Isolation and Analysis of Dominant *secA* Mutations in *Escherichia coli*

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The *secA* gene product is an autoregulated, membrane-associated ATPase which catalyzes protein export across the *Escherichia coli* plasma membrane. Previous genetic selective strategies have yielded *secA* mutations at a limited number of sites. In order to define additional regions of the SecA protein that are important in its biological function, we mutagenized a plasmid-encoded copy of the *secA* gene to create small internal deletions or duplications marked by an oligonucleotide linker. The mutagenized plasmids were screened in an *E. coli* strain that allowed the ready detection of dominant *secA* mutations by their ability to derepress a *secA-lacZ* protein fusion when protein export is compromised. Twelve new *secA* mutations were found to cluster into four regions corresponding to amino acid residues 196 to 252, 352 to 367, 626 to 653, and 783 to 808. Analysis of these alleles in wild-type and *secA* mutant strains indicated that three of them still maintained the essential functions of SecA, albeit at a reduced level, while the remainder abolished SecA translocation activity and caused dominant protein export defects accompanied by *secA* depression. Three *secA* alleles caused dominant, conditional-lethal, cold-sensitive phenotypes and resulted in some of the strongest defects in protein export characterized to date. The abundance of dominant *secA* mutations strongly favors certain biochemical models defining the function of SecA in protein translocation. These new dominant *secA* mutants should be useful in biochemical studies designed to elucidate SecA protein's functional sites and its precise role in catalyzing protein export across the plasma membrane.

Both genetic and biochemical approaches have been utilized to study the molecular mechanisms responsible for protein export in *Escherichia coli*. Genetic selections have been employed to identify a group of *sec* genes whose products catalyze secretion of cell envelope proteins across the plasma membrane (2). This process has been shown to require at least six proteins: the soluble SecB protein (12, 13, 28, 29), the membrane-dissociable SecA protein (3, 6, 18-20), and four integral membrane proteins, SecD (10), SecE (21, 24), SecF (1a), and SecY/PrfA (8, 11). In addition, two different signal peptidases involved in signal peptide processing have been characterized (27, 31). In vitro protein translocation systems have been used to demonstrate a direct requirement for some of these *sec* gene products in protein export as well as for other unidentified proteinaceous factors (3, 7, 8, 13, 30) and to reveal the central role of ATP hydrolysis in catalyzing protein translocation across the plasma membrane (4). In spite of this progress, most of the mechanistic details of the protein export process remain unsolved.

SecA protein appears to occupy a pivotal role in *E. coli* protein export. SecA protein must bind to the inner membrane prior to or concurrently with precursor proteins to allow for functional precursor protein binding (6). SecA protein has also been shown to possess an ATPase activity which is greatly stimulated when SecA interacts with both precursor proteins and inverted inner membrane vesicles containing functional SecY/PrfA protein, and this ATPase activity has been inferred to be essential for protein translocation across the plasma membrane (14). The large, 901-amino-acid-residue SecA protein (26) is a multifunctional protein. In addition to protein translocation and ATPase activities, the SecA protein also contains an autoregulatory

activity, as shown by the translational repression of a *secA-lacZ* protein fusion in cells overproducing the SecA protein (25). This autoregulatory property of the SecA protein is coupled to the protein export capability of the cell, since SecA protein synthetic levels are low during normal protein secretion but rise 10- to 20-fold when protein export is blocked (20, 22). The site of this regulation appears to be in the intergenic region of the *geneX-secA* operon (20, 24a). The molecular paradigm for *secA* regulation, the mechanism of its coupling with protein export proficiency, and its purpose in protein export are areas that require further study.

Despite the large size of the SecA protein and its apparent functional complexity, previous genetic selective strategies have yielded *secA* mutations at a limited number of sites. The available *secA*(Ts) mutations are contained only within the coding sequence for the first 170 amino acid residues of the SecA protein (26). Analysis of *secA* alleles which suppress signal sequence mutations showed that three such alleles resulted in alterations at amino acid residues 111, 373, and 488 of the SecA protein (9). In order to define additional regions of the SecA protein that are important in its functions, we have made use of the facts that *secA* regulation is tightly coupled to the protein-export-proficient state of the cell and that even relatively mild protein export defects derepress *secA* expression (20, 22). Accordingly, we have subjected a plasmid-encoded *secA* gene to in vitro mutagenesis and screened this plasmid library colorimetrically for new *secA* mutants that derepress a *secA-lacZ* fusion. We describe the isolation and characterization of these new *secA* alleles below.

