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Suppression of Signal Sequence Defects and Azide Resistance in *Escherichia coli* Commonly Result from the Same Mutations in *secA*

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The SecA protein of *Escherichia coli* is required for protein translocation from the cytoplasm. The complexity of SecA function is reflected by missense mutations in the *secA* gene that confer several different phenotypes: (i) conditional-lethal alleles cause a generalized block in protein secretion, resulting in the cytoplasmic accumulation of the precursor forms of secreted proteins; (ii) *azi* alleles confer resistance to azide at concentrations up to 4 mM; and (iii) *prlD* alleles suppress a number of signal sequence mutations in several different genes. To gain further insights into the role of SecA in protein secretion, we have isolated and characterized a large number of *prlD* mutations, reasoning that these mutations alter a normal function of wild-type SecA. Our results reveal a striking coincidence of signal sequence suppression and azide resistance: the majority of *prlD* alleles also confer azide resistance, and all *azi* alleles tested are suppressors. We suggest that this correlation reflects the mechanism(s) of signal sequence suppression. There are two particularly interesting subclasses of *prlD* and *azi* alleles. First, four of the *prlD* and *azi* alleles exhibit special properties: (i) as suppressors they are potent enough to allow PrlD (SecA) inactivation by a toxic LacZ fusion protein marked with a signal sequence mutation (suppressor-directed inactivation), (ii) they confer azide resistance, and (iii) they cause modest defects in the secretion of wild-type proteins. Sequence analysis reveals that all four of these alleles alter Tyr-134 in SecA, changing it to Ser, Cys, or Asn. The second subclass consists of seven *prlD* alleles that confer azide supersensitivity, and sequence analysis reveals that six of these alleles are changes of Ala-507 to Val. Both of the affected amino acids are located within different putative ATP-binding regions of SecA and thus may affect ATPase activities of SecA. We suggest that the four azide-resistant mutations slow an ATPase activity of SecA, thus allowing successful translocation of increased amounts of mutant precursor proteins.

Proteins such as LamB (maltoporin, λ receptor) and MalE (maltose-binding protein), which are destined for secretion to the outer membrane and the periplasm of *Escherichia coli*, respectively, are synthesized in precursor form with a cleavable signal sequence at the amino terminus. This sequence targets proteins to the secretion pathway for translocation out of the cytoplasm. Mutations that alter the signal sequence prevent proper targeting and result in accumulation of the precursor form of the molecule in the cytoplasm. A variety of genetic and biochemical methods have been employed to determine the role of the signal sequence and to understand the mechanism by which proteins are translocated across the cytoplasmic membrane (for reviews, see references 6, 48, and 61).

One approach that has been successfully employed in *E. coli* to identify components of the cellular secretion machinery involves the isolation and characterization of suppressors of signal sequence mutations. These suppressors, which are termed *prl* for protein localization, appear to broaden the specificity of the secretion machinery to allow recognition of precursor molecules with defective signal sequences. Three genes, *prlA*, *prlG*, and *prlD*, have been identified repeatedly in a variety of suppressor selections. Subsequent analysis revealed that these suppressors are alleles of *secY*, *secE*, and *secA*, respectively. Recessive, loss-of-function mutations in each of these three genes block the secretion pathway and cause a generalized precursor accumulation in the cytoplasm. Biochemical analysis supports the view that these proteins play a critical role in the translocation reaction; indeed, successful reconstitution of translocation in vitro with only these three proteins has been reported (reference 1, but also see references 10, 11, and 60).

SecY/PrlA and SecE/PrlG are integral cytoplasmic membrane proteins. Recently, homologs of these two proteins have been identified in other bacteria, archaebacteria, yeasts, and mammals, and it has been suggested that these proteins may form a channel through which secreted proteins pass as they leave the cytoplasm (16, 24, 26). This discovery, together with the universal presence of signal sequences in secreted proteins, argues that the basic mechanism of protein secretion has been conserved throughout nature. While homologs of SecA have been found in other bacteria, in plastids of algae, and in chloroplasts of plants (5, 14, 34, 35, 39, 46, 47, 59, 62), none has been discovered in yeasts or mammals. Whether this reflects an important difference between prokaryotes and eukaryotes remains to be determined.

The SecA protein is large (102 kDa) and complex. It is found both in soluble form in the cytoplasm and embedded in the cytoplasmic membrane (12). This unusual behavior in fractionation studies may reflect a role for SecA in the targeting of precursor proteins from the cytoplasm to the translocation sites (SecY/E) in the membrane (25). SecA functions as a dimer (3, 17), and it is known to exhibit ATPase activity (30). This hydrolytic activity, which is required for translocation, is stimulated by precursor proteins, acidic phospholipids, and SecY/E (31). Recently, ATP hydrolysis was shown to drive SecA insertion and deinsertion from inner membrane vesicles (18, 27), and this likely reflects a key role for SecA in the energetics of translocation.

Several years ago, mutations conferring resistance to azide

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were found to be located in *secA* (23, 37). Thus, as it turns out, the first *secA* mutations were actually isolated by Lederberg (29), who termed them *azi*. Azide is known to inhibit certain types of ATPases, consistent with the known activities of SecA. Apparently, SecA is the most azide-sensitive, essential ATPase in *E. coli* under the selection conditions employed. This resistance is low level (up to 4 mM azide); higher levels kill Az^r cells presumably by other mechanisms. Additionally, it is known that azide inhibits the ATPase activity required for both in vivo and in vitro translocation (37), although the nature of the interactions between azide and SecA remains to be elucidated.

The *prlA* and *prlG* suppressor alleles provide useful tools for analyzing the translocation reaction. For example, these alleles form the basis for a genetic technique termed suppressor-directed inactivation (SDI). This technique has been used to block the secretion pathway at various stages and works in the following manner: LamB-LacZ hybrid proteins that contain a functional signal sequence can cause a lethal jamming of the cellular secretion machinery. Signal sequence mutations in the hybrid gene prevent jamming. Certain *prlA* and *prlG* suppressors restore lethality, and under these conditions, it can be demonstrated with diploid analysis that the suppressor gene product is inactivated by the toxic fusion protein. Apparently the LamB-LacZ fusion protein tagged with a defective signal sequence can inactivate the suppressor protein that is trying to secrete it. Further analysis of these jammed complexes suggested temporal order in the secretion pathway: SecA targets precursor proteins to the membrane at SecE, and SecY is recruited into the complex at a later step (7–9).

