JOURNAL OF BACTERIOLOGY, Jan. 2002, p. 111–118 Vol. 184, No. 1
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.1.111–118.2002
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Genetic Screen Yields Mutations in Genes Encoding All Known Components of the *Escherichia coli* Signal Recognition Particle Pathway

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Received 10 July 2001/Accepted 2 October 2001

We describe the further utilization of a genetic screen that identifies mutations defective in the assembly of proteins into the *Escherichia coli* cytoplasmic membrane. The screen yielded mutations in each of the known genes encoding components of the *E. coli* signal recognition particle pathway: *ffh*, *ffs*, and *ftsY*, which encode Ffh, 4.5S RNA, and FtsY, respectively. In addition, the screen yielded mutations in *secM*, which is involved in regulating levels of the SecA component of the bacterium's protein export pathway. We used a sensitive assay involving biotinylation to show that all of the mutations caused defects in the membrane insertions of three topologically distinct membrane proteins, AcrB, MalF, and FtsQ. Among the mutations that resulted in membrane protein insertion defects, only the *secM* mutations also showed defects in the translocation of proteins into the *E. coli* periplasm. Genetic evidence suggests that the S382T alteration of Ffh affects the interaction between Ffh and 4.5S RNA.

In mammalian cells, much of protein targeting to the membrane of the endoplasmic reticulum depends on the signal recognition particle (SRP) and the membrane-bound SRP receptor (24, 36). The mammalian SRP complex consists of six proteins and an RNA (43, 44). As a nascent chain emerges from the ribosome, its amino-terminal signal sequence is recognized by the 54-kDa protein of the SRP (SRP54) (9, 13, 48). The SRP ribosome–nascent-chain complex is then delivered to the SRP receptor (6). The nascent chain is released to the membrane-embedded translocon and subsequently translocated into the endoplasmic reticulum (24).

The *Escherichia coli* SRP, first identified by searching for sequence homologs of the mammalian SRP subunits, is a simplified version of its mammalian counterpart (3, 21, 27, 35). Instead of six proteins and one RNA, the *E. coli* SRP consists only of a 48-kDa protein, Ffh, and the 4.5S RNA. Mature-form 4.5S RNA is 114 nucleotides long, much smaller than its mammalian homolog 7S L RNA (over 300 nucleotides long). The *E. coli* SRP receptor, FtsY, unlike the heterodimeric eukaryotic SRP receptor, consists of only one subunit. The *ffh*, *ffs*, and *ftsY* genes, which encode Ffh, 4.5S RNA, and FtsY, respectively, are all essential for cell viability (5, 14, 20).

Despite the presence of an SRP analog in *E. coli* and reports indicating its involvement in the translocation of several secreted proteins (14, 20, 21), the *E. coli* SRP does not appear to play a major role in the translocation of proteins into the periplasm and outer membrane. Instead, it appears to function mainly in the targeting and integration of cytoplasmic membrane proteins (7, 12, 15, 33, 39, 41). The SRP binds to the hydrophobic transmembrane segment of cytoplasmic membrane proteins (40, 41). The SRP ribosome–nascent-chain complex is targeted to the cytoplasmic membrane through interaction with FtsY, which is associated with the inner membrane (14, 22). Besides the SRP, the *E. coli* secretion machinery, including the membrane-bound SecYEG translocation channel as well as the ATPase SecA, has been demonstrated both genetically and biochemically to be important for the integration of membrane proteins (10, 38, 42, 45, 46). YidC, a homolog of the mitochondrial import protein Oxa1p (4), also appears to be required for the insertion process (28).

Evidence for *E. coli* SRPs in membrane protein assembly comes from in vitro studies and from in vivo studies examining the effects of depleting SRP components on the integration of membrane proteins. An in vivo genetic screen that yields *E. coli* mutants defective in membrane protein insertion has been described previously (37). The *E. coli* strain used expresses a hybrid protein, MalF–β-galactosidase 102, in which β-galactosidase is fused to the second periplasmic domain of the cytoplasmic membrane protein MalF. The insertion of MalF into the inner membrane and the export of its second periplasmic domain leads to the partial translocation of β-galactosidase, resulting in the loss of β-galactosidase activity (16). Mutations disrupting either the membrane protein insertion process or disulfide bond formation result in localization of β-galactosidase in the cytoplasm, where it folds into an active conformation (1, 37).