MATERIALS AND METHODS

Media and reagents. Media used for the growth of bacterial cultures have been described previously (17). DNA restric-

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tion and modifying enzymes were obtained from New England Biolabs, Inc. (Beverly, Mass.). DNase I was obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). All enzymes were used as recommended by the supplier. Radiochemicals were purchased as follows. [α - 32 P]dATP (600 to 800 Ci/mmol) and 125 I-donkey anti-rabbit immunoglobulin G (IgG) (750-3,000 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, Calif.), and Tran- 35 S label (1,114 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.). IgSorb was purchased from the New England Enzyme Center (Boston, Mass.). The Sequenase DNA sequencing kit was purchased from United States Biochemical Corp. (Rockville, Md.). The *Apa*I linker (GGGCCC) was purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). pZ523 columns were purchased from 5'→3', Inc. (Westchester, Penn.), and GeneClean kits were from Bio 101, Inc. (La Jolla, Calif.). M13mp18 and M13mp19 replicative-form DNAs and the *lac* universal primer (no. 1211) were purchased from New England Biolabs, Inc.

Strains and plasmids. MM171.3 [Φ (*secA-lacZ*)/*f181*(Hyb) *recA1* *srI*::Tn10 (λ PR9)] has been described previously (25). The following MC4100 ($F^- \Delta$ *lacU169 araD136 relA rpsL thi*) derivatives were constructed: MC4100.2 (*recA1* *srI*::Tn10), HS2081.1 [*malT*(Con) *rpoB* *recA*::*cat*], BA13.1 [*secA13* (Am) *trp*(Am) *supF*(Ts) *recA1* *srI*::Tn10], and DO1151.100 [*secA51*(Ts) *srI*::Tn10]. MC1000.12 (*recA1* *srI*::Tn10) and MC1000.72 [*secA51*(Ts) *pcnI* *leu*::Tn5 *recA1* *srI*::Tn10] are both derivatives of MC1000 [$F^- \Delta$ (*leu-ara*)7697 *araD139* *lacX74 rpsL galU galK*]. Plasmid pMF8 containing the *geneX-secA* operon has been described previously (25).

Plasmid constructions. Large- and small-scale preparations of plasmid DNA were purified as described previously (23), except that large-scale preparations were purified further by using pZ523 columns following the manufacturer's instructions. The *in vitro* mutagenesis of pMF8 was as follows. Plasmid DNA (1 to 2 μ g) in 20 μ l of 50 mM Tris (pH 7.5)–1 mM $MnCl_2$ was treated with 30 pg of DNase I per ml at 22°C for 30 min, which resulted in ~10% linearization, followed by extraction with phenol–chloroform and precipitation with ethanol. The DNA was resuspended in an equal volume of 10 mM Tris (pH 7.5)–10 mM $MgCl_2$ –50 mM NaCl–100 μ g of bovine serum albumin per ml–125 μ M each the four deoxynucleoside triphosphates and was treated with 5 U of Klenow fragment of DNA polymerase I at 37°C for 20 min. Full-length, linear plasmid DNA was purified by electrophoresis through an agarose gel and isolated by using a GeneClean kit according to manufacturer's instructions. The phosphorylated *Apa*I linker and linear plasmid DNA were ligated in a 500:1 or 1,000:1 molar ratio, respectively, and used to transform MC1000.12. Approximately 15,000 transformants were pooled and grown to stationary phase in LB supplemented with 25 μ g of ampicillin per ml, and plasmid DNA was isolated. The DNA was digested with *Apa*I, and full-length linear DNA was purified as described above, ligated, and retransformed. Approximately 15,000 transformants were pooled again, and the plasmid library was subjected to a second round of enrichment for plasmids containing the *Apa*I linkers as described above. The plasmid pool was used to transform MM171.3. The transformation mix was grown in LB at 37°C for 1 h and plated on lactose tetrazolium plates supplemented with ampicillin (100 μ g/ml) at 37°C. Colonies were scored after 24 h of incubation. Plasmids pGJ14, pGJ15, and pGJ16 are derivatives of plasmid pMF8 containing *Apa*I linker insertions at the *Kpn*I, *Bgl*II, and *Nru*I sites in the *secA* gene, respectively. For construction of pGJ14 and pGJ15, oligonucleotides contain-

FIG. 1. Genetic selection for *secA* mutants. MM171.3 containing a *secA-lacZ* protein fusion and an additional copy of the *geneX-secA* operon on an integrated transducing phage is depicted. Transformation by plasmid pMF8 containing an intact *geneX-secA* operon results in a Lac^- phenotype, while transformation by a pMF8 derivative containing a mutation in the *secA* gene (symbolized by the arrowhead) results in a Lac^+ phenotype.

ing the *Apa*I recognition sequence and the appropriate 5' or 3' overhang were synthesized, phosphorylated with polynucleotide kinase, and ligated to pMF8 DNA that had been cleaved with the appropriate restriction enzyme. For construction of pGJ16, the phosphorylated *Apa*I hexamer linker was ligated to full-length linear pMF8 DNA that had been partially cleaved with *Nru*I.