It seems likely that suppressor mutations alter a normal activity of the wild-type protein rather than creating an entirely new activity. Accordingly, an understanding of Prl suppressor action could reveal insights into the functions of these critical Sec proteins. It has long been assumed that these suppressors restore recognition of mutant signal sequences. However, recent work suggests an alternative model (15, 22, 38). This view posits a signal sequence proofreading activity for the wild-type secretion machinery, which normally causes rejection of precursor molecules with a defective signal. The *prlA* and *prlG* suppressors may act by bypassing this proofreading step, thus allowing defective molecules to proceed through the secretory pathway.

In order to gain insight into the complex nature of SecA function, we have undertaken an analysis of the *prlD* suppressor alleles. The results presented here show a striking correlation between signal sequence suppression and azide resistance. On the basis of these results, we propose that at least a subset of the *prlD* suppressors affect an activity of SecA related to ATP hydrolysis.

MATERIALS AND METHODS

Media and chemicals. Solid and liquid media have been described previously (52). Maltodextrins were obtained from Pfanstiel Laboratories, Inc. The purification of maltodextrins by dialysis has been described previously (58). For selecting suppressors, a concentration of the purified maltodextrin solution in M63 minimal agar was chosen such that MC4100 (*lamB*$^+$) grows and KB81 (*lamB14D leu*::Tn*10*) does not grow. Sodium azide was obtained from Sigma Chemical Co. [^{35}S]methionine was obtained from DuPont, NEN Research Products.

Bacterial strains. *E. coli* K-12 strain MC4100 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] (13) is the isogenic parent strain for pop3186 [*lamB-lacZ(42–1)*] (53), SE1073 [*lamB17D-lacZ(42–1)*] (20), and all *prlD* and *azi* derivatives of these strains constructed in this study. STA1000 is isogenic to MC4100 (57) and is the isogenic parent strain for STA14D (*lamB14D*), KB81 (*lamB14D leu*::Tn*10*) (K. Bieker), STA1013 (*lamB*Δ*78*), NT178 [*secA51*(Ts)] (N. Trun), and all *prlD* and *azi* derivatives of these strains constructed in this study. W208 (CGSC 1867; *azi-4*), χ148 (CGSC 6492; *azi-6*), Hfr Hayes (CGSC 5492; *azi-7*), and SS320 (CGSC 6414; *azi-9*), sources of the Lederberg (29) *azi* mutations, were obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University (B. Bachmann). Mapping markers *zac-3093*::Tn*10kan* and *zad-3094*::Tn*10kan* were obtained from the collection of Singer et al. (54). LP68 [MC4100 *nadA*::Tn*10* Δ(*gal-att-bio*)] was provided by L. Pratt. Strains were constructed by standard genetic techniques (32, 52). *prlD*, *azi*, and *secA* alleles were moved via P1 transduction with linkage to a Tn*10* insertion in *leuB* (1.7 min). The presence or absence of the alleles in each strain background was verified by screening for the azide phenotype and/or by moving the allele via P1 transduction into a *lamB14D* strain and checking for the suppressor phenotype (marker rescue).

Phages and plasmids. P1 transductions and preparation of P1*vir* and λ*vir* lysates were carried out as previously described (52). pMF8, a pBR322-derived plasmid carrying the *geneX-secA* operon, has been described previously (50). pJH1 was constructed by the removal of a *′geneX-secA* fragment from pMF8 by an *Eco*RI digest, gel purification of the vector, religation, and transformation into the appropriate strains. pJH1 was used as a negative control in all experiments with pMF8. λPR9 is a λ*imm*^{21} phage that carries the *secA*$^+$ and *envA*$^+$ genes (41), and it was provided by D. Oliver. Strains with λPR9 were constructed by plating the phage on the desired strain and purifying lysogens by screening for immunity to the λ phage B500 (λ *h*80 *imm*^{21} *c*I) and screening for sensitivity to phage φ80*vir* to ensure that the cells are still phage sensitive (52). To check whether λPR9 integrated at the chromosomal *attB* λ integration site and not into the *secA* locus, *nadA*::Tn*10* linked to a *gal-att-bio* deletion was transduced into these strains from LP68. The resulting Gal$^-$ transductants had lost immunity to the phage B500 and also lost the λPR9-mediated phenotypes, thus verifying the correct integration of λPR9.

Localized spontaneous mutagenesis. Localized spontaneous mutagenesis was previously described by Trun and Silhavy (58). *prlD* mutants were selected in the strain KB81 (*lamB14D leu*::Tn*10*). On two separate occasions, KB81 was streaked on Luria-Bertani agar containing tetracycline and incubated at 37°C overnight. Each time, 10 tubes containing 5 ml of Luria-Bertani agar each were then inoculated from 10 individual colonies and grown overnight at 37°C. The independent cultures were pelleted and resuspended in one-half volume of 10 mM $MgSO_4$–5 mM $CaCl_2$, and 0.1 ml from each was spread on maltodextrin M63 minimal agar and incubated at 37°C. Dex$^+$ suppressors arose at a frequency of 10^{-6}. The Dex$^+$ colonies were then pooled for each plate, and a P1 phage lysate was prepared from each pool. The lysates were used to transduce STA14D (*lamB14D*) to tetracycline resistance (Tet^r) on dextrin-tetracycline MacConkey plates at 37°C (thus selecting for P1 phage carrying regions of the chromosome containing *leu*::Tn*10*). Tet^r Dex$^+$ transductants (red or pink colonies on dextrin-tetracycline MacConkey agar) were purified for further characterization. These strains were screened for λ sensitivity by cross-streaking against λ*vir* on glucose (LamB repressed), glycerol (LamB not induced), and maltose (LamB induced) minimal agar plates. Strength of suppression was determined with these phenotypic assays.

Isolation of azide resistant mutants. Azide resistant mutants (*azi*) were selected by plating independent cultures on Luria-Bertani agar plates containing 3.5 mM sodium azide at 37°C. The strain used for selection was the same as that used for the procedure described above (KB81), and the azide resistance and suppression phenotypes were reassessed after moving these mutations into STA14D and MC4100 via P1 transduction; mapping was also performed with these strains.

Maltose sensitivity and azide sensitivity disk assays. Cells of the appropriate strains were grown in Luria-Bertani media (with antibiotics when necessary) to saturation, pelleted, and resuspended in one-half volume of 10 mM $MgSO_4$–5 mM $CaCl_2$. A total of 0.1 ml of the cell suspension and any supplemental amino acids or antibiotics was then mixed with 3 ml of molten F-top agar and plated on glycerol M63 minimal agar for the maltose sensitivity assay or Luria-Bertani agar for the azide sensitivity assay. Seven-millimeter-diameter paper filter disks (Schleicher and Schuell) were then placed on top of the hardened F-top agar. For the maltose sensitivity assay, 10 μl of 2.5% maltose was added to the disks, and for the azide sensitivity assay, 10 μl of 1 M azide was added to the disks. The plates were incubated overnight at 30°C (maltose assay) or 37°C (azide assay). The diameter of the zone of sensitivity was measured, subtracting the diameter of the filter disk (7 mm).