Colonies of the strain used for the screen appear white on minimal agar containing the substrate dye X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) due to the lack of active β-galactosidase. Colonies of a *dsbA*- or *dsbB*-null mutant appear dark blue due to high levels of β-galactosidase activity. Mutagenized cells were screened for those that exhibited a less intense blue color than that of the *dsbA* or *dsbB* phenotypically null cells, and 108 mutants with various degrees of β-galactosidase activity were found. Twenty-five of these mutants were the result of *ffs* mutations that cause defects in membrane

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
HPT57	MC1000 *phoA*$^+$ *phoR leu*$^+$ λ102 (MalF-LacZ102 Amp^r)	37
HPT129	HPT57 *zih*::Tn*10*	37
HPT130	HPT57 *fadR*::Tn*10*	37
HPT264	JP313 *leu*::Tn*10*, arabinose sensitive	This study
HPT265	JP313 *secM73 leu*::Tn*10*, arabinose sensitive	This study
HPT301	JP313 *leu*::Tn*10 secM93*	This study
HPT302	JP313 *leu*::Tn*10 secM98*	This study
HPT299	JP313 *zhg*::Tn*10*	This study
HPT300	JP313 *zhg*::Tn*10 ftsY70*	This study
HPT369	HPT264, arabinose resistant	This study
HPT370	HPT265, arabinose resistant	This study
HPT404	JP313 *pheA3141*::Tn*10*Kan	This study
HPT405	JP313 *pheA3141*::Tn*10*Kan *ffh-77*	This study
HPT406	JP313 *pheA3141*::Tn*10*Kan *ffh-87*	This study
HPT407	JP313 *pheA3141*::Tn*10*Kan *ffh-103*	This study
HPT-UV23	HPT57 *ffs-23*	This study
HPT-UV46	HPT57 *ffs-46*	This study
HPT-UV70	HPT57 *fts Y70*	This study
HPT-UV73	HPT57 *secM73*	This study
HPT-UV77	HPT57 *ffh-77*	This study
HPT-UV87	HPT57 *ffh-87*	This study
HPT-UV89	HPT57 *ffh-89*	This study
HPT-UV93	HPT57 *secM93*	This study
HPT-UV98	HPT57 *secM98*	This study
HPT-UV103	HPT57 *ffh-103*	This study
CAG18608	MG1655 *pheA3141*::Tn*10*Kan	34
CAG599	F$^-$ *zhg*::Tn*10 lacZ*(Am) *trp*(Am) *pho*(Am) *supC* (Ts) *mal*(Am) *rpsL*	Harris D. Bernstein
EC294	MG1655 *leu*::Tn*10*	Lab collection
JP313	MC4100 Δ*ara714*	Lab collection
SG20253	MG1655 *zba-3054*::Tn*10*	34
Plasmids		
CJ1	pACYC184-*secM* Cm^r	Don B. Oliver
pBAD42-*secA*	pBAD42-*secA* Cm^r	Jan-Willem de Gier
pBE2	*gene X-secA* in pACYC, Cm^r	Don B. Oliver
pGJ78 series (J and K)	PSBT fusions at positions J (periplasmic) and K (cytoplasmic) of MalF in pBR origin plasmid under control of the IPTG-inducible promoter, Kan^r	10
pHP5	pAM238-*ffs* Spc^r	37
pHP42	pBAD18-*ftsQ*-PSBT Amp^r	37
pHP44	pBR322-*acrR' acrA acrB576*-PSBT	37
pMS421	p*lacI*^{q} Spc^r	Lab collection
pTRC-*ftsY*	pTRC-*ftsY* Cm^r	39
RB11-*ffh*	p*lac-ffh*, with *lacI*^{q} on the same plasmid, Amp^r	Harris D. Bernstein