Mapping and sequencing of the *secA* mutations. The mutations were located by standard restriction enzyme mapping techniques by using the following digests: *Apa*I, *Eco*RI, *Kpn*I, *Sna*BI, *Apa*I and *Eco*RI, *Apa*I and *Kpn*I, and *Apa*I and *Sna*BI. Single-stranded DNA was prepared for DNA sequence analysis by one of the following methods. Plasmid DNA (1 to 2 μ g) was digested with either *Sna*BI or *Bam*HI, extracted with phenol–chloroform, and precipitated with ethanol; the DNA was resuspended in 70 mM Tris (pH 8.0)–1 mM $MgCl_2$ –10 mM dithiothreitol and was incubated overnight with 4 U of exonuclease III at room temperature, followed by extraction with phenol and precipitation with ethanol. Alternatively, an appropriate DNA fragment containing the *secA* mutation was subcloned into M13mp18 or M13mp19, and single-stranded DNA was isolated as described previously (26). DNA sequencing reactions were done by using the Sequenase kit and the appropriate *secA* or M13 *lac* primer according to the manufacturer's instructions.

RESULTS

***In vitro* mutagenesis and isolation of *secA* mutations.** We have previously described a strain, MM171.3, which contains a *secA-lacZ* protein fusion gene at the normal *secA* locus and an additional copy of the *geneX-secA* operon on an integrated transducing phage (25). Introduction of pMF8, which contains the *geneX-secA* operon and which overproduces SecA protein 10-fold, into this strain resulted in a 3-fold reduction of expression of the *secA-lacZ* fusion due to autogenous superrepression (25). Figure 1 shows that such

FIG. 2. In vitro mutagenesis scheme. The steps for constructing the mutagenized pMF8 plasmid library are outlined.

repression was easily detected on lactose tetrazolium plates, since MM171.3(pMF8) displayed a Lac⁻ phenotype (red colonies), whereas MM171.3 containing a plasmid lacking a functional geneX-*secA* operon displayed a Lac⁺ phenotype (pink colonies). MM171.3 containing a plasmid that produced nonfunctional SecA protein that interfered with chromosomal *secA* function displayed a stronger Lac⁺ phenotype on this medium (white colonies). We reasoned that this genetic system would be an ideal starting point for the isolation of plasmid-encoded *secA* mutations.

We wanted to use a form of mutagenesis that results in a high frequency of mutations at randomly located sites, produces substantial changes in the SecA protein sequence, and results in mutations that are easily mapped on the large, 2,703-bp *secA* gene (26). Two-codon mutagenesis (1) fulfills two of these criteria, since in vitro treatment of plasmid DNA with DNase I in the presence of Mn²⁺ results in essentially random linearization and since ligation with a hexanucleotide linker encoding a unique restriction enzyme cleavage site allows ready determination of the site of the mutation. Furthermore, we have found that repair of the plasmid DNA after DNase I treatment with the Klenow fragment of DNA polymerase I resulted in the production of small deletions and duplications, presumably because of the action of the polymerase on staggered nicks produced during DNase I treatment (see below). This repair process allowed a wider variety of mutations to be created.

Plasmid pMF8 was subjected to the mutagenesis and screening procedures shown in Fig. 2. In addition to the steps outlined above, we had to enrich for plasmids that contained the hexanucleotide *ApaI* linker since they constituted only ~5% of the plasmids obtained after transformation. Therefore, we pooled a large number of transformants,

FIG. 3. Screen for *secA* mutants allowing accumulation of SecA protein by using Western blot analysis. Samples (1 ml) of early stationary phase cultures grown in LB supplemented with 20 µg of ampicillin per ml at 37°C were sedimented, resuspended in 300 µl of sample buffer (2% sodium dodecyl sulfate, 125 mM Tris [pH 6.8], 5% β-mercaptoethanol, 15% glycerol, 0.005% bromophenol blue), and boiled for 2 min. Samples (40 µl) were loaded onto 10% polyacrylamide gels which were subjected to electrophoresis, and the relevant portions of the gel were electrophoretically transferred onto nitrocellulose strips. Strips were incubated with either rabbit anti-SecA or anti-OmpA primary antisera followed by addition of ¹²⁵I-donkey anti-rabbit IgG secondary antisera and processed for autoradiography as described previously (3).

isolated the mutagenized pool of plasmid DNA, and cleaved with *ApaI*. Only those plasmids containing the *ApaI* linker were cleaved by this treatment. Isolation of linear plasmid DNA, ligation, and retransformation resulted in a population of plasmids enriched for the presence of the *ApaI* linker. After two rounds of enrichment, more than 85% of all plasmids contained the *ApaI* linker. This pool of plasmids was used to transform MM171.3, and transformants were selected on lactose tetrazolium plates containing ampicillin. One-third of all colonies arising on these plates were white or pink in color, unlike the MM171.3(pMF8) parent strain, indicating that the mutagenesis procedure had been efficient. Colonies displaying this altered phenotype were picked, purified, and subjected to further analysis.