Pulse-chase assay for MalE processing in LacZ fusion strains. *lamB-lacZ* (with or without the *17D* signal sequence mutation) strains were grown to saturation in glycerol M63 liquid medium at 37°C and subcultured (1:20) in fresh glycerol M63 liquid medium at 30°C to an optical density at 600 nm of ~0.25. LamB-LacZ fusion protein and MalE synthesis were then induced with 0.2% maltose for 40 min, after which the cells were labeled with [^{35}S]methionine for 20 s, followed by chase time points of 10 s through 4 min. LamB-LacZ and MalE were immunoprecipitated with anti-LacZ and MalE antibodies, respectively. LacZ antibodies (rabbit anti-β-galactosidase) were obtained from 5 prime→3 prime, Inc. Samples were subjected to electrophoresis with sodium dodecyl sulfate (SDS)–10% polyacrylamide gels, followed by autoradiography and imaging with a PhosphorImager (Molecular Dynamics).

Pulse-chase assay for MalE and LamB processing. Cells were grown to saturation in glycerol M63 liquid medium at 37°C and subcultured (1:20) in fresh glycerol M63 liquid medium at 30°C, with 0.4% maltose to induce synthesis of MalE and LamB, to an optical density at 600 nm of approximately 0.30. This procedure has been described previously (55). [^{35}S]methionine labeling was performed with a 20-s pulse followed by chase time points of 10 s to 4 min, and

TABLE 1. Phenotypic characterization of the *prlD* suppressors

Allele(s)		Phage sensitivity of λ*vir* on[a]:			Use of maltodextrins[b]
lamB	*prl*	Glu	Gly	Mal	
lamB$^+$	*prlD*$^+$	S	S	S	Rd
lamB14D	*prlD*$^+$	R	R	R	Wh
lamB14D	*prlD2*, *-4*, *-5*, *-20–24*, *-26*, *-27* and *azi-17*	S/R	S/R	S	Rd
lamB14D	*prlD28–33*	R	R/S	S	DPk
lamB14D	*prlD25*, *-34–49* and *azi-4*, *-6*, *-7*, *-9*, *-11–16*	R	R	S/R	Pk or Wh/Rd centers
lamB14D	*prlA4*	S	S	S	Rd
lamB14D	*prlG1*	S/R	S/R	S	Rd

[a] Phage sensitivity was determined by cross-streaking of each strain across λ*vir* on M63 minimal agar supplemented with glucose (Glu), glycerol (Gly), or maltose (Mal). R, resistant; S, sensitive; R/S, more resistant than sensitive; S/R, more sensitive than resistant.

[b] Ability to utilize maltodextrins was scored on dextrin MacConkey agar. Rd, red (LamB$^+$); Wh, white (LamB$^-$); DPk, dark pink; Pk, pink; Wh/Rd centers, white with red centers (LamB$^\pm$).

samples were immunoprecipitated with anti-MalE, anti-LamB, and/or anti-SecA polyclonal antibodies, as described in reference 58. Anti-SecA antibody was provided by D. Oliver. Samples were subjected to electrophoresis with SDS–10% polyacrylamide gels, followed by autoradiography and imaging with a PhosphorImager.

Quantitation of the percentage of mMalE. Densitometry was performed with the ImageQuant system (Molecular Dynamics) to analyze images obtained from the PhosphorImager. The percentage of mature MalE (mMalE) as a function of total MalE protein (including precursor [pMalE]) was obtained by the following equation: mMalE − bkgd/[(mMalE + pMalE) − 2(bkgd)], where bkgd is the background for each lane in the gel. The amounts of background, mMalE, and pMalE in each time point lane in the gel were determined by placing rectangular boxes (identical in size) around each band and subsequent integration of the boxes with ImageQuant. mMalE has lost three of its original nine methionines by signal sequence cleavage; to correct for the corresponding loss in radioactivity of mMalE from pMalE, the amount of mMalE from each time point was multiplied by 9/6 (40).

PCR amplification and DNA sequencing of *prlD* and *azi* alleles. The *secA* gene was amplified by PCR with the single colony method as described previously (43), with four primers in order to amplify overlapping halves of the gene: SecA1 (nucleotides [nt] 635 to 654, 5′GGCACGCCGTCTGAAAAGGG3′) and SecA6 (nt 2396 to 2377, 5′CGCGGCAACTTCTGCCTGCC3′) and SecA10 (nt 2088 to 2105, 5′CTGCCGGACCTGGTCTAC3′) and SecA4 (nt 3668 to 3648 5′GCG CATCTGCTGCGCGACGCG3′), with nt designating the nucleotide position within the *E. coli geneX-secA* DNA sequence (51). Standard DNA sequencing of the PCR products was then accomplished with primers spaced approximately every 200 to 300 bp within *secA*. The *prlD20*, *-21*, *-22*, *-23*, *-28*, and *-43* mutations are the only alterations that were found within the entire *secA* coding sequence, including the Shine-Dalgarno site. Because *prlD24*, *-26*, *-27*, and *-31* are identical to *prlD20* and *-28*, the complete DNA sequence was not determined.

RESULTS

Previously characterized *prlD* suppressors. The first *prlD* alleles, *prlD1*, *-2*, *-3*, *-4*, and *-5*, were isolated as suppressors of mutations that alter the hydrophobic core of the signal sequence of MalE (4, 44, 45). DNA sequence analysis verified that *prlD2*, *-4*, and *-5* are unique alleles of *secA* and are located in three widely spaced regions of SecA (21). *prlD3* is the same mutation as *prlD2*. The *prlD1* allele appeared to lie outside of *secA* (4); however, cells carrying this mutation grew poorly, and the mutation has been lost.

Isolation of additional *prlD* suppressors. LamB functions in *E. coli* as the maltoporin and also as the phage λ receptor. Cells carrying *lamB* signal sequence mutations cannot grow with maltodextrins as a sole carbon source (Dex$^-$), are resistant to λ phage (λ^r), and accumulate the precursor form of LamB in the cytoplasm. Suppressors that restore export of the mutant precursor protein are easily selected by demanding growth on maltodextrin minimal agar (Dex$^+$ phenotype). Recently, in a large screen for *prl* suppressors of the *lamB14D* allele (a change of valine to aspartate at amino acid position 14 in the signal sequence), our laboratory isolated 17 extragenic suppressors at or near the *secA* locus. Linkage was shown by transductional mapping with *leu*::Tn*10*, which is approximately 50% linked to *secA* (56).

