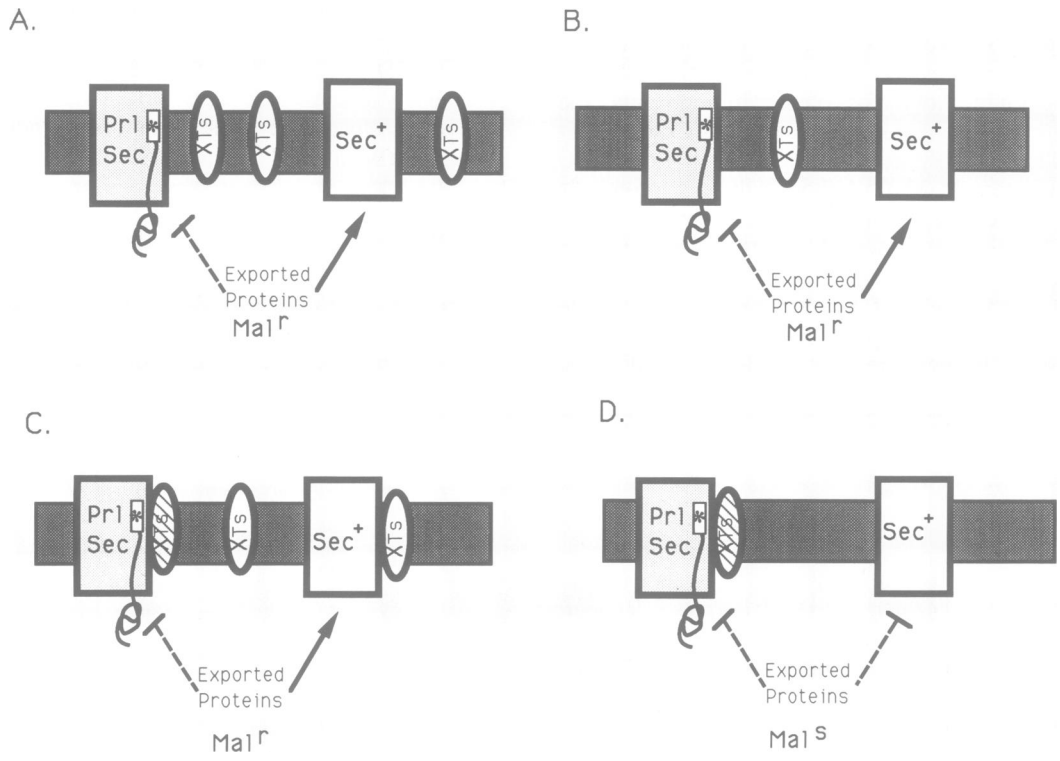

Fig. 2. Sec titration. Symbols are as described in Figure 1. except that 'X' indicates the temperature-sensitive Sec component being tested for titration. The panels represent the situation in a Sec titration strain following maltose induction of hybrid protein synthesis. (A) The permissive temperature when X is not a stable component of the SDI complex. (B) The semi-permissive temperature when X is not stable component of the SDI complex. (C) The permissive temperature when X is a stable component of the SDI complex. (D) The semi-permissive temperature when X is a stable component of the SDI complex. See text for additional details.

type *secE*$^+$ gene. Strains used to test for Sec titration at the translocation complex are basically identical except that they are merodiploid and heterozygous for *secY*; *secY*$^+$ is on the chromosome and *prlA4* is on a low copy number plasmid vector. This strategy was used to facilitate comparisons between Sec titration results obtained with both jammed complexes. The only obvious difference between strains in the two sets of experiments is the plasmid carrying the different suppressor alleles, *prlGl* or *prlA4*.

Sec titration with SecY

It was observed previously that SecE is present in functional excess to SecY. However, SDI at the PrlGl (SecE) suppressor is recessive to *secE*$^+$ alone (Bieker and Silhavy, 1990). Accordingly, we had predicted that SecY would not be present in the pretranslocation complex. To test directly for the stable association of the SecY protein in the pretranslocation complex, we utilized the *secY39*(Cs) allele (Riggs *et al.*, 1988) in the Sec titration assay. The DNA sequence of this allele has been determined and the cold sensitive defect is the result of an Arg357→His substitution in the fourth cytoplasmic loop (Baba *et al.*, 1990). The presence of this mutation causes an export defect at temperatures below 37°C and the defect is lethal at 23°C (Baba *et al.*, 1990). As shown in Table III, the Sec titration strain carrying the *secY39* allele (KB388) was maltose-resistant at both temperatures tested, 30°C and 35°C, indicating that the SecY protein cannot be titrated in the pretranslocation complex under these conditions.