protein insertion (37). In this study, we describe nine additional mutants with mutations in each component of the *E. coli* SRP-SRP receptor complex and mutations in *secM*, a gene involved in the regulation of SecA synthesis (8, 26, 30, 32). This study represents the first genetic approach that identifies mutations in all known components of the *E. coli* SRP. We further demonstrate that the *ffs*, *ffh*, *ftsY*, and *secM* mutants exhibit defects in the membrane assembly of three cytoplasmic membrane proteins: FtsQ, MalF, and AcrB. Among the mutations affecting membrane protein insertion, only *secM* mutations also cause defects in protein secretion.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. The *E. coli* strains and plasmids used in this study are listed in Table 1. Cells were grown at 37 or 30°C in NZY rich medium (10) or M63 minimal medium with appropriate supplements and antibiotics (37). Additional supplements used in this study included nicotinic acid (2 ng/ml), leucine, and phenylalanine (both at 50 μg/ml).

Mapping of *ffs*, *secM*, *ffh*, and *ftsY* mutations. Possible mutations linked to *ffs*, *ffh*, *ftsY*, and *secM* were determined by P1 transduction using transposons linked to each of the genes; Tn*10* in strain SG20253 was 50% linked to the wild-type *ffs*, Tn*10* in strain EC294 was 50% linked to *secM-secA*, Tn*10* Kan from strain CAG18608 was 60% linked to *ffh*, and Tn*10* in strain CAG599 was 30% linked to *ftsY*.

Pulse-chase, immunoprecipitation, and steady-state protein level. Pulse-chase and immunoprecipitation experiments were carried out as described previously (37). Anti-OmpA and anti-maltose binding protein (anti-MBP) antibodies (laboratory collection) were used to immunoprecipitate OmpA and MBP. Steady-state SecA, Ffh, and FtsY levels were examined by carrying out a standard Western blot analysis. Whole-cell proteins were precipitated by treating cells grown to an optical density at 600 nm (OD_{600}) of 0.2 with 6% trichloroacetic acid. Anti-SecA (Jan-Willem de Gier), anti-Ffh, and anti-FtsY (both from Harris Bernstein) were used for the detection of the protein of interest.

Preparation and detection of total PSBT fusion proteins and biotinylated fusion proteins. The expression and preparation of the MalF-*Proprionibacterium shermanii* transcarboxylase (PSBT), AcrB-PSBT, and FtsQ-PSBT fusion proteins from wild-type and mutant strains were carried out according to procedures described previously (37). Polyclonal anti-MalF PhoA J and anti-FtsQ antibodies (laboratory collection), as well as the anti-AcrB antibody (Hiroshi Nikaido), were

TABLE 2. Summary of 104 UV-induced mutants

Class	Position(s) of the mutation(s)	No. of mutants identified
I (defective in disulfide bond formation)	*dsbA* and *dsbB*	28 (*dsbA*) and 21 (*dsbB*)
II (defective in membrane protein insertion)	*ffs*	27
	ffh	4
	ftsY	1
	secM	3
	Unmapped	1
III	λ102 (*malF-lacZ102*)	14
IV	90-min region	1
	Unmapped	4

used for the detection of total MalF-PSBT, FtsQ-PSBT, and AcrB576-PSBT fusion proteins. Streptavidin-horseradish peroxidase (HRP) (Amersham) was used to detect the biotinylated fusion proteins.