In this study, we employed a different method, localized spontaneous mutagenesis (described in Materials and Methods), to obtain 13 additional *prlD* suppressors of *lamB14D*. In transductional crosses, the 13 new alleles and the previously isolated 17 alleles are tightly linked to *secA51*(Ts) and similarly linked to outside markers: 48% linkage to *zac-3093*::Tn*10kan* (2.0 min), 42% to *leu*::Tn*10* (1.7 min), 13% to *araC* (1.4 min), and 4% to *zad-3094*::Tn*10kan* (3.5 min).

The 30 *prlD* alleles were numbered on the basis of the strength of their suppressor phenotypes (Dex$^+$ and λ^s) in the *lamB14D* strain. These data are summarized in Table 1. For comparison, the three unique *prlD* mutations isolated by the Bassford group are also included. Thus, the total number of *prlD* suppressors examined in this study is 33. As can be seen in Table 1, 10 of the *prlD* suppressors are comparable in strength to *prlG* suppressors, while the rest are weaker. In general, *prlA* suppressors are more potent than either *prlG* or *prlD* suppressors. Suppressor strength correlates directly with the amount of LamB in the outer membrane.

Suppression and azide resistance are highly coincident. Azide resistance, which maps to the *secA* locus (23, 37), was used for fine structure mapping to more firmly establish the linkage observed between the new alleles and *secA51*(Ts). Surprisingly, we found that most of the suppressors already confer azide resistance (Az^r). Not only are 20 of 33 of the *prlD* alleles resistant to 3 mM azide on L agar, but all 3 of the original *prlD* alleles also confer resistance.

To quantitate the level of Az^r, we used a disk assay as described in Materials and Methods. The results revealed that almost one-fourth (7 of 33) of all the *prlD* suppressors cause another azide phenotype, supersensitivity to azide (Az^{ss}), compared with the same strain containing *secA*$^+$. Almost an additional quarter of the total number of *prlD* alleles (6 of 33) confer similar azide sensitivity (Az^s) to *secA*$^+$ strains.

To further investigate the correlation between suppression and Az^r, we obtained (from B. Bachmann) Az^r mutations (*azi-4*, *-6*, *-7*, and *-9*) that were isolated by Lederberg (29). These *secA* alleles also turn out to be suppressors of *lamB14D*, as judged by the Dex$^+$ and λ^s phenotypes of these strains (Table 1). Suppression is similar to that of the other *prlD* alleles. Thus, the first *prlD* suppressors were actually isolated in 1950 as *azi* mutations.

In addition, we isolated seven new *azi* mutations (*azi-11*, *-12*, *-13*, *-14*, *-15*, *-16*, and *-17*) by selecting for resistance to 3.5 mM azide. All contain mutations that map to *secA*. These mutations are also suppressors of *lamB14D*; they confer similar Dex$^+$ and

TABLE 2. Azide resistance is highly coincident with suppression

Allele(s)	Suppression[a]	Azide phenotype (disk assay)[b]
secA$^+$		S
prlD2, *-4*, *-5*, *-21–23*, *-29*, *-30*, *-34*, *-36–42*, *-45–48*	+	R
prlD20, *-24*, *-26–28*, *-31*, *-43*	+	SS
prlD25, *-32*, *-33*, *-35*, *-44*, *-49*	+	S
azi-4, *-6*, *-7*, *-9*, *-11–17*	+	R

[a] See Table 1.

[b] Resistance was scored with a disk assay as described in Materials and Methods. R, resistance (0- to 5-mm diameter of the zone of sensitivity at 37°C to 10 μl of 1 M azide after subtraction of the disk diameter [7 mm]); S, sensitive (15 to 17 mm); SS, supersensitive (20 to 28 mm).

λ^s phenotypes to the *prlD* alleles (Table 1). One of these, *azi-17*, is comparable to the strongest subset of *prlD* suppressors.

As summarized in Table 2, the majority (82%) of *prlD* suppressors exhibit altered azide sensitivity. In addition, all *azi* alleles, both new and previously isolated, suppress *lamB14D* with the efficiency of *prlD* alleles. We think it improbable that the high degree of correlation of these two phenotypes is a coincidence, and we believe it provides insight into the mechanism of *prlD* suppression.

Diploid analysis: the azide-resistant suppressors are allelic to *secA*. Fikes and Bassford (21) found that suppression of *malE14-1* by *prlD2* is dominant, similar to the dominance observed with *prlA* and *prlG* (6, 56). This is as expected, because *prl* alleles are gain-of-function mutations. All of the new *prlD* suppressors are dominant in diploid analysis with λPR9 (λ*secA*$^+$).

We were concerned that dominance could be attributed to a simple increase in the levels of SecA because slight suppression of *malE14-1* could be effected by a high-copy-number plasmid carrying *secA*$^+$ (21). We observed that pMF8, a similar plasmid carrying *secA*$^+$, allows weak suppression of *lamB14D*. Nevertheless, λPR9 lysogens, which are true *secA* diploids, do not exhibit detectable levels of suppression, confirming that suppression by the new *prlD* alleles is indeed dominant.

Az^r, however, is recessive (23, 37), and this allows mapping by complementation. The Az^r phenotype of all of the *prlD* and *azi* suppressors is recessive to *secA*$^+$ in *trans* (λPR9), proving that these mutants are allelic to *secA*. Thus, the new Az^r *prlD* suppressors and the seven new *azi* alleles are all *secA* alleles that merit the name *prlD*. The Az^{ss} phenotype conferred by *prlD* alleles is dominant, and the Az^s alleles also cannot be used in complementation tests. However, because their linkage to *secA* is indistinguishable from that of the Az^r alleles, we think it likely that these are *secA* alleles as well.

Biochemical characterization of suppression. To quantitate suppression of signal sequence mutations, we utilized a pulse-chase assay to monitor precursor maturation over time. This assay, while useful for quantitation, is not as sensitive an indicator of suppression as the phenotypic plate assays. For example, *prlA* suppressors are so potent that increased processing of mutant precursors can be observed in the pulse-chase assay even with strongly defective signal sequence mutations; processing cannot be observed with the same mutations in strains containing *prlG* (19, 55, 56). Suppression by *prlG* alleles can be observed in the pulse-chase assay with a signal sequence mutation that causes a less-severe secretion defect, such as *lamBΔ78*.

