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secG and Temperature Modulate Expression of Azide-Resistant and Signal Sequence Suppressor Phenotypes of *Escherichia coli secA* Mutants

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SecA is a dynamic protein that undergoes ATP-dependent membrane cycling to drive protein translocation across the *Escherichia coli* inner membrane. To understand more about this process, azide-resistant (*azi*) and signal sequence suppressor (*prlD*) alleles of *secA* were studied. We found that azide resistance is cold sensitive because of a direct effect on protein export, suggesting that SecA-membrane interaction is regulated by an endothermic step that is azide inhibitable. *secG* function is required for expression of azide-resistant and signal sequence suppressor activities of *azi* and *prlD* alleles, and in turn, these alleles suppress cold-sensitive and export-defective phenotypes of a *secG* null mutant. These remarkable genetic observations support biochemical data indicating that SecG promotes SecA membrane cycling and that this process is dependent on an endothermic change in SecA conformation.

Export of preproteins across the inner membrane of *Escherichia coli* has been studied extensively during the past decade. Both biochemical and genetic approaches have resulted in the identification of many if not all of the proteinaceous components of the translocation machinery (30, 36). Precursor proteins, synthesized with an amino-terminal signal peptide, associate with chaperones, such as SecB protein, which maintains them in an export-competent conformation (29). They are then targeted to the plasma membrane, where they associate with the translocase complex, which is composed of the membrane-dissociable SecA protein and the integral membrane protein, SecYEG, which are thought to form a translocation channel (5, 9, 14, 19). Central to preprotein assembly at the translocase complex is SecA protein, a 204-kDa homodimeric protein, which has been shown to bind the signal peptide and mature region of the preprotein, SecB protein, anionic phospholipids, and the amino-terminal portion of SecY protein (1, 4, 8, 15, 17, 20, 32). SecA regulates these diverse interactions and drives protein translocation by its ATPase activity (23, 34). ATP binding to SecA catalyzes the initial insertion of preprotein into the membrane, while hydrolysis promotes translocation across the membrane (31). Protein translocation appears to depend on the ability of SecA to undergo multiple cycles of membrane insertion and retraction, and such SecA-membrane cycling has been shown to depend on the function of the high-affinity ATP-binding domain of SecA (11, 28). This biochemical behavior of SecA has led to a model in which SecA has been hypothesized to act like a molecular ratchet, utilizing its membrane cycling activity to translocate proteins (12). Recently, however, it has been argued that protein translocation can occur under conditions in which SecA is permanently imbedded in the plasma membrane (6). In addition to SecA-membrane cycling, it has been found that SecG protein undergoes a topology inversion during protein translocation, and this event appears to be coupled to the insertion and retraction cycle of SecA protein, suggestive of a mechanistic linkage of these two processes (25).

Sodium azide is a known inhibitor of many ATPases, and azide-resistant mutants of *Escherichia coli*, denoted *azi*, have been found to be alleles of *secA* (13, 16, 26). Previous studies indicate that azide inhibits the translocation ATPase activity of SecA (26). Azide has been shown to trap SecA in the membrane-inserted state, as judged by the formation of a protease-resistant and membrane-protected 30-kDa fragment of SecA (35). In addition, previous genetic studies of signal sequence suppressor alleles of *secA*, denoted *prlD*, found that most such strains are altered in their azide sensitivity or resistance, thus indicating a strong interconnection between these two properties of SecA protein (16). Most *prlD* alleles are located in or adjacent to the ATP-binding domains of SecA, which govern SecA membrane cycling (16, 17, 23). In order to understand more about SecA function and its regulation, we have performed further genetic characterization of *azi* and *prlD* mutants.

The strains used in this study are described in Table 1, and where necessary they were constructed by P1 transduction (22). The growth medium employed in this study has been described previously (22). The concentrations of ampicillin, kanamycin, and tetracycline used were 100, 15, and 10 μg ml^{-1}, respectively. Sodium azide was purchased from Mallinckrodt.