Strains showing an altered phenotype could have resulted from a variety of mutations that were not of immediate interest (e.g. nonsense or frameshift mutations in *secA*, *secA* mutations affecting protein stability, geneX mutations polar on *secA* expression, mutations reducing plasmid copy number). In order to focus on mutations of interest, we screened these strains by Western immunoblot analysis for those that still overproduced the SecA protein. An example of these results is shown in Fig. 3. In this example, 6 of 16 strains had SecA levels equal to or higher than those of MM171.3 (pMF8) (Fig. 3, compare lanes 6, 7, 9, 16, 17, and 18 with lane 2). In addition, several of these strains produced forms of SecA protein with altered electrophoretic mobilities (Fig. 3, lanes 6, 7, and 18), presumably because of deletion or insertion of additional amino acid residues in the SecA protein or more subtle alterations affecting its electrophoretic behavior. Of a total of approximately 100 strains screened in this manner, 21 were found that still produced high levels of SecA protein.

Mapping and DNA sequence analysis of the *secA* alleles. Plasmid DNA was isolated from all strains that displayed an altered phenotype in MM171.3 and overproduced SecA protein, and the position of the *ApaI* linker was determined by restriction enzyme analysis. The results are shown in Fig. 4. Of 21 plasmids characterized, 18 were found to contain mutations in the *secA* gene, while 3 contained mutations in geneX. The analysis of the geneX mutations will be described elsewhere (11a). The mutations in *secA* tended to be

FIG. 4. Location of *secA* mutations. The locations of mutations isolated by the MM171.3 colorimetric screen which produced high levels of SecA protein are shown above the bar depicting the geneX-*secA* operon. The locations of mutations unable to complement a *secA*(Am) mutant are shown below the bar. The numbers in parentheses indicate the number of mutations obtained at that site. A scale in base pairs is given below the figure.

clustered. In order to demonstrate that our initial mutagenized plasmid library contained mutations throughout the *secA* gene, we transformed our starting library into the *secA*(Am) mutant, BA13.1, and collected plasmid derivatives that were unable to complement this mutation. Since this latter group of plasmids had not been subjected to the more stringent genetic and biochemical screening methods employed formerly, they should be more representative of the total mutational complexity found in our plasmid library. Figure 4 shows that the plasmids which were collected solely by their inability to complement BA13.1 showed a more random distribution of mutations. This result confirms that the nonrandom distribution of mutations obtained with the MM171.3-Western blot screening method was due to the imposition of this screening method.

We sequenced the 18 new *secA* mutations isolated from the MM171.3-Western blot screening method, and the results are summarized in Fig. 5 and Table 1. Only 12 of the 18 *secA* mutations were unique; the remaining 6 *secA* mutations were identical to 3 of the 12 unique mutations. The 12 unique mutations clustered into four regions of *secA* corresponding to amino acid residues 196 to 252, 352 to 367, 626 to 653, and 783 to 808 (for construction of pGJ14, pGJ15, and pGJ16, see below). Of the 12 mutations, 7 were small deletions averaging ~40 bp and 5 were small insertions averaging ~8 bp in addition to the *Apa*I linker insertion. It is important to note that these mutational events were not exceptional, since similar small deletions and insertions which did not result in SecA overproduction were found in a randomly selected group of plasmids from our initial library (data not shown). There was also concern that other mutagenic events occurred during the mutagenesis procedure that, in conjunction with the *Apa*I linker-marked mutation, could add to or cause the phenotypes observed. To address this possibility, we reconstructed a small DNA fragment containing the *Apa*I linker mutation from plasmid pGJ3 into pMF8. MM171.3 harboring this new plasmid displayed a phenotype similar to that of the original strain (data not shown).

Since our genetic and biochemical screening method had resulted in a very nonrandom distribution of *secA* mutations of the desired phenotype, we decided to construct three mutations in the large middle region of the *secA* gene where no mutations had been obtained. Three derivatives of pMF8 were constructed and verified by DNA sequence analysis that had *Apa*I linkers at the *Bgl*II, *Nru*I, and *Kpn*I sites of the *secA* gene; these mutations resulted in alterations of the SecA protein sequence between amino acid residues 421 and 422, 502 and 504, and 558 and 559, respectively (Fig. 5

and Table 1). Western blot analysis of MM171.3 containing each of these three plasmids indicated that only one (pGJ14 containing the mutation at the *Kpn*I site) produced high levels of SecA protein (data not shown). This result indicates that the absence of mutations in certain regions of *secA* was probably due to the involvement of these regions in protein folding and stability. In addition, the absence of mutations in other *secA* regions could be due to the fact that such mutations caused dominant lethal defects (see below).