Spheroplast preparation and trypsin sensitivity assay. *E. coli* strains carrying plasmids pGJ78-J or -K and pMS421 were grown overnight at 37°C in NZY medium containing spectinomycin (100 μg/ml) and kanamycin (40 μg/ml). After being diluted 1:100 in the same medium and grown to an OD_{600} of 0.3, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM to induce the fusion protein. After the culture reached an OD_{600} of 0.6, cells were chilled on ice for at least 20 min and 1.5 ml of cells was spun down and resuspended in ice-cold 18% sucrose–100 mM Tris-HCl (pH 8.0). After addition of EDTA (0.01 M) and lysozyme (100 μg/ml) to the resuspended cells and incubation on ice for 20 min, cells were split into three parts. To the first, trypsin was added at a final concentration of 10 μg/ml. To the second, only the sucrose buffer mentioned above was added. To the third, before addition of trypsin at 10 μg/ml, Triton X-100 was added at a final concentration of 1%. All three samples were incubated on ice for 15 min before the addition of phenylmethylsulfonyl fluoride to a final concentration of 35 mg/ml. Proteins were then precipitated with 10% trichloroacetic acid. After the protein pellets were washed with acetone and dried, sodium dodecyl sulfate sample buffer containing 0.7 M β-mercarptoethanol was added to resuspend the proteins. Whole-cell proteins were first separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis before being transferred to a nitrocellulose membrane using a Semi-Dry protein transfer apparatus (Bio-Rad). After incubation of the membrane with streptavidin-HRP (Amersham) diluted at 1:1,500 and detection of the biotinylated proteins using an ECL kit (Amersham), the nitrocellulose membrane was incubated in 0.5 M NaOH for 5 min. The membrane was then washed several times before incubation with anti-MalF-PhoA J antibody (laboratory collection) for subsequent detection of the total MalF-PSBT fusion proteins.

RESULTS

Three classes of mutational events might cause cytoplasmic localization of the β-galactosidase of the MalF–β-galactosidase 102 fusion protein: disruption of disulfide bond formation, interference with membrane protein insertion, and removal of portions of the MalF protein, altering the location of the attached β-galactosidase from the periplasm to the cytoplasm. Eighty-eight of 108 mutations obtained were characterized previously (37). Mutants with 4 of the 20 remaining mutations grew poorly and were difficult to analyze genetically. To determine to which category the other 16 mutations belonged, P1 transduction was carried out to detect linkage of the mutations to each of the genes involved in disulfide bond formation, to those genes required for membrane protein insertion, or to the *malF-lacZ102* fusion (reference 37 and Materials and Methods). Four of these mutations were linked to *ffh*, one was linked to *ftsY*, two were linked to *ffs*, and two were linked to *secM*. An additional mutation that caused MalF membrane assembly defects was linked to none of these genes and was not studied further. The remainder of the mutations resulted in mutants that showed no defects in MalF assembly, being mutations either in *dsbA* or in none of the genes tested (Table 2).

The *ffh* mutations. The four *ffh* mutants exhibited low levels of β-galactosidase activity (<10-fold higher than for the wild-type parent). The faint-blue-colony phenotype on X-Gal plates was fully complemented by plasmid RB11-*ffh*, carrying only *ffh* (results not shown). DNA sequencing revealed that *ffh-77* has a single nucleotide change of G109C (counting from the ATG start site), resulting in an alanine 37-to-proline change (Table 3). *ffh-87* and *ffh-103* share the same T1144A nucleotide alter-

TABLE 3. Mutations in *secM, ffs, ffh*, and *ftsY* mutants

Mutated gene(s)	Nucleotide change[a]	Amino acid change[b]
secM73, secM93	C181T	Q60 stop site
secM98	Deletion of one T from TT at positions 170 and 171	Alteration of amino acids at positions 58–88 before stop site at position 89
ffs-23	TG to AA from −1 and +1 positions[c]	
ffs-46	GG to AA from +57 and +58 positions[c]	
ffh-77	G109C	A37P
ffh-87, ffh-103	T1144A	S382T
ffh-89	A355G	K119E
ftsY70	A1241G	E414G

[a] Nucleotide numbering starts from the start codon.
[b] Amino acid numbering starts from the first translated amino acid (including the signal sequence).
[c] Positions are relative to the first base of the mature RNA.