Azide resistance is cold sensitive. Since previous studies showed that the insertion of SecA into the membrane involves a temperature-dependent unfolding of the protein (33) and azide prevents retraction of SecA from the membrane (35), we were interested in determining the effect of temperature on azide resistance. *azi* and *prlD* mutants that are normally azide resistant at 37°C were tested for their ability to form colonies on Luria-Bertani (LB) plates containing azide at various temperatures. Remarkably, none of the *azi* or *prlD* mutants that were azide resistant at 37 or 42°C were able to grow on LB plates containing 1 mM azide at 20°C, although growth was normal on LB plates (Table 2). The *azi* and *prlD* mutants showed reduced azide resistance at 30°C, where single-colony formation was inhibited at 1 mM azide and growth was blocked

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TABLE 1. Characteristics of the bacterial strains used in this study

Strain	Genotype or description	Source or reference
CK1801	MC4100 Δ(*uncB-uncC*)	Carol Kumamoto
DG100	KN370 *leu*::Tn*10*	This study
DG100.2	KN370 *secG*$^+$ *argG*::Tn*10*	This study
DG101	KN370 *prlD22 leu*::Tn*10*	This study
DG101.2	KN370 *prlD22 secG*$^+$ *argG*::Tn*10*	This study
DG104	KN370 *prlD20 leu*::Tn*10*	This study
DG309	KN370 *azi-4 leu*::Tn*10*	This study
DG309.2	KN370 *azi-4 secG*$^+$ *argG*::Tn*10*	This study
DG311	KN370 *prlD5 leu*::Tn*10*	This study
DG313	KN370 *prlD2 leu*::Tn*10*	This study
DG313.2	KN370 *prlD2 secG*$^+$ *argG*::Tn*10*	This study
DG1801	CK1801 Δ*secG*::Kan	This study
DO168	MC4100 *leu*::Tn*10*	Laboratory stock
DO309	MC4100 *azi-4*	26
DO312	MC4100 *azi-7*	26
DO315	MC4100 *azi-6*	26
DO318	MC4100 *azi-9*	26
JH101	STA14D *prlD22 leu*::Tn*10*	16
JH104	STA14D *prlD20 leu*::Tn*10*	16
KB311	STA14D *prlD5 leu*::Tn*10*	16
KB313	STA14D *prlD2 leu*::Tn*10*	16
KB315	STA14D *prlD4 leu*::Tn*10*	16
KN370	C600 *recD1009* Δ*secG*::Kan	24
LG800	STA14D *prlD43*	16
MC4100	F$^-$ *araD139 relA1 thi rpsL150 flB5301* Δ*(argF-lac)U169 deoC7 ptsF25 rbsR*	26
MM2	MC4100 *malE14-1*	2
MM100	MM2 *leu*::Tn*10*	This study
MM101	MM2 *prlD22 leu*::Tn*10*	This study
MM104	MM2 *prlD20 leu*::Tn*10*	This study
MM309	MM2 *azi-4 leu*::Tn*10*	This study
MM311	MM2 *prlD5 leu*::Tn*10*	This study
MM313	MM2 *prlD2 leu*::Tn*10*	This study
RV100	MM100 Δ*secG*::Kan	This study
RV101	MM101 Δ*secG*::Kan	This study
RV104	MM104 Δ*segG*::Kan	This study
RV309	MM309 Δ*secG*::Kan	This study
RV311	MM311 Δ*secG*::Kan	This study
RV313	MM313 Δ*secG*::Kan	This study
RV400	STA14D *leu*::Tn*10* Δ*secG*::Kan	This study
RV401	STA14D *leu*::Tn*10 azi-4* Δ*secG*::Kan	This study
RV402	STA14D *leu*::Tn*10 prlD5* Δ*secG*::Kan	This study
RV403	STA14D *leu*::Tn*10 prlD20* Δ*secG*::Kan	This study
RV404	STA14D *leu*::Tn*10 prlD22* Δ*secG*::Kan	This study
RV405	STA14D *leu*::Tn*10 prlD43* Δ*secG*::Kan	This study
STA14D	MC4100 *lamB14D*	16

completely at 2 mM azide. These findings indicate that azide resistance is cold sensitive.

To determine whether the cold sensitivity of azide resistance correlates with reduction in the rate of protein secretion under these conditions, we determined the effect of temperature and azide on the rate of processing of maltose-binding protein (MBP) and OmpA in *azi* and *prlD* mutants. The rate of protein processing is a valid measure of the rate of protein secretion, given the topology of signal peptidase I (7). Even in the absence of azide, lowering the growth temperature of the *prlD2* mutant to 20°C reduced the rate of protein secretion, particularly for OmpA (Fig. 1), indicating that protein export was somewhat cold sensitive in this case. Furthermore, while protein secretion in the *prlD* and *azi* mutants was substantially resistant to the effects of azide at 37°C, it was not resistant at 20°C. These data demonstrate that protein export in *azi* and *prlD* strains is phenotypically azide sensitive at low growth temperatures.