We were unable to detect suppression of *lamB14D* by the *prlD* alleles in the pulse-chase assay. However, all of the suppressors checked, including *prlD2*, *-4*, *-5*, *-21*, *-22*, and *-23* and *azi-7* and *-17*, show suppression of *lamBΔ78* (Fig. 1 [data shown for *prlD2* and *-21*]). Suppression of *lamBΔ78* is relatively weak compared with that of *prlA* suppressors, coincident with the weak Dex$^+$ and λ^s phenotypes exhibited by these strains. We were unable to reliably detect differences in strength of suppression between different *prlD* and *azi* alleles with the pulse-chase assay. However, the *prlD21*, *-22*, and *-23* and *azi-17* strains appear to produce mature LamB only by 2 min, while the *prlD2*, *-4*, and *-5* and *azi-7* strains show significant suppression by 10 s (Fig. 1, compare *prlD2* and *-21*). The difference in kinetics may reflect a difference in the mechanism of suppression between these sets of alleles (described below).

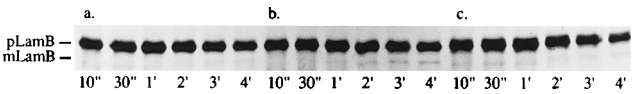

FIG. 1. Suppression of *lamBΔ78* by *prlD21* and *prlD2*. Cells were pulse-labeled with [^{35}S]methionine, and immunoprecipitations were performed as described in Materials and Methods. Shown is the processing of LamBΔ78 in the following strains: STA1013 (a), STA1013 containing *prlD21* (b), and STA1013 containing *prlD2* (c). p represents the precursor form of LamB, and m represents the mature form of this protein. Time points indicate the time of the trichloroacetic acid precipitation after the addition of chase.

We also used the pulse-chase assay to measure suppression of *lamBΔ78* in λPR9 lysogens and found that suppression by these representative alleles is dominant (data not shown). As noted above, wild-type λPR9 lysogens do not exhibit a suppressor phenotype. Overexpression of SecA, with pMF8, allows suppression of *lamBΔ78* (data not shown). However, the level of suppression is even less than that observed with *prlD2*, *-4*, *-5*, *-21*, *-22*, and *-23* and *azi-7* and *-17*.

As a whole, biochemical assays support our phenotypic observations. Suppression by the representative alleles tested, *prlD2*, *-4*, *-5*, *-21*, *-22*, and *-23* and *azi-7* and *-17*, is similar to that observed with the *prlG* suppressors, and it can only be detected in the pulse-chase assay with the leaky signal sequence mutation *lamBΔ78*.

Four of the *prlD* and *azi* alleles allow SDI. An additional test of suppression measures the ability of a *prl* allele to restore proper targeting of a mutant fusion protein, LamB17D-LacZ, into the secretion pathway (7–9). LamB-LacZ is lethal when induced to high levels with maltose (maltose sensitivity [Mal^s]) (53). However, the signal sequence mutation *17D* relieves this toxicity (maltose resistance [Mal^r]) (20). A certain number of the *prlA* and *prlG* suppressors redirect the fusion protein into the secretion pathway and restore Mal^s, while other suppressor alleles fail this test.

We found that 4 of the 44 suppressor alleles (9%) are able

TABLE 3. Suppression of *lamB17D-lacZ* by *prlD21* and *prlD2*

Strain	Diam (mm) of zone of maltose sensitivity[a]
prlD$^+$ *lamB-lacZ*	22
prlD$^+$ *lamB17D-lacZ*	0
prlA4 lamB17D-lacZ	15
prlG1 lamB17D-lacZ	14
prlD21 lamB17D-lacZ	14
prlD2 lamB17D-lacZ	0

[a] Values were obtained with a disk assay as described in Materials and Methods and indicate the diameter of the zone of sensitivity at 30°C to 10 μl of 2.5% maltose after subtraction of the disk diameter (7 mm).

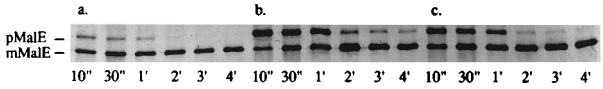

FIG. 2. *prlD21* causes a severe, recessive secretion defect in a *lamB17D-lacZ* fusion strain. Cells were pulse-labeled and immunoprecipitated with antibody to MalE and otherwise prepared as described in Materials and Methods. Shown is the processing of MalE in the following strains: SE1073 (a), SE1073 containing *prlD21* (b), and SE1073 containing *prlD21* and λPR9 (*secA*$^+$) (c). p represents the precursor form of MalE, and m represents the mature form of this protein. Time points indicate the time of the trichloroacetic acid precipitation after the addition of chase.

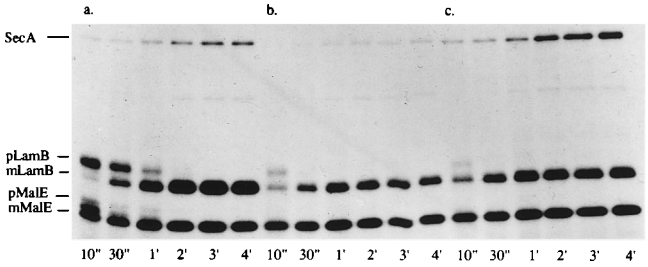

FIG. 3. Effect of *prlD21* on the secretion of wild-type LamB and MalE precursors. Cells were pulse-labeled and immunoprecipitated with antibodies to LamB, MalE, and SecA and otherwise prepared as described in Materials and Methods. Strains are as follows: MC4100 containing *prlD21* (a), MC4100 (b), and MC4100 carrying the *secA*$^+$ plasmid pMF8 (c). p represents the precursor forms of LamB and MalE, and m represents the mature forms of these proteins. Time points indicate the time of the trichloroacetic acid precipitation after the addition of chase.

to restore proper targeting of LamB17D-LacZ into the secretion pathway (Mal^s): *prlD21*, *-22*, and *-23* and *azi-17* (Table 3 [data shown for *prlD21*; *prlD22* and *-23* and *azi-17* are similar]). These alleles are all Az^r and are among the strongest *prlD* suppressors of *lamB14D* (Table 1). However, the three original *prlD* alleles, *prlD2*, *-4*, and *-5*, are also Az^r and equally strong suppressors but do not restore Mal^s to the *lamB17D-lacZ* strain. In addition, Az^r does not correlate with strength of suppression; for example, some Az^{ss} suppressors are stronger than some Az^r suppressors. Therefore, we conclude that although all of the alleles capable of restoring Mal^s confer Az^r and are strong suppressors, Az^r and strength of suppression are not generally correlated with the ability to restore Mal^s.