The *secY39* mutation does increase maltose-sensitivity in the haploid parent strain at both temperatures tested (compare KB215, Table II with KB217, Table III). This increase in maltose-sensitivity probably reflects a synergy between the SDI defect and the *secY* defect and indicates that SecY function has become limiting and is in the range of potential titration.

Table III. Pretranslocation complex Sec titration: SecY

Strain	Chromosome	Plasmid	Diameter of sensitivity[a] 30°C	35°C
KB371	*prlG*$^+$, *lamB17D−lacZ, secY39(Cs)*		0	0
KB217	*prlG1, lamB17D−lacZ, secY39(Cs)*		22	3
KB389	*prlG*$^+$ *lamB17D−lacZ, secY39(Cs)*	*prlG*$^+$	0	0
KB388	*prlG*$^+$ *lamB17D−lacZ, secY39(Cs)*	*prlG1*	0	0

[a]Numbers indicate diameter of sensitivity (mm) to 0.01 ml 10% maltose after the subtraction of disk diameter (7 mm) at the indicated temperature.

The equivalent titration experiment was also performed using the *secY24* (Shiba *et al.*, 1984) temperature-sensitive mutation (Gly240→Asp in the third cytoplasmic loop) and the same results were obtained (data not shown). The results presented in this section support the prediction that SecY is not a stable component of the pretranslocation complex.

Sec titration with SecD and SecF

Three *secD* alleles, *secD1, secD29* and *secD57*, and the *secF62* allele were used to test for the titration of the SecD and SecF proteins (Gardel *et al.*, 1990). The DNA sequence alterations caused by these mutations has not yet been determined, so the exact nature of the defects are not known. However, all four alleles are cold-sensitive, causing

Table IV. Pretranslocation complex Sec titration: SecD and SecF

Strain	Chromosome	Plasmid	Diameter of sensitivity[a] 30°C	37°C
KB184	*prlG*$^+$, *lamB17D–lacZ, secD1(Cs)*		0	0
KB216	*prlG1, lamB17D–lacZ, secD1(Cs)*		29	31
KB385	*prlG*$^+$, *lamB17D–lacZ, secD1(Cs)*	*prlG*$^+$	0	0
KB384	*prlG*$^+$, *lamB17D–lacZ, secD1(Cs)*	*prlG1*	0	0
KB302	*prlG*$^+$, *lamB17D–lacZ, secD29(Cs)*		0	0
KB305	*prlG1, lamB17D–lacZ, secD29(Cs)*		27	10
KB398	*prlG*$^+$, *lamB17D–lacZ, secD29(Cs)*	*prlG*$^+$	0	0
KB397	*prlG*$^+$, *lamB17D–lacZ, secD29(Cs)*	*prlG1*	0	0
KB303	*prlG*$^+$, *lamB17D–lacZ, secD57(Cs)*		0	0
KB306	*prlG1, lamB17D–lacZ, secD57(Cs)*		28	23H
KB402	*prlG*$^+$, *lamB17D–lacZ, secD57(Cs)*	*prlG*$^+$	0	0
KB401	*prlG*$^+$, *lamB17D–lacZ, secD57(Cs)*	*prlG1*	0	0
KB304	*prlG*$^+$, *lamB17D–lacZ, secF62(Cs)*		0	0
KB307	*prlG1, lamB17D–lacZ, secF62(Cs)*		36	0
KB406	*prlG*$^+$, *lamB17D–lacZ, secF62(Cs)*	*prlG*$^+$	0	0
KB405	*prlG*$^+$, *lamB17D–lacZ, secF62(Cs)*	*prlG1*	0	0

[a]Numbers indicate diameter of sensitivity (mm) to 0.01 ml 10% maltose after the subtraction of disk diameter (7 mm) at the indicated temperature. H indicates a hazy, rather than clear, zone of inhibition to growth around the disk.

Table V. Translocation complex: Sec titration of SecD and SecF

Strain	Chromosome	Plasmid	Diameter of sensitivity[a] 30°C	37°C
KB136	*prlA*$^+$, *lamB–lacZ, secD1(Cs)*		34	26
KB350	*prlA*$^+$, *lamB17D–lacZ, secD1(Cs)*	*prlA*$^+$	0	0
KB351	*prlA*$^+$, *lamB17D–lacZ, secD1(Cs)*	*prlA4*	22.5	19.5
KB368	*prlA*$^+$, *lamB–lacZ, secD29(Cs)*		30	23
KB352	*prlA*$^+$, *lamB17D–lacZ, secD29(Cs)*	*prlA*$^+$	0	0
KB353	*prlA*$^+$, *lamB17D–lacZ, secD29(Cs)*	*prlA4*	27	0
KB369	*prlA*$^+$, *lamB–lacZ, secD57(Cs)*		38	27
KB354	*prlA*$^+$, *lamB17D–lacZ, secD57(Cs)*	*prlA1*$^+$	0	0
KB356	*prlA*$^+$, *lamB17D–lacZ, secD57(Cs)*	*prlA4*	8	2
KB370	*prlA*$^+$1, *lamB–lacZ, secF62(Cs)*		37	29
KB357	*prlA*$^+$, *lamB17D–lacZ, secF62(Cs)*	*prlA*$^+$	0	0
KB358	*prlA*$^+$, *lamB17D–lacZ, secF62(Cs)*	*prlA4*	12	2H