TABLE 2. Azide resistance is cold sensitive[a]

Allele	Growth at temp[b]:			
	20°C	30°C	37°C	42°C
secA$^+$	−	−	−	−
prlD2	−	+/−	+	+
prlD4	−	+/−	+	+
prlD5	−	+/−	+	+
prlD22	−	+/−	+	+
azi-4	−	+/−	+	+
azi-6	−	+/−	+	+
azi-7	−	+/−	+	+
azi-9	−	+/−	+	+

[a] Strains were streaked on LB plates containing 1 mM sodium azide and incubated overnight at the indicated temperature. The following strains were used: DO168 (*secA*$^+$), KB313 (*prlD2*), KB315 (*prlD4*), KB311 (*prlD5*), JH101 (*prlD22*), DO309 (*azi-4*), DO315 (*azi-6*), DO312 (*azi-7*), and DO318 (*azi-9*).

[b] −, no growth; +, growth with single colonies; +/−, growth without single colonies. Parallel controls showed that all strains grew well at these temperatures on LB plates lacking sodium azide.

Azide resistance is SecG dependent. Since both SecG and azide have been suggested to affect SecA membrane cycling (25, 35), we were interested in studying the effect of *secG* function on azide resistance. Using P1 transduction, *azi* and *prlD* alleles were introduced into KN370 containing a *secG* deletion. Remarkably, all *azi* or *prlD* Δ*secG* double mutants were unable to form colonies on LB plates containing 1 mM azide at 37°C (Table 3). To show that this result was directly due to the lack of *secG* function and was not an effect of the general strain background, these strains were transformed with plasmids containing *secG*, *secE* and *secY*, or *secE*, *secY*, and *secG* expressed from the *trc* promoter (10). An azide-resistant phenotype was recovered only in strains containing plasmids with *secG*, demonstrating the importance of this gene in azide resistance. Since overproduction of SecYE protein alone was insufficient to promote an azide-resistant phenotype, it seems unlikely that the involvement of *secG* in this case was indirect,

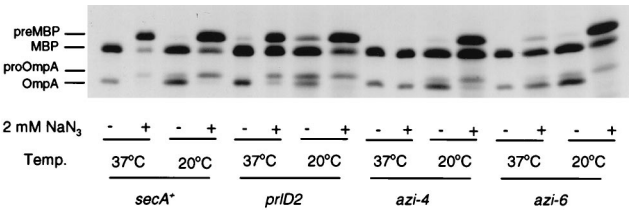

FIG. 1. Analysis of protein secretion of *azi* and *prlD* mutants at low temperature in the presence or absence of azide. Strains (left to right: DO168, KB313, DO309, and DO315) were grown in M63 minimal medium (22) containing 0.4% glycerol, 0.4% maltose, and 20 μg (each) of 18 amino acids (lacking cysteine and methionine) ml^{-1} at 37°C until the mid-logarithmic phase, when portions of each culture were shifted to the indicated temperature. After 20 min, sodium azide was added to a final concentration of 2 mM to the indicated cultures. Five minutes later, a 0.5-ml aliquot of each culture was pulse-labeled with 10 μCi of Tran ^{35}S-label (>1,000 Ci mmol^{-1}; ICN) for 1 min, followed by the addition of an equal volume of ice-cold 10% trichloroacetic acid. MBP and OmpA were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described previously (26). Three times more sample was loaded on the gel for the samples labeled at 20°C. The positions of the precursor and mature forms of MBP (preMBP and MBP, respectively) and OmpA (proOmpA and OmpA, respectively) are given.