Physiological characterization of the *secA* mutants. In order to assess the biological activities of the SecA proteins produced by the new *secA* mutants, we transformed the plasmids shown in Table 1 into four different strain backgrounds in which such activities could be monitored more readily. MM171.3 containing the *secA-lacZ* fusion was used to quantitate SecA autoregulatory activity *in vivo* by assaying β -galactosidase activity. The *secA*(Am) *supF*(Ts) and the *secA*(Ts) mutants, BA13.1 and DO1151.100, respectively, were used to measure the SecA protein's essential translocation activity by monitoring cell growth and *in vivo* protein export at 42°C. The wild-type strain, MC4100.2, or its isogenic derivative constitutive for *mal* regulon expression, HS2081.1, was used to investigate any dominant effects the *secA* mutations had on protein export. In constructing these strains we noticed that introduction of plasmid pGJ1, pGJ2, or pGJ3 into wild-type or *secA* mutant strains resulted in a dominant, conditional-lethal, cold-sensitivity phenotype for growth at 30°C. Therefore, for subsequent analysis we divided the *secA* mutations into nonconditional (class I) and conditional-lethal (class II) categories. To allow the reader to more easily follow the properties of the new *secA* mutants throughout our subsequent studies, we have summarized many of the pertinent results in Table 2.

Analysis of SecA autoregulatory activities for the class I mutations is shown in Table 3. The nine mutations fell into three categories. First, the β -galactosidase activity was lower in MM171.3 containing plasmid pGJ4, pGJ11, or pGJ12 than in MM171.3(pBR322) but not as low as in MM171.3(pMF8). This result indicated that the SecA protein produced by these plasmids retained some residual autoregulatory activity. Second, MM171.3(pGJ15) and MM171.3(pGJ16) exhibited β -galactosidase activities similar to those of MM171.3(pBR322), consistent with these two plasmids producing an unstable form of SecA protein. Third, the β -galactosidase activities of MM171.3 containing each of the remaining six plasmids were even higher than that of MM171.3(pBR322), indicating that these plasmid-encoded

FIG. 5. Schematic representation of the *secA* mutations contained on the pGJ plasmids. The horizontal bar depicts the *secA* gene contained in each plasmid. The location and type of *secA* mutation are indicated; the solid bar indicates the *Apal* linker(s) or a duplication, and a break in the bar indicates a deletion. A scale in amino acid residues is given at the top of the figure.

SecA proteins compromised protein export and derepressed the *secA-lacZ* fusion.

Complementation analysis of the class I mutations in the *secA*(Am) and *secA*(Ts) mutants is given in Table 4. Only the *secA* alleles contained on pGJ4, pGJ11, and pGJ12 complemented these *secA* mutants, indicating that they encoded SecA protein that possessed essential protein translocation activity. Two plasmids, pGJ5 and pGJ10, could not be introduced into these *secA* mutant strains, even at the permissive temperature, presumably because of interference with SecA function. We found that these two plasmids could be introduced into but would not complement a *secA*(Ts) *pcn* mutant strain, MC1000.72, in which plasmid copy number was reduced approximately 10-fold (16).

To further characterize the translocation activities of the class I mutants, we analyzed the export and processing of precursors to the periplasmic maltose-binding protein (MBP) and the outer membrane protein, OmpA, in BA13.1 containing the appropriate plasmids. By assaying protein export from strains grown at 30 or 42°C, we could assess the translocation activity of SecA protein produced from these plasmids in the presence or absence of SecA protein produced by the chromosome, respectively. However, it should be noted that even at 30°C, the level of SecA protein produced by the *secA* chromosomal copy is insufficient to

support wild-type rates of protein export (19). The results are shown in Fig. 6. At 30°C, both positive and negative effects were seen on protein export in BA13.1 containing the class I plasmids compared with that in BA13.1(pBR322). However, none of the strains showed restoration of wild-type export rates equivalent to those of BA13.1(pMF8) (Fig. 6a, compare lanes 3 through 9 with lanes 1 and 2; Fig. 6b, compare lanes 3 through 5 with lanes 1 and 2). Shift of these strains to 42°C revealed that only BA13.1 containing plasmid pGJ4, pGJ11, or pGJ12 had normal export rates for MBP and OmpA (Fig. 6a, lanes 10 through 18; Fig. 6b, lanes 6 through 10). However, since the SecA level was at least 10-fold greater than that of the wild-type in these three strains, the specific activity of the SecA proteins produced by these three plasmids may still be quite low. These results are in complete agreement with the complementation results discussed above.

We investigated whether the class I *secA* mutations had dominant effects on protein export in a wild-type strain background, HS2081.1. These results are shown in Fig. 7. All merodiploid strains containing class I mutations showed reduced rates of protein export, as indicated by the presence of precursors to MBP and OmpA. However, HS2081.1 containing pGJ4, pGJ11, or pGJ12, which encoded functional forms of SecA protein as indicated by the complemen-