[a]Numbers indicate diameter of sensitivity (mm) to 0.01 ml 10% maltose after the subtraction of disk diameter (7 mm) at the indicated temperature. H indicates a hazy, rather than clear, zone of inhibition to growth around the disk.

secretion-specific defects at temperatures as high as 37°C and lethality at 23°C.

Results obtained for Sec titration of SecD and SecF by the pretranslocation complex are shown in Table IV. Assay strains KB384, KB397, KB401 and KB405 remain maltose-resistant, implying that neither SecD nor SecF interacts stably with the pretranslocation complex. In contrast, identical experiments to test for SecD and SecF interaction with the translocation complex show a positive titration result, i.e. strains KB351, KB353, KB356 and KB358 exhibit pronounced maltose-sensitivity at the semi-permissive growth temperature, 30°C (Table V). These results suggest that both SecD and SecF are stable components of the translocation complex.

It is important to reiterate that the only relevant difference between strains used for titration experiments with the pretranslocation complex (Table IV) and those used for titration experiments with the translocation complex (Table V) is the plasmid bearing the suppressor allele, *prlG1* or *prlA4*. Accordingly, these two sets of experiments serve as controls for each other and it is clear that positive titration results are suppressor-specific.

At the translocation complex, titration with the *secD29* allele was considerably stronger than that seen with the *secD57* allele. This difference is consistent with the relative severity of the secretion defects these alleles confer at the semi-permissive temperatures utilized (Gardel *et al.*, 1990; K.Johnson and J.Beckwith, personal communication).

A number of other control experiments are presented in Tables IV and V to demonstrate further the specificity of the titration assay. In haploid strains that permit hybrid protein export, the *secD* and secF mutations always cause substantially increased maltose-sensitivity relative to their *sec*$^+$ counterparts (Table II). As noted above, this probably reflects a synergy between the secretion defect caused by the hybrid protein and the SecD or SecF limitation caused by the conditional mutation. However, in haploids or diploids, when hybrid protein export is blocked by the signal sequence mutation and no suppressor allele is present, the *secD* or *secF* mutant strains always remain maltose-resistant. Maltose-sensitivity requires hybrid jamming.

Our results indicate that both SecD and SecF are stable components of the translocation complex. Because the proteins do not titrate in the pretranslocation complex, it would appear that they function late in the secretion pathway as predicted by Gardel *et al.* (1990).

Sec titration with SecA

Previous attempts to determine if SecA was titrated by the translocation complex yielded negative results (Bieker and Silhavy, 1990). In contrast, positive results were obtained using the same method to determine if SecA was titrated by the pretranslocation complex. However, repeated retesting showed that these results were somewhat unpredictable; occasionally a negative result was obtained. This erratic behavior led us to suspect an epigenetic mechanism. Titration experiments with temperature-sensitive *secA* mutations are difficult because conditions that compromise the secretion machinery derepress synthesis of SecA (Oliver and Beckwith, 1982). Accordingly, these experiments set up a race between competing mechanisms, induction of the toxic hybrid protein and derepression of SecA synthesis. In some cases derepression of SecA synthesis may occur to a degree sufficient to override the lethal effects of hybrid protein induction.

To test the epigenetic explanation, we sought to ensure partial loss of SecA function by exposing cells to the semi-permissive growth condition for 1 h prior to the induction of hybrid protein synthesis. When assays are performed in this manner, the results are consistent; SecA can be titrated by both the pretranslocation and the translocation complexes (Table VI). These results indicate that SecA interacts stably with both.