TABLE 3. *secG* is required for azide resistance, while *azi* and *prlD* alleles suppress the cold sensitivity of Δ*secG* strains[a]

Strain genotype	Growth at[b]:				
	37°C with 1 mM azide				20°C with no plasmid
	No plasmid	*secG*	*secEY*	*secEYG*	
Δ*secG secA*$^+$	−	−	−	−	−
Δ*secG prlD2*	−	+	−	+	+
Δ*secG prlD5*	ND	ND	ND	ND	+
Δ*secG prlD20*	ND	ND	ND	ND	−
Δ*secG prlD22*	−	+	−	+	+
Δ*secG azi-4*	−	+	−	+	+

[a] To test for azide resistance, strains were streaked onto LB plates without (control) or with 1 mM sodium azide and incubated overnight at 37°C. To test for cold sensitivity, logarithmic-phase cultures were plated at a 10^6 dilution onto duplicate LB plates and incubated either overnight at 37°C (control) or for 3 days at 20°C. Colony formation was then scored. The following strains were used: DG100 (Δ*secG*), DG313 (Δ*secG prlD2*), DG311 (Δ*secG prlD5*), DG104 (Δ*secG prlD20*), DG101 (Δ*secG prlD22*), and DG309 (Δ*secG azi-4*). The plasmids used were pTrcG (*secG*$^+$), pTrcHA-EY (*secE*$^+$ *secY*$^+$), and pTrcHA-EYG (*secE*$^+$ *secY*$^+$ *secG*$^+$).

[b] +, growth; −, no growth; ND, not done.

for example, by promoting a higher level of activity of SecYE protein.

To determine whether the observed azide-sensitive phenotype of *azi* or *prlD* Δ*secG* double mutants correlates with a reduced rate of protein export under these conditions, the rate of MBP and OmpA secretion was investigated. While azide addition caused a significant inhibition of MBP and OmpA secretion in the *azi* and *prlD* single mutants, more severe inhibition of protein export was noted for the *azi* or *prlD* Δ*secG* double mutants (Fig. 2). These effects were not due to a decrease in SecA protein levels in any of these strains as monitored by pulse-labeling and immunoprecipitation or by Western blotting (up to 2 h in the presence of 2 mM azide [data not shown]). These data are consistent with the observed azide sensitivity of growth of the *azi* or *prlD* Δ*secG* strains.

Deletion of *secG* results in a cold-sensitive phenotype in certain strain backgrounds, such as C600 and W3110 (24). In some cases, the cold-sensitive phenotype is manifested only when the *unc* genes encoding F_1F_0-ATPase are deleted also (10). MC4100 Δ*secG* mutants were not cold sensitive, whether they contained *unc*$^+$ or ΔuncB-C alleles (results not shown), indicating that the status of the *unc* locus need not determine the cold-sensitive phenotype of *secG* mutants. The reason for such variation among different strain backgrounds is unclear. Because MC4100 Δ*secG* derivatives are not cold sensitive, we tested *azi* and *prlD* derivatives of this strain for the dependence of azide resistance on *secG* function. Even in this strain background, we found that *secG* function affected the level of azide

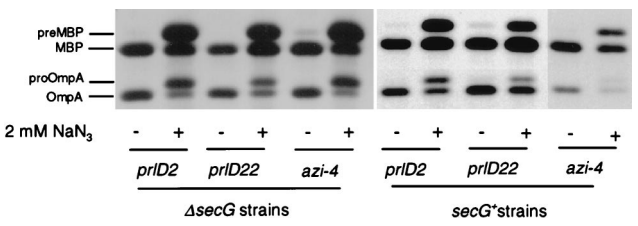

FIG. 2. Analysis of the azide sensitivity of protein secretion of *azi* or *prlD* Δ*secG* double mutants. Strains (from left to right: DG313, DG101, DG309, DG313.2, DG101.2, and DG309.2) were grown at 37°C, treated with sodium azide (where indicated), and radiolabeled, and MBP and OmpA were analyzed as described in the legend to Fig. 1.

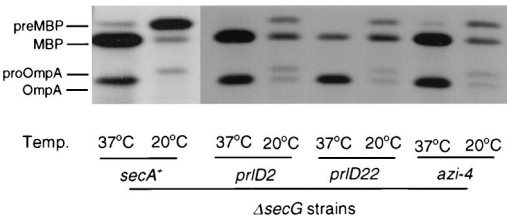

FIG. 3. Analysis of protein secretion of *azi* or *prlD* Δ*secG* double mutants at low temperature. Strains (from left to right: DG100, DG313, DG101, and DG309) were grown at 37°C, shifted to the temperature indicated for 20 min, and subjected to radiolabeling, and MBP and OmpA were analyzed as described in the legend to Fig. 1, except that an equal amount of each sample was loaded on the gel.

resistance, although not as severely as KN370 derivatives. *azi* and *prlD* MC4100 derivatives were able to form colonies on LB plates containing 3.5 mM azide, while their isogenic Δ*secG* counterparts were only able to form colonies on LB plates containing 1 mM azide, with the exception of the *prlD22* Δ*secG* mutant, which formed colonies on LB plates containing up to 2 mM azide (results not shown).