TABLE 1. DNA and protein sequence alterations for the *secA* mutations

Plasmid	DNA and protein sequence alterations ^a																
pGJ1	GCG	CGG	GGC	CCG	AAT												
	ala219	arg	gly	pro	asn240												
pGJ2	GCG	GGC	CCG	AAT													
	ala219	gly	pro	asn240													
pGJ3	ATG	GGG	GCC	CGG	GCC	CCA	CAG										
	met782	gly	ala	arg	ala	pro	gln796										
pGJ4	GAA	GGG	GCC	CGG	GCC	CGG	GCC	CGG	GCC	CGG	GCC	CGT	CAG	GAA	AAA	GAA	GAC
	glu252	gly	ala	arg	ala	arg	ala	arg	ala	arg	gly	arg	gln	glu	lys	glu	asp253
pGJ5	GAT	GGG	CCC	GAA													
	asp351	gly	pro	glu368													
pGJ6	ACT	AAG	GGC	CCC	GAT												
	thr625	lys	gly	pro	asp654												
pGJ7	GGT	AGG	GCC	CGG	GCC	CGC	TAC										
	gly788	arg	ala	arg	ala	arg	tyr794										
pGJ8	CAG	GGG	GGC	CCG	CGT												
	gln787	gly	gly	pro	arg792												
pGJ9	CAG	GGC	CCG	GGC	CCC	TCC											
	gln801	gly	pro	gly	pro	ser809											
pGJ10	CAG	GGG	GGC	CCG	GGT	ATC											
	gln788	gly	gly	pro	gly	ile789											
pGJ11	CCT	GAG	GGC	CCG	GGC	CCT	GAA	GAA									
	pro195	glu	gly	pro	gly	pro	glu	glu196									
pGJ12	CCT	GAG	GGC	CCT	GAA	GAA											
	pro195	glu	gly	pro	glu	glu196											
pGJ14	GGT	ACG	GCC	CGG	GCC	CGT	ACC										
	gly558	thr	ala	arg	ala	arg	thr559										
pGJ15	AAA	GAT	CGG	CCC	GGG	CCC	GAT										
	lys421	asp	arg	pro	gly	pro	asp422										
pGJ16	ATC	GGG	GCC	CGG	GCC	CCG	ACC										
	ile502	gly	ala	arg	ala	pro	thr504										

^a The relevant DNA and protein sequence regions encompassing the mutations are shown. Numbered amino acid residues indicate wild-type information immediately preceding and following the altered protein sequence. Amino acid residues encoded by the hexamer or 15-mer *ApaI* linkers are in boldface type, amino acid residues due to sequence duplications are in italics, and amino acid residues attributable to both are in boldface italics.

tation analysis, displayed nearly normal protein export profiles (Fig. 7a, compare lanes 3 through 11 with lanes 1 and 2; Fig. 7b, compare lane 3 with lanes 1 and 2). HS2081.1 containing pGJ5 or pGJ10, plasmids which were toxic in *secA* mutant strain backgrounds, displayed the greatest protein export defects (Fig. 7a, lanes 4 and 9). As pGJ15 and pGJ16 encode unstable SecA proteins, no dominant effect on protein export was found for HS2081.1 containing either of these plasmids (Fig. 7b, lanes 4 and 5).

We have extended our analysis of protein export physiology to the class II *secA* mutants which displayed a cold-sensitive phenotype. MC4100.2 containing pGJ1, pGJ2, or pGJ3 was grown at 38°C and shifted to 30°C for various

lengths of time, and the extent of MBP and OmpA processing was determined by Western blot analysis. The results are shown in Fig. 8. Protein export appeared to be normal in these three strains at 38°C (Fig. 8, lanes 3 through 5) but was increasingly defective with longer incubation times at 30°C (Fig. 8, lanes 8 through 10, 13 through 15, and 18 through 20). When the export defects seen in Fig. 6, 7, and 8 are compared, it must be kept in mind that a given export defect would appear less severe when analyzed by Western blotting. In fact, when these three strains were analyzed by pulse-labeling and immunoprecipitation techniques, more than 80% of MBP and OmpA were in the precursor form after a shift to 30°C for only 1 h (data not shown).

TABLE 2. Summary of the properties of the *secA* mutants

Plasmids	Class ^a	Protein stability ^b	Complementation ^c	Autoregulatory activity ^d	Translocation activity ^e	Dominance ^f
pGJ1, pGJ2, pGJ3	II	S	NV	—	NV	Very strong
pGJ4, pGJ11, pGJ12	I	S	+	±	+	Weak
pGJ5, pGJ10	I	S	—	—	—	Strong
pGJ6, pGJ7, pGJ8, pGJ9, pGJ14	I	S	—	—	—	Moderate
pGJ15, pGJ16	NA	U	—	—	—	None

^a I, Nonconditional; II, conditional-lethal cold sensitivity for growth; NA, not applicable.

^b S, Stable; U, unstable. SecA protein stability was assessed by Western blot analysis.

^c +, Complementation; —, no complementation of *secA*(Ts) and *secA*(Am) mutant defects; NV, strain not viable.

^d ±, Partial activity; —, no SecA autoregulatory activity, as assessed by repression of a *secA-lacZ* fusion.

^e +, Presence of translocation activity; —, absence of translocation activity, as assessed by the processing of precursors of MBP and OmpA; NV, strain not viable.