In diploid analysis with λPR9 lysogens, the Mal^s phenotype generated by the four alleles is recessive, shown as follows. The diameters (millimeters) of the zones of maltose sensitivity (as described in Materials and Methods) were 0 for *prlD*$^+$ *lamB17D-lacZ*, λPR9 (*secA*$^+$); 14 for *prlD21 lamB17D-lacZ*; and 0 for *prlD21 lamB17D-lacZ*, λPR9. (Data for *prlD22* and *-23* and *azi-17* were similar.) This implies that the LamB17D-LacZ fusion protein is specifically inactivating the PrlD21, -22, and -23 and *Azi-17* proteins (SDI). If this is true, we would expect the Mal^s phenotype to result from a secretion defect caused by the loss of PrlD (SecA) function. Pulse-chase experiments support this view (Fig. 2 [data shown for *prlD21*; *prlD22* and *-23* and *azi-17* are similar]). The *prlD21* allele causes a pronounced secretion defect for MalE in the *lamB17D-lacZ* strain (35% mMalE produced at the 10-s time point compared with 84% with *secA*$^+$). This defect is relieved by the presence of *secA*$^+$ in *trans* (57% mMalE at 10 s).

Thus, despite weak suppression of *lamB14D* and *lamB*Δ*78* by the *prlD* and *azi* alleles, four of these alleles are able to restore Mal^s and a secretion defect to the *lamB17D-lacZ* strain. The results are similar to those obtained with certain *prlA* and *prlG* suppressors. In all cases, it appears that the toxic fusion protein is inactivating the Prl suppressor protein that is trying to secrete it.

A secretion defect is associated with the four special alleles. While performing the pulse-chase assays described above, we noticed a modest defect in the secretion of wild-type MalE in strains containing *prlD21* and *lamB*Δ*78* but not in isogenic strains containing *prlD2*, *-4*, or *-5* or *azi-7*. This secretion defect is due solely to the presence of the *prlD21* allele. When the suppressor is introduced into a wild-type strain, a decrease in the rate of signal sequence processing is seen for both LamB and MalE, compared with strains containing wild-type SecA or SecA overexpressed (pMF8) (Fig. 3). The test was repeated with 11 suppressors representative of each of the phenotypic classes found except Az^s. This list includes *prlD21*, *-22*, and *-23* and *azi-17* (allowing SDI and conferring Az^r); *prlD2*, *-4*, *-5*, and *-37* and *azi-7* (conferring Az^r); and *prlD20* and *-28* (conferring Az^{ss}). Only *prlD21*, *-22*, and *-23* and *azi-17*, those that allow SDI, cause a secretion defect. The percentage of MalE processed after 10 s ranges from 69 to 77% for *prlD21*, *-22*, and *-23* and *azi-17*, compared with 92 to 100% for the wild type and the other seven *prlD* and *azi* strains. The secretion defects are recessive to a single extra copy of *secA*$^+$ on the chromosome (data not shown). Thus, these four mutant SecA proteins do not actively interfere with secretion. Rather, they appear to have reduced function.

It is known that SecA synthesis is regulated according to the secretion needs of the cell (42, 50). Consistent with the secretion defect conferred by *prlD21*, *-22*, and *-23* and *azi-17* is derepression of *secA* (Fig. 3, for example, as seen for *prlD21*). The other Az^r and Az^{ss} alleles that were checked do not cause derepression of *secA*.

Thus, *prlD21*, *-22*, and *-23* and *azi-17*, which allow SDI, are the only alleles that confer significant secretion defects. We suggest that the secretion defect is closely correlated and perhaps integral to the ability of these alleles to restore proper targeting of LamB17D-LacZ. The fact that only a subset of the suppressor alleles confer both a secretion defect and SDI argues that these four mutations cause distinctive changes in SecA function.

DNA sequence analysis of a subset of the *prlD* and *azi* alleles. We performed DNA sequence analysis of the four special alleles as well as of the Az^{ss} class of *prlD* alleles with the purpose of identifying a specific region of SecA that mediates these phenotypes.

First, sequence analysis of *prlD21*, *-22*, and *-23* and *azi-17* revealed that these four alleles all alter a single amino acid in SecA: Tyr-134. *prlD21* causes a change of Tyr-134 to Ser and, additionally, Glu-148 to Lys; the codon changes (underlined) are from T<u>A</u>C to T<u>C</u>A and G<u>A</u>A to <u>A</u>AA, respectively. *prlD23* also causes a change of Tyr-134 to Ser (T<u>A</u>C to T<u>C</u>T), but without the second mutation. *prlD22* and *azi-17* change Tyr-134 to Cys (T<u>A</u>C to T<u>G</u>C) and Asn (<u>T</u>AC to <u>A</u>AC), respectively. *prlD21* and *prlD23* were obtained by UV mutagenesis, so the multiple mutations observed are not unusual. Second, sequence analysis showed that six of the seven Az^{ss} *prlD* alleles (*prlD20*, *-24*, *-26*, *-27*, *-28*, and *-31*) are alterations of Ala-507 to Val (G<u>C</u>G to G<u>T</u>G). *prlD43* is an alteration of His-484 to Gln (CA<u>C</u> to CA<u>G</u>).

In sum, the four special alleles *prlD21*, *-22*, and *-23* and *azi-17* all alter the same amino acid in the N terminus of *secA*; six of the seven Az^{ss} alleles alter a particular amino acid in the C terminus of *secA*. Thus, genetic analysis revealed significant clustering of mutations in *secA* with distinct and identical phenotypes.