Cold sensitivity caused by *secG* deletion can be suppressed by *azi* and *prlD* alleles. Loss of *secG* function leads to cold sensitivity of growth and an accumulation of preproteins at low temperature (24). Overexpression of acidic phospholipids has been shown to suppress this phenotype (18), suggesting that the loss of *secG* function may relate to a defect in SecA-membrane binding or insertion (which requires anionic phospholipids [3, 33]). Since purified SecA proteins containing the *azi* and *prlD* mutations displayed increased membrane ATPase activity, even at 28°C (28a), indicating enhanced SecA-membrane interaction, we speculated that *azi* and *prlD* alleles may be able to suppress the cold sensitivity of *secG* mutants. To this end, the growth property of the *azi* or *prlD* Δ*secG* derivatives was investigated. Nearly all of these double mutants were able to form colonies on LB plates at 20°C (Table 3), indicating that *azi* and *prlD* alleles suppressed the cold sensitivity caused by the *secG* mutation. One exception to this pattern of suppression was the *prlD20* Δ*secG* mutant, which was unable to form colonies at 20°C. The lack of suppression in this case seems to relate to the cold sensitivity of *prlD20* strains (data not shown). Our findings are consistent with those of an earlier study showing that *secA36*, which leads to azide resistance, can suppress a *secG* defect in protein export at 20°C (21).

In order to determine whether the growth observed for *azi* or *prlD* Δ*secG* double mutants at low temperatures correlates with an increase in the rate of protein export, secretion of MBP and OmpA was investigated. The *azi* or *prlD* Δ*secG* mutants displayed an increased rate of protein secretion at 20°C compared to the isogenic Δ*secG* parent (Fig. 3). These results suggest that a direct mechanism of suppression of the Δ*secG* growth defect by these *secA* alleles is most probable.

Signal sequence suppressor activity of *prlD* alleles is *secG* dependent. Since *secG* function is needed for azide resistance, we were interested whether it was also required for the signal sequence-suppressor activity of *prlD* alleles, since both of these properties would result in an increased demand on the protein translocation system. To this end, *prlD* or *azi* alleles were introduced into *secG*$^+$ or Δ*secG* derivatives of MM2 that contain the *malE14-1* allele, which is a defect in the signal sequence of MBP (2). As expected, *prlD secG*$^+$ strains were able to suppress the signal sequence defect, resulting in a Mal$^+$ phenotype, while the *azi-4 secG*$^+$ strain was Mal$^-$ (Fig. 4). Remarkably, all *prlD* Δ*secG* double mutants were Mal$^-$, indi-