^f Dominance was assessed by the severity of protein export defects in wild-type strains and growth defects in *SecA*(Ts) and *secA*(Am) mutants carrying the plasmid indicated.

TABLE 3. Autoregulatory defects of class I *secA* mutants

Plasmid	Mean β -galactosidase activity $\pm$ SD ^a
pBR322	363 $\pm$ 6.4
pMF8	108 $\pm$ 1.1
pGJ4	214 $\pm$ 3.0
pGJ11	197 $\pm$ 3.7
pGJ12	316 $\pm$ 3.0
pGJ5	888 $\pm$ 81.8
pGJ6	406 $\pm$ 15.3
pGJ7	565 $\pm$ 9.8
pGJ8	689 $\pm$ 17.9
pGJ9	579 $\pm$ 9.2
pGJ10	457 $\pm$ 6.0
pGJ14	464 $\pm$ 10.0
pGJ15	346 $\pm$ 22.1
pGJ16	339 $\pm$ 5.4

^a Strains were subcultured in duplicate into LB supplemented with 25 μ g of ampicillin per ml, grown at 30°C until mid-logarithmic phase of growth, and placed on ice. Dilutions were plated on TYE (10 g of Bacto-tryptone, 5 g of yeast extract, 8 g of NaCl, and 15 g of Bacto-Agar per liter) and TYE-ampicillin plates to score for plasmid loss, which was less than 5% in all cases. β -Galactosidase assays were performed in triplicate by the method of Miller (17), and β -galactosidase activity is indicated in Miller units.

DISCUSSION

Since previous genetic selections have resulted in a limited spectrum of *secA* mutations, we have developed a new genetic method for isolating novel *secA* mutants. Our strategy relied on the observation that the *secA* gene is autoregously repressed during normal protein export but becomes derepressed when protein export is compromised. This allowed us to mutagenize a plasmid-encoded copy of the *geneX-secA* operon and colorimetrically detect dominant *secA* mutants, since they resulted in derepression of a *secA-lacZ* fusion. This genetic system was also capable of detecting *secA* autoregulatory mutants, since such mutants would fail to superrepress the *secA-lacZ* fusion and would result in a Lac⁺ phenotype in our assay. As part of this screening procedure it was necessary to use Western blot-

TABLE 4. Complementation analysis of class I mutations

Plasmid	Complementation of ^a		
	BA13.1	DO1151.100	MC100.72
pBR322	—	—	—
pMF8	+	+	+
pGJ4	+	+	+
pGJ11	+	+	+
pGJ12	+	+	+
pGJ6	—	—	—
pGJ7	—	—	—
pGJ8	—	—	—
pGJ9	—	—	—
pGJ14	—	—	—
pGJ15	—	—	—
pGJ16	—	—	—
pGJ5	NV	NV	—
pGJ10	NV	NV	—

^a The transformed strains have the following *secA* alleles: BA13.1, *secA13*(Am); DO1151.100, *secA51*(Ts); MC100.72, *secA51*(Ts) *pcn*. Strains containing the plasmids indicated were streaked onto two TYE-ampicillin plates, and growth and colony formation were scored after incubation overnight at either 42 or 30°C. Complementation was indicated by growth and colony formation at 42°C. +, Complementation; —, no complementation; NV, strain was not viable at either 30 or 42°C.

FIG. 6. Analysis of protein export in the class I *secA* mutants. Strains were grown in M63 minimal medium containing 0.4% glycerol, 0.4% maltose, 20 μ g of ampicillin per ml, and other supplements when needed to an A_{600} of 0.4 to 0.5, when aliquots of each culture were shifted to the temperature indicated for 1 h. Aliquots (0.5 ml) of each culture were labeled with 10 μ Ci of Tran-³⁵S label for 1 min followed by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid. Proteins were analyzed by immunoprecipitation, polyacrylamide gel electrophoresis, and autoradiography as described previously (26). (a) BA13.1 containing the plasmid indicated grown at 30 (lanes 1 to 9) or 42 (lanes 10 to 18) °C for 1 h prior to labeling. (b) BA13.1 containing the plasmid indicated grown at 30 (lanes 1 to 5) or 42 (lanes 6 to 10) °C for 1 h prior to labeling. The positions of SecA, MBP, and OmpA and the respective precursors, pMBP and pOmpA, are indicated.

ting methods to eliminate all trivial mutants that failed to synthesize stable forms of SecA protein. This combined strategy resulted in the identification of 12 new *secA* mutations contained in four regions of *secA* corresponding to amino acid residues 196 to 252, 352 to 367, 626 to 653, and 783 to 808. Physiological analyses of strains carrying these mutations indicated that three mutations (contained on pGJ4, pGJ11, and pGJ12) reduced both SecA translocation and SecA autoregulatory activities, while the remainder abolished SecA translocation activity and caused stronger dominant protein export defects accompanied by *secA* depression. When three *secA* mutations were constructed in a large region lacking any mutations, two of them (contained on pGJ15 and pGJ16) resulted in SecA protein instability, while the remaining one (contained on pGJ15) elicited properties identical to those of our other mutants.