Control experiments similar to those described in previous sections (Table VI) yield expected results with one exception. Strain KB241 (*prlG1 secA51*) is less maltose-sensitive at 30°C than the corresponding *secA*$^+$ strain KB215 (Table II). Whether or not this reflects a derepression of SecA synthesis

Table VI. Sec titration with SecA

Strain	Chromosome	Plasmid	Diameter of sensitivity[a] 30°C	35°C
Controls				
KB46	*lamB–lacZ, secA51(Ts)*		21	28
KB107	*lamB17D–lacZ, secA51(Ts)*		0	0
Pretranslocation complex				
KB241	*prlG1, lamB17D–lacZ, secA51(Ts)*		2.5	19
KB393	*prlG*$^+$, *lamB17D–lacZ, secA51(Ts)*	*prlG1*$^+$	0	0
KB392	*prlG1*$^+$, *lamB17D–lacZ, secA51(Ts)*	*prlG1*	0	16
Translocation complex				
KB471	*prlA*$^+$, *lamB17D–lacZ, secA51(Ts)*	*prlA*$^+$	0	0
KB472	*prlA*$^+$ *lamB17D–lacZ, secA51(Ts)*	*prlA4*	0	23

[a]Numbers indicate diameter of sensitivity (mm) to 0.01 ml 10% maltose after the subtraction of disk diameter (7 mm) at the indicated temperature. Plates were prewarmed for 60 min at the test temperature before adding maltose (see Materials and methods).

remains to be determined. Nonetheless, in merodiploids, the maltose-sensitivity is suppressor-dependent and is observed only under semi-permissive growth conditions. Thus, we think it likely that SecA is present in both the pretranslocation and translocation complexes.

Discussion

Two secretion-related, integral membrane complexes have been defined by suppressor-directed inactivation (SDI). These two complexes were distinguished both by their unique Sec protein composition and by the biochemical state of the toxic LacZ hybrid protein that entraps these presumptive translocation intermediates. When the pretranslocation complex is generated, the hybrid protein signal sequence is uncleaved, suggesting that translocation has not yet started. When the translocation complex is generated, the hybrid protein is oriented in a transmembrane fashion; signal sequence cleavage has occurred, signifying partial translocation of the LacZ hybrid protein across the inner membrane (Bieker and Silhavy, 1990). To probe the nature and composition of both the pretranslocation and translocation complexes further, we have utilized recessive, conditionally lethal mutations in all known *sec* genes to reduce functional levels of each of these proteins in turn. This method should allow the detection of Sec proteins which stably interact with, and are thus titrated by, these complexes *in vivo*. We realize that the strategy of Sec titration is complex; the interaction of many components is inferred from the manipulation of subtle experimental conditions. However, the data obtained show a satisfying degree of internal consistency.

Previous studies using SDI suggested that SecY was not a stable component of the pretranslocation complex. The Sec titration assays presented here support this suggestion. Thus, it appears that SecE acts at a step in the secretion pathway which precedes a stable association with SecY.

In the Sec titration experiments for SecD and SecF, the sets of strains utilized differ only in the *prl* suppressor allele supplied *in trans*, and lethality (Mal^s), when it is observed, always results from a defect in protein secretion. Thus, results obtained with the pretranslocation and translocation complexes serve as controls for each other, making both positive and negative results more meaningful by ruling out many alternative explanations. Successful titration of SecD and SecF at the translocation complex ensures that functional levels of these proteins are low enough to have been titrated at the pretranslocation complex. Similarly, the negative titration results at the pretranslocation complex ensure that the positive result at the translocation complex is not false because of a non-titration-specific phenomenon that affects the Mal^s phenotype indirectly. Taken together, the data suggest that SecD and SecF function at a late step in the secretion pathway.

Titration results with SecA have proven the most difficult to interpret. In order to obtain data that were consistently reproducible, we incubated *secA5l* mutant strains at the semi-permissive conditions for an hour prior to hybrid protein induction. We believe this step negates, at least partially, the known derepression of SecA synthesis that occurs when protein secretion is compromised. Even so, certain control experiments show unexpected results as noted in Results. Despite these qualifications, we suspect that the positive titration results are meaningful. Maltose-sensitivity is a phenotype that is characteristic of secretion defects and alternative explanations are not easy to envision. Moreover, our prediction that SecA is present in both complexes is consistent with available biochemical data as noted below.

Our interpretation of the Sec titration experiments is perhaps best summarized with a model which is shown schematically in Figure 3: (i) Initially, SecA interacts with the precursor, which is probably associated with SecB or an equivalent chaperonin (not shown); at this stage, the components of the translocation machinery are disassembled (Figure 3, I). (ii) SecA then directs the protein precursor to SecE at the cytoplasmic face of the inner membrane to form the pretranslocation complex (Figure 3, II). SecY, SecD and SecF are not stable components of this complex. (iii) SecE then recruits SecY, vectorial movement of the precursor across the membrane occurs and the signal sequence is cleaved by the signal peptidase on the periplasmic face of the inner membrane. *In vitro* translocation studies suggest that SecA is associated with the translocation machinery at an intermediate step that does not require SecD and SecF (III). (iv) A translocation complex which includes SecA, SecD, SecE, SecF and SecY is assembled and the cleaved mature form of the exported protein is translocated and released (IV and V). (v) Finally, the translocator disassembles and becomes ready for the next round of translocation (I). This model, which is based on data obtained