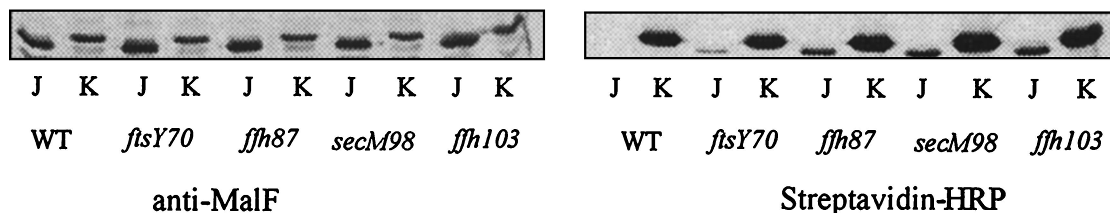

FIG. 1. *ffh*, *ftsY*, and *secM* mutants are defective in the membrane insertion of MalF. The MalF-PSBT periplasmic J fusion and cytoplasmic K fusion were expressed from pGJ78-J and pGJ78-K in HPT57 (wild type) and HPT-UV70, HPT-UV87, HPT-UV98, and HPT-UV103 containing the *ftsY70, ffh-87, secM98*, and *ffh-103* mutations, respectively. Anti-MalF antibody was used to detect total fusion proteins, and streptavidin-HRP was used to detect the biotinylated fusion proteins. WT, wild type.

ation, which results in a serine 382-to-threonine change. *ffh-89* contains a single nucleotide change of A355G, leading to a lysine 119-to-glutamate change. Western blot analysis indicated that the steady-state Ffh levels in mutants containing the *ffh-77, ffh-87*, or *ffh-103* mutation were similar to that in the wild type (results not shown). For some of the subsequent analyses, *ffh* mutations were moved to a JP313 strain background by P1 transduction.

The *ftsY* mutation. The single *ftsY* mutant showed a faint-blue-colony phenotype with an intensity like that of the *ffh* mutants and was complemented by a plasmid (pTRC-*ftsY*) carrying only the *ftsY* gene. Sequencing analysis revealed a single A1241G nucleotide alteration (numbering starts from the ATG start site) causing a glutamate 414-to-glycine change (Table 3). Western blot analysis indicated no significant difference between the steady-state FtsY protein levels for this mutant and the wild type (results not shown). This *ftsY* mutant allele, *ftsY70*, was moved by P1 transduction to a new strain background for some of the subsequent analyses.

The *ffs* mutants. Two mutations linked to the *ffs* region (Table 3) were complemented by introducing plasmid pHP5 carrying only *ffs*. The *ffs-23* mutation converts a TG to an AA at the −1 and +1 positions relative to the starting point of the mature RNA. *ffs-46* has GG-to-AA nucleotide changes at the 157 and 158 positions, corresponding to a region between the apical tetraloop and the symmetric internal loop of the 4.5S RNA. *ffs-46* differs from our other *ffs* alleles in that it caused significantly slowed cell growth both on plates and in liquid culture.

The *secM* mutants. The two new mutations linked to the *secM-secA-mutT* operon were complemented for the pale-blue-colony phenotype by plasmid pBE2 carrying *secM* and *secA*. *secM93* shares the same nucleotide alteration with the previously identified *secM73*, which results in a C181T change (counting from the GTG start site), resulting in a stop codon at the amino acid residue at position 60 (with the signal sequence included in the numbering). *secM98* has a frameshift mutation that deletes a T from two T's at positions 170 and 171, resulting in a stop codon at the amino acid residue at position 89 (Table 2).

Initially termed *gene X, secM* regulates SecA and does not play a direct role in protein secretion (23). *secM, secA*, and *mutT* are cotranscribed; known nonsense mutations in *secM* have a strong polar effect on *secA* expression (8, 26, 30, 32). *secM* encodes a 170-amino-acid-long protein which, upon secretion to the periplasm, is processed to its final mature form of 133 amino acids (23, 29). In wild-type cells, SecM is rapidly degraded by the periplasmic-tail-specific protease (17). SecM is also subjected to a transient-translation pause at a position close to the carboxyl terminus, and this elongation arrest involves the nascent SecM itself. This pausing is found to be significantly enhanced when SecM translocation is retarded (17).