Although our genetic strategy was aimed at obtaining dominant *secA* mutations, it is clear from the number of sites in which such mutations can occur that this type of mutation is common. This result favors two possible models for SecA function: either this protein functions as a multimer at some point in protein translocation or it forms a stable association with some other component or site during protein export. In these two scenarios, overproduction of the mutant form of the SecA protein effectively poisons wild-type subunits in the former model or titrates out a protein that interacts with

FIG. 7. Class I mutations result in dominant protein export defects. HS2081.1 containing the plasmid indicated was grown at 37°C, and radiolabeling and protein analysis were performed as described in the legend to Fig. 6. The positions of SecA, MBP, and OmpA and the respective precursors, pMBP and pOmpA, are indicated.

SecA in the latter model. An association of the SecA protein with the integral membrane SecY/PrfA protein is supported by the observations that SecA protein can suppress a *secY24(Ts)* protein translocation defect in vitro (7) and that a functional SecY protein must be present on membrane vesicles in order to stimulate SecA ATPase activity (14). A direct association of the secretory precursor protein with SecA protein is supported by the ability of proOmpA to stimulate SecA ATPase activity in liposomes (15) and by the isolation of *secA* alleles that suppress signal sequence mutations (9). Genetic approaches utilizing multicopy suppres-

sor analysis or extragenic suppressor analysis of the conditional lethal, class II *secA* mutants could be useful to discriminate between these two models. Since both models predict that the dominant *secA* mutations result in the production of proteins retaining certain SecA functions, biochemical analysis of these altered proteins should be informative. For example, analysis of at least one class II mutant indicates that a stable precursor protein-SecA complex accumulates specifically during the export block (3a). Further biochemical analysis of the dominant *secA* mutants could help to localize the SecA protein's functional sites of interaction with the precursor protein and components of the export machinery.

Although our genetic selection was also capable of detecting *secA* regulatory mutants, no mutations were obtained that solely affected the autoregulatory function of SecA. We feel that this result is not due to inadequate mutagenesis of our starting plasmid library, since we demonstrated that this library contained a reasonably random distribution of mutations of substantial severity prior to any selective procedures employed. One type of model which would explain our results assumes that an autoregulatory region of SecA protein exists but has not been detected because it overlaps with a region needed for protein folding or protein translocation activity or because it is of sufficiently small size to have escaped detection by our methods. In this regard, one good way of assuring a tight coupling between protein export proficiency and *secA* regulation would be to have these two properties tightly linked at a structural level. If this is indeed the case, then a more subtle type of mutagenesis may be needed to genetically uncouple these two properties. Another model which would explain our results assumes that another protein senses the protein translocation activity of SecA and is itself the repressor of *secA* translation. In this scenario, the superrepression of *secA* expression that is seen in MM171.3(pMF8) would be due to such an effector sensing excess SecA protein translocation activity and causing additional repression. We have demonstrated recently that the SecA protein specifically binds to its own mRNA by using either photo-cross-linking or filter-binding methods (24a). Although this finding strongly favors models where SecA protein directly participates in its autoregulation, additional studies will be needed to resolve this issue.

Finally, our results extend the number of regions on the SecA protein implicated in its protein translocation activity. Previous studies showed that the available *secA(Ts)* mutations are contained only within the coding sequence for the first 170 amino acid residues of the 901-amino-acid-residue SecA protein (26). Analysis of *secA* alleles which suppress signal sequence mutations showed that three such alleles resulted in alterations at amino acid residues 111, 373, and 488 of the SecA protein (9). Analysis of *azi* mutations, conferring resistance of cell growth and protein export to inhibition by sodium azide, showed that these were alleles of *secA*, two of which resulted in alterations at amino acid residues 179 and 645 of the SecA protein (19a). Finally, analysis of predicted ATP-binding sites in the SecA sequence which could mediate SecA ATPase activity (14) identified three A-type elements and one B-type element (5) located at SecA amino acid residues 160 to 167, 341 to 348, 503 to 511, and 266 to 283, respectively (20). Given the large size and presumed complexity of the SecA protein, future detailed studies will be required to make sense of these different regions of SecA protein on a structural and functional level.

FIG. 8. Analysis of protein export in the class II *secA* mutants. Samples (1 ml) were collected from early stationary phase cultures grown in LB supplemented with 20 µg of ampicillin per ml at 38°C or shifted to 30°C for the times indicated and processed and analyzed by Western blotting as described in the legend to Fig. 3. MC4100.2 containing the indicated plasmid grown at 38°C (lanes 1 to 5) or shifted to 30°C for 1 (lanes 6 to 10), 2 (lanes 11 to 15), or 3 (lanes 16 to 20) h prior to analysis of SecA, MBP, and OmpA and the respective precursors, pMBP and pOmpA, by Western blotting.

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