Table VII. Strain list

Strain	Genotype	References
MC4100	F-*araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Casadaban (1976)
C648	MC4100 *secD1(Cs) zaj*Tn*10*	Beckwith collection
KJ173	MC4100 *phoR secD29(Cs) zaj*Tn*10*	Beckwith collection
KJ178	MC4100 *phoR secD57(Cs) zaj*Tn*10*	Beckwith collection
KJ184	MC4100 *phoR secF62(Cs) zaj*Tn*10*	Beckwith collection
pop3186	MC4100 *lamB−lacZ42-1*	Emr and Silhavy (1980)
SE1073	MC4100 *lamB17D−lacZ42-1*	Emr and Silhavy (1980)
STA1000	F-*araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoCl ptsF25 rbsR*	Stader and Silhavy (1988)
LG9	STA1000 *prlG1*	Stader *et al.* (1989)
BKR73A4	SE1073 *prlA4* 90% linked Tn*10*	Bieker and Sihavy (1989)
KB35	SE1073 *prlG1* 90% linked Tn*10*	Bieker and Silhavy (1990)
KB46	pop3186 *secA51(Ts) recA*::Tn*5*	Bieker and Silhavy (1990)
KB94	SE1073 *recA*::Tn5	Bieker and Silhavy (1990)
KB107	SE1073 *secA51(Ts) recA*::*CAM*	This study
KB136	pop3186 *secD1(Cs) zaj*Tn*10*	This study
KB184	SE1073 *secD1(Cs) zaj*Tn*10 recA*::*CAM*	This study
KB196	SE1073 *prlG1*	This study
KB211	SE1073 *secA51(Ts)*	This study
KB215	SE1073 *prlG1 recA*::*CAM*	This study
KB216	SE1073 *prlG1 secD1(Cs) zaj*Tn*10 recA*::*CAM*	This study
KB217	SE1073 *prlG1 secY39(Cs) zhc*Tn*10 recA*::*CAM*	This study
KB241	SE1073 *prlG1 secA51(Ts) recA*::*CAM*	This study
KB302	SE1073 *secD29(Cs) zaj*Tn*10 rec*::*CAM*	This study
KB303	SE1073 *secD57(Cs) zajTn10 recA*::*CAM*	This study
KB304	SE1073 *secF62(Cs) zaj*Tn*10 recA*::*CAM*	This study
KB305	KB196 *secD29(Cs) zaj*Tn*10 recA*::*CAM*	This study
KB306	KB196 *secD57(Cs) zaj*Tn*10 recA*::*CAM*	This study
KB307	KB196 *secF62(Cs) zaj*Tn*10 recA*::*CAM*	This study
KB350	pJS100(*prlA*$^+$)/KB184	This study
KB351	pTA204(*prlA4*)/KB184	This study
KB352	pJS100(*prlA*$^+$)/KB302	This study
KB353	pTA204(*prlA4*)/KB302	This study
KB354	pJS100(*prlA*$^+$)/KB303	This study
KB356	pTA204(*prlA4*)/KB303	This study
KB357	pJS100(*prlA*$^+$)/KB304	This study
KB358	pTA204(*prlA4*)/KB304	This study
KB368	pop3186 *secD29(Cs) zaj*Tn*10*	This study
KB369	pop3186 *secD57(Cs) zaj*Tn*10*	This study
KB370	pop3186 *secF62(Cs) zaj*Tn*10*	This study
KB371	SE1073 *secY39(Cs) zhc*Tn*10 recA*::*CAM*	This study
KB374	pAF23(*secE*$^+$)/KB211	This study
KB375	pAF21 (*prlG1*)/KB215	This study
KB382	pAF21(*prlG1*)/KB94	This study
KB384	pAF21(*prlG1*)/KB184	This study
KB385	pAF23(*secE*$^+$)/KB184	This study
KB388	pAF21(*prlG1*)/KB371	This study
KB389	pAF23(*secE*$^+$)/KB371	This study
KB392	pAF21(*prlG1*)/KB107	This study
KB393	pAF23(*secE*$^+$)/KB107	This study
KB397	pAF21(*prlG1*)/KB302	This study
KB398	pAF23(*secE*$^+$)/KB302	This study
KB401	pAF21(*prlG1*)/KB303	This study
KB402	pAF23(*secE*$^+$)/KB303	This study
KB405	pAF21(*prlG1*)/KB304	This study
KB406	pAF23(*secE*$^+$)/KB304	This study
KB471	pJS100(*prlA*$^+$)/KB47	Bieker and Silhavy (1990)
KB472	pTA204(*prlA4*)/KB47	Bieker and Silhavy (1990)
KB941	pJS100(*prlA*$^+$)/SE1073 *recA*::Tn5	Bieker and Silhavy (1990)
KB942	pTA204(*prlA4*)/SE1073 *recA*::Tn5	Bieker and Silhavy (1990)