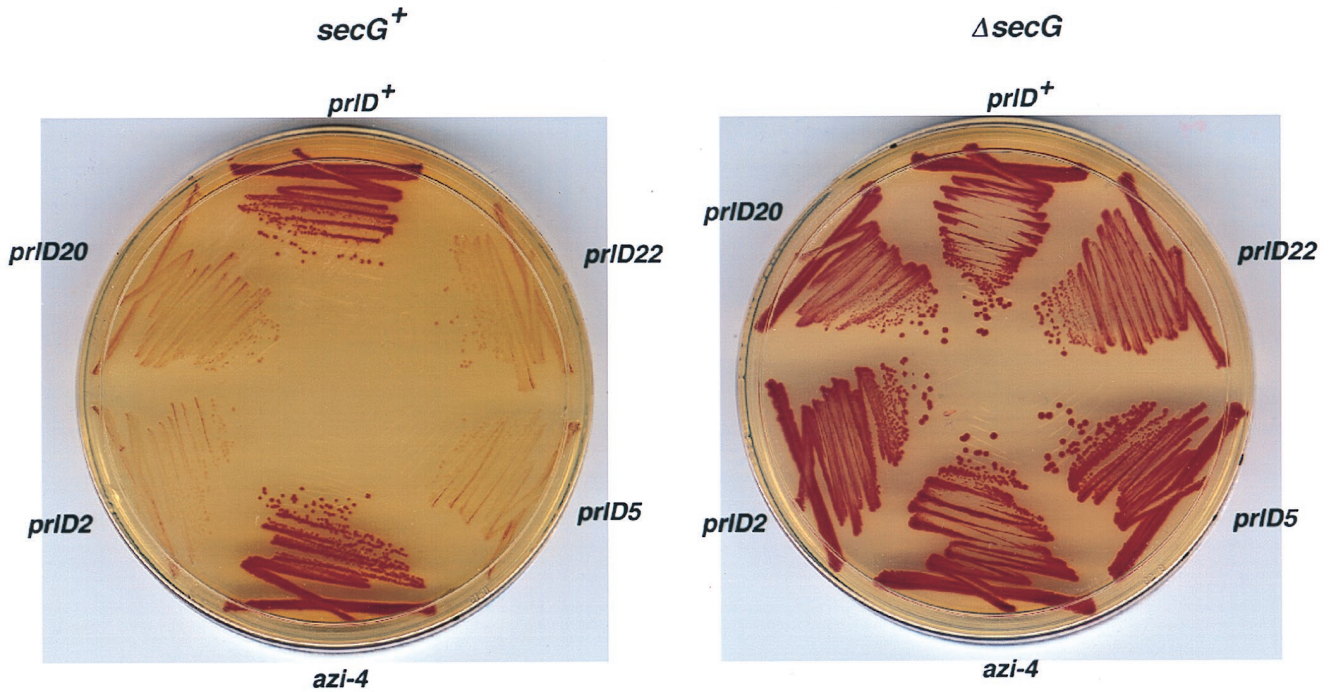

FIG. 4. *secG* function is required for signal sequence suppression by *prlD* alleles. *prlD* or *azi* MM2 derivatives with (*secG*$^+$) or without (Δ*secG*) *secG* function were tested for their ability to suppress the *malE14-1* signal sequence mutation by overnight growth on maltose-tetrazolium plates at 37°C. Red colonies (Mal$^-$) indicate little or no suppressor activity, while white colonies (Mal$^+$) indicate good suppressor activity.

cating that *secG* function is required for *prlD*-mediated signal sequence suppression. In a second assay system that measures *lamB14D* signal sequence suppressor activity by the strain's sensitivity to lambda phage adsorption and killing by cross-streaking, all *prlD* Δ*secG* double mutants had a lambda-resistant phenotype, except for the *prlD22* Δ*secG* double mutant (*prlD22* is the strongest signal sequence suppressor [16]), which was weakly lambda sensitive (data not shown). We conclude that *secG* function is needed for the expression of the signal sequence suppressor activity of *prlD* alleles.

In this work, we have shown a remarkable genetic interaction between the SecA and SecG proteins. This conclusion is based on the fact that the azide-resistant and signal sequence-suppressor properties of *azi* and *prlD* mutants are SecG dependent, and yet *azi* and *prlD* alleles suppress the cold sensitivity of *secG* mutants. The requirement of SecG for expression of the *azi* and *prlD* phenotypes presumably relates to the ability of SecG to increase the pool of biochemically activated, SecYEG-bound SecA protein. Thus, in the *azi* or *prlD secG* double mutants, there is insufficient activated SecA protein to promote the azide-resistant and signal sequence suppression activities of SecA, which place an unusual demand on the translocation process. However, there is sufficient activated SecA protein under this circumstance to promote normal protein translocation at low temperature in the absence of *secG* function. The ability of these mutations to activate SecA protein in the absence of *secG* function as well as to promote suppression of signal sequence defects may relate to their predicted destabilization of the compact quaternary structure of SecA protein that is likely to require a conformational change to promote biochemical activation (based on the atomic structure of SecA) (16a).

A second important conclusion from our study is that azide resistance is cold sensitive. It is tempting to speculate that this cold sensitivity is another manifestation of the inherent cold sensitivity of the protein export process that has been observed previously (27) and that its biochemical basis rests on an endothermic transition of SecA conformation that has been noted previously (3, 33), and which is required to promote SecA membrane interaction and cycling. Presumably SecG, as well as perhaps other Sec components, normally helps to overcome this cold-sensitive step, which is azide inhibitable and which can be partially compensated for by *azi* and *prlD* mutations, thereby bypassing the strict requirement for SecG function. This step is likely to correspond to one in the membrane insertion-retraction cycle of SecA, consistent with the ability of azide to block SecA membrane retraction and SecG to promote SecA membrane cycling (25, 35). Further elucidation of this complex system will require biochemical studies that are currently under way.

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