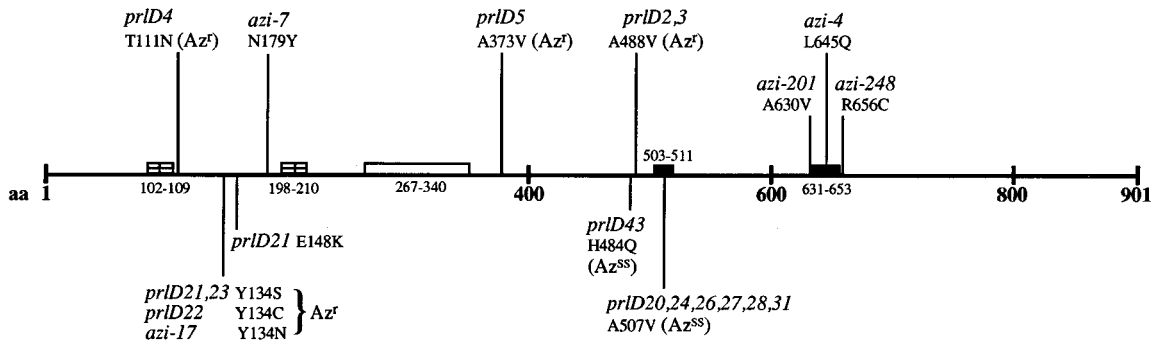

FIG. 4. Compilation of the known *prlD* and *azi* mutational alterations of the *E. coli* SecA protein. The horizontal line represents the linear amino acid (aa) sequence of the protein, and numbers indicate specific residues. Changes caused by previously identified mutations are indicated above the line; changes caused by mutations identified in this study are indicated below the line. Azide phenotypes are indicated for *prlD* alleles. Boxes indicate functional regions of the molecule (see text for details): ⊞, ABC I (high-affinity ATP-binding site); ■, ABC II (low-affinity ATP-binding site); □, putative signal sequence-binding site.

DISCUSSION

The SecA protein is an essential component of the cellular secretion machinery. It binds the signal sequence and mature regions of precursor proteins and, in an ATP-dependent fashion, catalyzes their membrane insertion (26, 31, 49, 61). In an effort to better understand the function(s) of SecA, we undertook a detailed study of the *prlD* suppressor alleles. One possible mechanism of PrlD-mediated suppression posits a broadened specificity of signal sequence recognition. Viewed in this manner, the *prlD* mutations might be expected to alter the signal sequence recognition domain of SecA, allowing it to recognize mutant sequences it would normally ignore. However, our results demonstrate a high correlation between suppression and azide resistance: the majority of the *prlD* suppressors confer azide resistance (Az^r), and all of the *azi* alleles are suppressors. Another class of *prlD* alleles confers azide supersensitivity (Az^{ss}). Because the broadened specificity model does not clearly predict or easily explain this correlation, we suggest that the mechanism of suppression may be more complex. Available DNA sequence data, shown in Fig. 4, support this view.

A putative ATP-binding and hydrolysis region consisting of two subsites is located in the N terminus of SecA (Fig. 4) (33). This region is called the ATP-binding cassette (ABC I) and corresponds to a high-affinity site identified by Mitchell and Oliver (33). Intriguingly, ATP binding at this site appears to cause a conformational change that drives membrane insertion of SecA (18, 27) (Fig. 5, step iii). All *prlD* and *azi* mutations in this region confer Az^r and are suppressors of signal sequence mutations, including *prlD4* (T111N) (21) and *azi-7* (N179Y) (29, 37). Particularly interesting is the clustering of *prlD21* (Y134S and E148K), *-22* (Y134C), and *-23* (Y134S) and *azi-17* (Y134N), which have been shown in this work to confer unique phenotypes. These mutations all alter Tyr-134. As will be discussed further below, we believe that these special alleles alter the ATPase activity of ABC I. Consistent with this view, Nakane et al. (36) have shown that alterations of Thr-128 in *Bacillus subtilis* SecA confer Az^r and cause increased ATPase activity. None of the *prlD* or *azi* mutations are located within the actual subsites of ABC I (amino acids 102 to 109 and 198 to 210); perhaps, as Mitchell and Oliver (33) suggested, alterations of amino acids in these sites abolish ATP binding and/or hydrolysis entirely and are therefore lethal.

The second ABC (ABC II) is located in the C-terminal half of SecA and was identified as a low-affinity site (33). It has been proposed that ATP hydrolysis at this second site allows SecA to remove itself (deinsert) from the membrane (18, 27). All azide supersensitive (Az^{ss}) alleles alter amino acids near or within the first subsite of ABC II (amino acids 503 to 511): *prlD20*, *-24*, *-26*, *-27*, *-28*, and *-31* (all A507V) and *-43* (H484Q). It is tempting to speculate that Az^{ss} may be related to a defect in the membrane deinsertion reaction. An azide-induced block at this step would leave SecA embedded in the membrane, where it may cause lethality by titrating other essential components of the secretion machinery. This model could explain the dominance of Az^s and Az^{ss} in diploid analysis.

In addition to all of the Az^{ss} alleles, Az^r is also conferred by suppressor mutations within ABC II. These Az^r mutations are located within or near either subsite of the cassette, rather than in the intervening sequence, as is the case for mutations in ABC I. *prlD2* (A288V) (21) is located near the first subsite. *azi-4* (L645Q) (29, 37), *azi-201* (A630V), and *azi-248* (R656C) (23) are all alterations of amino acids near or within the second subsite (amino acids 631 to 653), but the suppressor phenotype of *azi-4* is particularly weak and those of *azi-201* and *-248* are unknown.

An additional suppressor, *prlD5* (A373V), is located near a region defined by Kimura et al. (28) as the signal sequence binding site (amino acids 267 to 340). Evidence for such a site came from cross-linking of proteolytic fragments of SecA with proOmpF-Lpp, a model precursor protein. Perhaps *prlD5* alters the interaction of SecA with signal sequences (2). Note, however, that *prlD5* confers Az^r. This result, coupled with the fact that Az^r mutations are also found in both ABC I and II, demonstrates the complexity of the azide resistance phenotype. Clearly, mutational alterations in several different functional domains of SecA can confer Az^r, and the mechanism of signal sequence suppression for the different alterations is probably not the same.

We have identified four alleles (out of the total of 44) that exhibit special properties. These alleles, *prlD21*, *-22*, and *-23* and *azi-17*, confer azide resistance, are potent enough with LamB17D-LacZ to allow SDI, and cause defects in the secretion of wild-type proteins. Strikingly, all alter Tyr-134, a residue conserved in all known SecA proteins (5, 14, 34, 35, 39, 46, 47, 59, 62). We suspect that these four mutations may slow an ATPase activity of SecA.