in vivo, is largely consistent with, and does not contradict, biochemical analyses obtained *in vitro* (Brundage *et al.*, 1990, 1992; Hartl *et al.*, 1990; Akimura *et al.*, 1991). Rather it develops and extends current views as noted below.

The targeting of precursors to the membrane

Early genetic studies predicted an interaction between SecA and SecY (Brickman *et al.*, 1984). However, careful analysis fails to support this conclusion (Baba *et al.*, 1990).

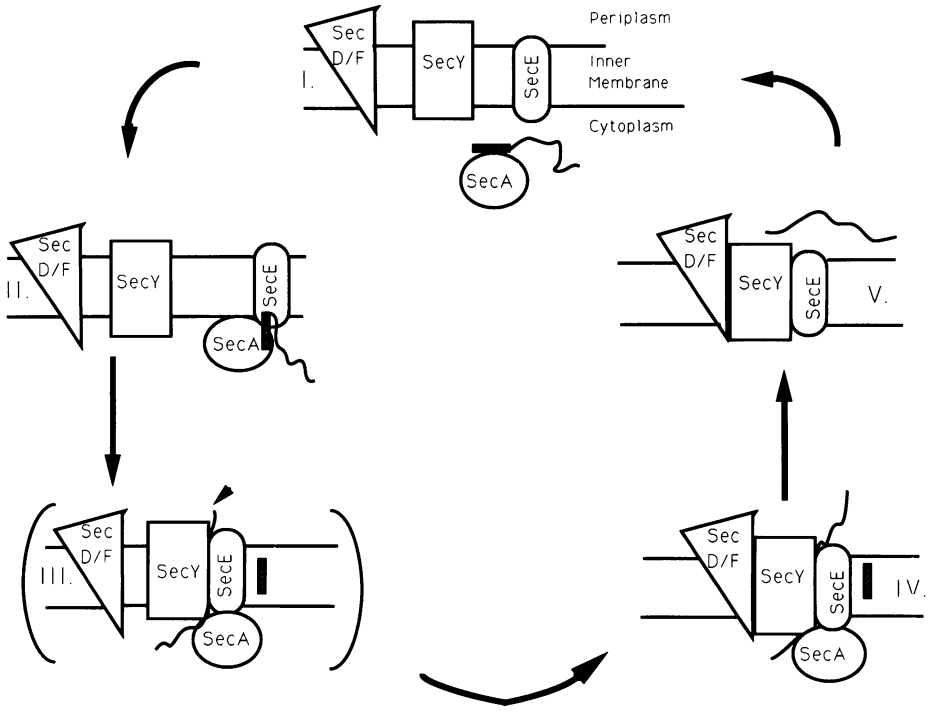

Fig. 3. A model for protein translocation. See Discussion for details.

Nonetheless, biochemical studies support such an interaction. *In vitro*, high levels of SecA can suppress the translocation defect associated with the SecY(Ts) mutant protein (Fandl *et al.*, 1988). In addition, Hartl *et al.* (1990) have shown that antibodies to an amino-terminal peptide of SecY disrupt binding of SecA to vesicles. Moreover, the binding of SecA to vesicles confers protease protection to the SecY protein.

While we believe that SecA interacts first with SecE, our results do not warrant comment on the proposed interaction between SecA and SecY. SecA appears to be present in both the pretranslocation and translocation complexes. Perhaps a more important question is why the pretranslocation complex has not been detected biochemically. In this context, we can suggest two explanations. First, the pretranslocation complex may not normally be stable without hybrid protein jamming. If this is the case, the step assayed biochemically by SecA binding would be the first stable interaction in the membrane, i.e. that which involves both SecE and SecY. Second, although SecE does not stably interact with SecY at the pretranslocation step, these two proteins may be maintained in close proximity through a less stable interaction. This would allow disruption of SecA binding by SecY antibodies and the protection of SecY by SecA in protease protection assays.

SecE interaction with SecY

The model clearly predicts an interaction between SecE and SecY, and this has been amply supported by both genetic (Bieker and Silhavy, 1990) and biochemical analyses (Brundage *et al.*, 1990, 1992; Matsuyama *et al.*, 1991; Akimaru *et al.*, 1991). Furthermore, we would predict an essential involvement of these two proteins in the translocation reaction (see also Bieker and Silhavy, 1989, 1990) and this prediction has also received substantial biochemical support (Brundage *et al.*, 1990, 1992; Akimaru *et al.*, 1991). However, Watanabe *et al.* (1990) reach a different conclusion, suggesting that SecY is not required for translocation. The significance of their results is not entirely clear (see below).

A role for SecD and SecF

Evidence for the involvement of both SecD and SecF in protein secretion is based on the general accumulation of the precursor forms of exported proteins in the cytoplasm and derepression of SecA synthesis that are observed with conditionally lethal mutants (Gardel *et al.*, 1990). Such results could be due to indirect effects. Our data strongly support a direct involvement of these proteins in the pathway of protein secretion. Indeed, these two proteins clearly appear as components of the translocation complex. Yet, there remains no biochemical evidence for function. This lack of congruence between genetics and biochemistry raises what is perhaps the most pressing question in the field of protein secretion: what is the function of SecD and SecF?

Gardel *et al.* (1990) have suggested that SecD and SecF function late in the secretion pathway based on two observations. First, it is predicted that both of these integral membrane proteins have large periplasmic domains, suggesting that they act subsequent to translocation initiation. Second, extensive searches for extragenic suppressors of signal sequence defects have not revealed mutations in either structural gene, consistent with the idea that the encoded proteins may function after signal sequence cleavage. We have found that both SecD and SecF can be titrated in the translocation complex. This finding is in contrast to the observation that neither is a stable component of the pretranslocation complex. Taken together, these results support the suggestion that SecD and SecF function late in the translocation process.

We cannot discern whether SecD and SecF participate in the same or different functions. However, the coregulation

of the encoding genes (Gardel *et al.*, 1990) and the identical titration patterns suggest that their functions are tightly linked. Two observations lead us to suggest that these two proteins may be involved in the release and recycling steps of translocation. First, neither the SecD nor the SecF protein appears to be required for translocation into vesicles *in vitro*, suggesting that they do not play a direct role in the movement of proteins across the membrane (Brundage *et al.*, 1990, 1992; Akimaru *et al.*, 1991). However, it has not been shown that these vesicles function catalytically with rates and efficiencies comparable with those observed *in vivo*. If SecD and SecF are only required for release and recycling of the translocator, this may account for the apparent expendability of these essential gene products *in vitro*. Second, defects in the SecD and SecF proteins have a profound effect on the lethality caused by hybrid protein jamming of the translocation machinery, exacerbating the lethality far more than comparable export defects in SecE, SecY or SecA. The translocation complex in which SecD and SecF reside may be a terminally jammed complex, representing the very last stage of translocation and release of the exported protein. In wild-type cells, these two proteins may catalyze inefficient release of the toxic hybrid protein from the jammed complex. If this is the case, defects in SecD and SecF would have a synergistic effect, thus enhancing the lethality of hybrid jamming.

The question of recycling

We propose a translocation machinery that assembles on demand. Indeed, we submit that Sec titration experiments would not be feasible if the translocation machinery were preassembled into a static complex. If this were the case, it would be impossible to sequester all of the Sec protein required for a positive titration result.

Mixed biochemical results have been obtained regarding the SecE and SecY interaction and recycling. The Wickner laboratory isolates the two proteins in a complex (Brundage *et al.*, 1990); the Mizushima laboratory can isolate both proteins independently and reconstitute activity (Akimaru *et al.*, 1991; Tokuda *et al.*, 1991). A recent, collaborative publication attempts to address this issue, but fails to reach a conclusion regarding recycling (Brundage *et al.*, 1992). More detailed, biochemical analysis of the catalytic mechanism is required to address this question.

As noted above, the Blobel lab has reported successful translocation in the absence of SecY (Watanabe *et al.*, 1990). We question this conclusion, but note that our model predicts significant progress of precursor proteins through the secretion pathway in the absence of SecY. Under these circumstances, the precursor protein could proceed to the pretranslocation complex and so a certain degree of protease resistance might be expected. Since current biochemical assays measure protease resistance rather than complete translocation, this remains an open question.

Blobel and Dobberstein (1975) anticipated that precursor proteins may traverse the membrane through a water-filled channel. Recently, evidence supporting the hypothesis that there is such a channel has been presented for mammalian systems (Simon and Blobel, 1991). If this prediction is verified, we think it likely that bacteria will utilize a similar channel based on the overall similarity of the translocation reaction. In bacteria, an open, water-filled channel of this size would be lethal owing to membrane depolarization. A recycling mechanism, like the one we propose, offers a convenient mechanism to inactivate such a channel when not in use.