Our model for suppression (Fig. 5) is based on recent studies with *prlA* and *prlG* (15, 22, 38) and biochemical studies performed by Schiebel et al. (49). The *prlA* and *prlG* suppressors appear to act by reducing a signal sequence proofreading function. In wild-type strains, defective signal sequences are re-

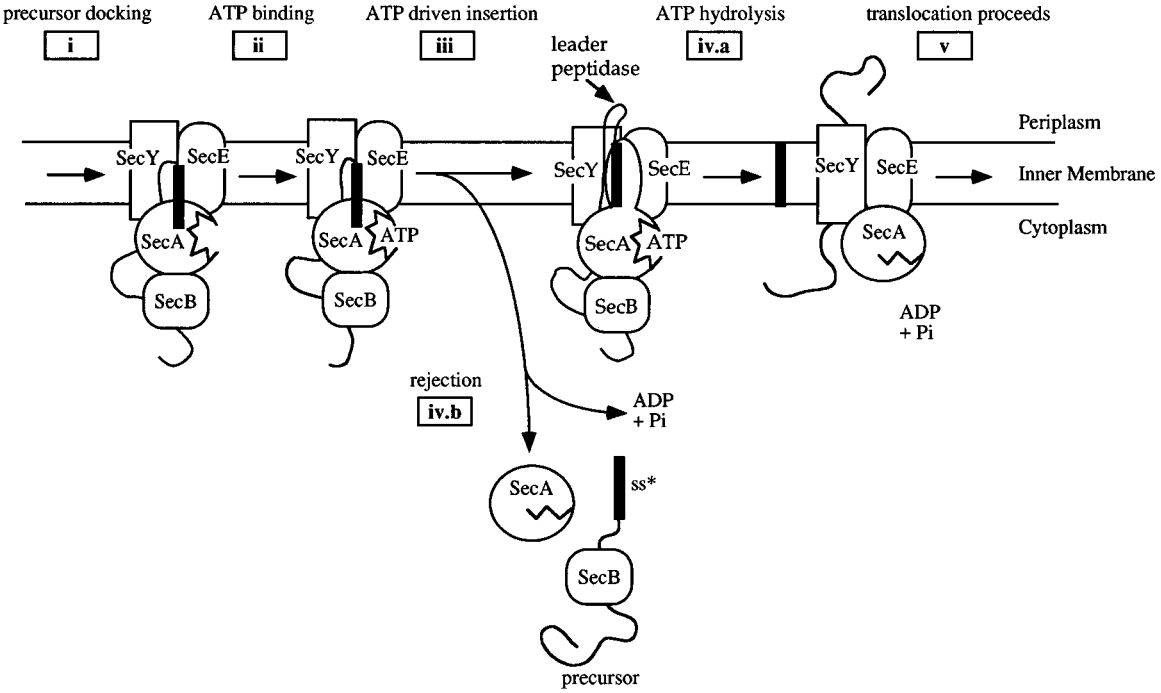

FIG. 5. Model for the mechanism of action of the four *prlD* suppressors. (i) SecB and SecA are recruited to maintain the precursor protein in an export-competent state and to target it to the translocator, composed of SecY and SecE. (ii) After docking, the precursor protein, SecA, binds ATP. (iii) Binding of ATP provides the energy for a conformational change that drives the precursor into the translocator, provided that the signal sequence is read correctly. (iv.a and v) ATP hydrolysis allows translocation to proceed. (iv.b) If the signal sequence is defective (ss*), the mutant precursor is rejected from the pathway. We propose that the *prlD* suppressors act to slow the step of ATP hydrolysis, the proofreading step, thus providing the translocator with additional time to engage. This allows successful translocation of increased amounts of mutant precursor.

jected from the secretion pathway. In suppressor strains, compromised proofreading allows mutant precursor secretion. The biochemical studies indicate that ATP binding by SecA causes a dramatic conformational change that drives precursor translocation to the extent that signal sequence processing occurs (18, 27, 49) (Fig. 5, iii). We suggest that signal sequence proofreading occurs at this ATP-dependent step. If SecY recognizes the signal sequence, the conformational change is allowed, and a functional translocase forms; ATP hydrolysis then drives further translocation (Fig. 5, iv.a) (49). However, if SecY rejects the signal sequence, ATP hydrolysis instead releases the defective precursor and translocation is aborted (Fig. 5, iv.b).

All signal sequence mutations, even deletions, are leaky. For example, a complete signal sequence deletion in *phoA* still allows secretion of 1% of PhoA protein (15). If these special *prlD* suppressors slow the ATPase activity, the extra time allotted by the increased half-life of the SecA-ATP complex should permit the successful translocation of increased amounts of mutant precursor. This putative decreased ATPase activity can explain the other properties of these *prlD* suppressors. First, azide is known to affect certain types of ATPases, and it seems likely that mutations which confer azide resistance will alter this activity. Second, if these mutations slow the ATPase activity, the mutant SecA protein might remain complexed with the toxic LamB17D-LacZ fusion protein for longer periods of time, and this would explain suppressor-directed inactivation of PrlD. Third, if the ATPase activity is slowed, normal function of the protein is then slowed, and this would explain the defects observed for the secretion of wild-type precursor proteins.

Our model predicts that mutant signal sequences can still be recognized by wild-type SecA. As noted above, the coincidence of suppression and azide resistance argues that few, if any, of the Az^r *prlD* suppressors act by restoring mutant signal sequence recognition. This is understandable if SecA can recognize defective signal sequences already, and it also explains why *prlA* suppressors are so potent. In *prlA* strains, suppression is SecA dependent (15). Thus, SecA must still be able to bind and properly direct both wild-type and mutant precursors into the secretion machinery efficiently.

A second prediction of the model relates to the proposed increased half-life of the precursor-SecA-ATP complex. If this is true, then it should be possible to effect suppression by simply increasing the concentration of this complex. Thus, we would predict that an increase in the amount of SecA should promote mutant precursor secretion by mass action. Indeed, increased levels of SecA do cause suppression of signal sequence mutations, albeit at low levels (reference 21 and this study). Additionally, we found that increased levels of SecA restore a modest level of Mal^s in the *lamB17D-lacZ* fusion strain. This suggests increased targeting of the toxic fusion protein as well.

The secretion defects caused by the four special *prlD* alleles result in derepression of PrlD synthesis. Accordingly, we have considered the possibility that the mechanism of action of these suppressors could be simply attributed to increased levels of PrlD (SecA). We think this unlikely for the following reasons. First, neither the suppressor nor the SDI phenotypes conferred by SecA overexpression are as strong as those conferred by these four alleles, despite the fact that SecA levels in the overexpression strain are two- to threefold higher than those in the suppressor strains (Fig. 3 [similar results obtained with *prlD22* and *-23* and *azi-17*]). Second, increased levels of SecA do not cause a secretion defect or azide resistance, as do

the suppressors. Third, the Mal^s conferred by the suppressor alleles is recessive to a single extra copy of *secA* (λPR9). In this case, increasing the gene dosage of *secA* relieves SDI.

The model is based on and offers explanation for only the four special *prlD* alleles. The remaining 40 are more difficult to explain. We think that the complexity of PrlD phenotypes reflects the multiple functions of SecA, and certain of the other alleles may provide further insights into the role of SecA and its ATPase activities in protein translocation.

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