Testing predictions of the model

The utility of any model stems from the testable predictions it makes. Clearly the interaction(s) between SecA and the membrane-bound components of the secretion machinery needs examination in greater detail. In this regard, both genetic and biochemical approaches can be envisioned. Our proposal for a recycling mechanism requires biochemical verification. Although currently available data concerning recycling are inconclusive, efforts to address this issue have already been made (Brundage *et al.*, 1992). Determining the functions of SecD and SecF will probably prove more problematic. However, the model suggests specific roles that should facilitate experimental design. Given the sophistication of the *E.coli* experimental system, we can expect rapid resolution of these important questions.

Materials and methods

Media and chemicals

Media and chemicals have been described elsewhere (Silhavy *et al.*, 1984).

Bacterial strains

All strains are derivatives of the *E.coli* K-12 strains MC4100 and STA1000, and are described in Table VII. Strains were constructed using the standard genetic techniques of P1 transduction and plasmid transformation (Silhavy *et al.*, 1984; Miller, 1972). With the exception of *secA* all *sec* alleles were moved using a genetically linked Tn*10*, selecting for resistance to tetracycline and screening for the appearance of the appropriate conditionally lethal phenotypes. *secA* alleles were moved by transduction from the *secA* donor strain into a *leu::Tn10* strain, selecting for Leu$^+$ and screening for the appearance of the conditionally lethal phenotype. *prlGl (secE)* strains were constructed by selecting for loss of the *argE::Tn10* in the recipient strain or by transduction using a *Tn10* insertion linked to the desired *secE* allele, following by marker rescue of the *prlGl* suppressor allele into the strain bearing *lamB14D*. The presence of the suppressor in this strain restores growth on dextrin as a sole carbon source. All strains were constructed and tested a minimum of two times, to ensure accuracy. In addition, cultures from strains bearing conditionally lethal mutations were screened at the non-permissive temperature in parallel to the experiment to guard against reversion. This precaution is especially relevant for strains bearing the *secD29* and *secD57* alleles, which yield pseudorevertants at high frequency.

In general strains were constructed in haploid, with the plasmid added at the last step, where appropriate. The one exception is the *secF62* mutation, which could only be added to strains bearing at least one copy of *secY*$^+$. Further testing revealed that the *secF62* allele caused a severe growth defect when present in haploid with the *prlA4 (secY)* allele, even when no fusion was present. This sickly phenotype was completely recessive to the *secY*$^+$ allele.

Plasmids

Two *prlA* bearing low copy plasmids were utilized in these studies. Both are derived from pSC101 vectors, and carry the *bla*$^+$ gene (Bieker and Silhavy, 1990). pJS100 bears a 16.2 kb *Bam*HI fragment which includes the *spc* and α operons cloned into pKA101. In this vector *secY*$^+$ is under P*spc* control. pTA204 carries a 2440 bp fragment bearing the *prlA4* allele cloned into a unique site in pTA108. The inserted fragment includes the genes for ribosomal proteins L30 and L15. In this vector *prlA4* is under regulation of the *lac* promoter. Both plasmids have been described previously and behave in a manner indistinguishable for the chromosomal alleles in all tests of complementation and suppresssion that have been performed.

The two isogenic *secE* bearing plasmids, pAF21 and pAF23 were constructed by A.Flower. pAF23 was constructed by subcloning a 1.1 kb fragment from pJS51 (carries *secE*$^+$, Schatz *et al.*, 1991) into the pSC101 derivative vector pLG339 (Stoker *et al.*, 1982). pAF23 is identical except that it carries the *prlGl (secE)* allele.

Maltose-sensitivity disk array

Sensitivity to maltose was determined using a disk array technique, analogous to that used to test sensitivity to antibiotics. Cells were grown overnight

in LB medium at the permissive temperature, centrifuged and resuspended in 0.5 vol 5 mM $MgSO_4$, 10 mM $CaCl_2$. 0.1 ml of the cell suspension and any supplemental amino acids were then added to 2.5 ml molten F-top agar, mixed and plated on glycerol M63 minimal agar. 10 μl of 10% and 20% maltose were added to separate 7 mm analytical filter paper disks. Plates were incubated overnight at the temperatures indicated in Results. When the *secA51* mutation was present, plates were prewarmed for 60 min to ensure partial loss of SecA function at the time of maltose addition. Maltose-sensitivity was calculated as the diameter of inhibition to growth (clearing around the disk) following incubation overnight, and is expressed as the diameter of sensitivity measured minus the diameter of the disk. When no sensitivity was observed, a score of 0 mm was given. Sensitivity scores presented in tables represent the average of at least five assays.

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