The *ffh, ftsY, ffs*, and *secM* mutants are all defective in membrane protein insertion. We considered it likely that the *ffh, ftsY*, and *ffs* mutations caused the cytoplasmic localization of β-galactosidase in the fusion as a result of their effects on membrane protein insertion; *secM* mutations might cause the same defects due to their polar effect on the downstream *secA*. We used a biotinylation assay, which was previously employed for detecting membrane protein insertion defects in the other *ffs* mutants, to test this possibility (37). In our assay system, a biotinylatable domain (80 amino acids in size) from the 1.3S subunit of PSBT was fused to the periplasmic domains of MalF, AcrB, and FtsQ (37). If the fusion proteins failed to be inserted into the cytoplasmic membrane, the PSBT domain was biotinylated by biotin ligase, an enzyme found only in the cytoplasm in *E. coli*. However, if efficiently inserted, the PSBT domain was translocated into the periplasm rapidly enough so that it would remain unbiotinylated.

ffh, ftsY, ffs, and *secM* mutants are all defective in the insertion of MalF into the cytoplasmic membrane, as indicated by the biotinylation of a fraction of the MalF-PSBT periplasmic fusion J (results for *ffh-87, ffh-103, ftsY70*, and *secM98* mutants are shown in Fig. 1; other results are not shown). These mutants also exhibited defects in the insertion of AcrB and FtsQ (results for *ffh-87, ftsY70*, and *secM73* are shown in Fig. 2; other results are not shown). In the wild-type parent strain, we observed no biotinylated signal for the PSBT fused to the periplasmic domains of MalF, AcrB, and FtsQ (Fig. 1 and 2). A biotin carboxyl carrier protein, which resides in the cytoplasm, showed the same levels of biotinylation in all strain backgrounds (results not shown).

Only *secM* mutations cause defects in the secretion of periplasmic and outer membrane proteins. We asked whether the mutations affecting membrane protein assembly also exhibited defects in protein translocation across the cytoplasmic membrane by monitoring OmpA signal sequence processing in pulse-chase experiments (37). After a 1-min chase with cold methionine, while the wild-type cells and *ffs, ffh*, and *ftsY* mutant cells all showed only mature OmpA, the *secM98* mutant still had significant amounts of precursor OmpA (Fig. 3). From

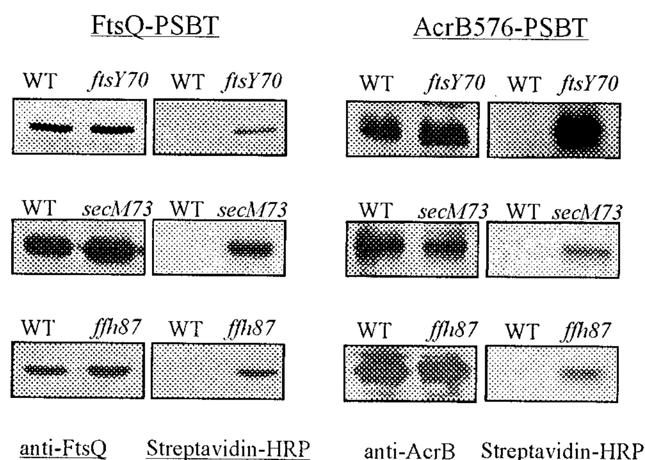

FIG. 2. *ffh*, *ftsY*, and *secM* mutants are defective in the membrane insertion of AcrB and FtsQ. AcrB576-PSBT and FtsQ-PSBT were expressed from pHP44 and pHP42 in HPT404 (wild-type *ffh*), HPT406 (*ffh-87*), HPT299 (wild-type *ftsY*), and HPT300 (*ftsY70*). AcrB576-PSBT was expressed from pHP44 in HPT264 (wild-type *secM*) and HPT265 (*secM73*). FtsQ-PSBT was expressed from pHP42 in HPT369 (wild-type *secM*) and HPT370 (*secM73*). Anti-AcrB and anti-FtsQ antibodies were used to detect AcrB576-PSBT and FtsQ-PSBT, respectively, and streptavidin-HRP was used to detect the biotinylated fusion proteins. Blots on different rows were exposed for different lengths of time, and the band intensities, therefore, should not be compared. WT, wild type.

this experiment, we conclude that while the *secM* mutations cause a defect in protein translocation into the periplasm, the same process remains unaffected by the *ffh*, *ftsY*, and *ffs* mutations.

The *secM* mutants produce apparently normal amounts of SecA. Because the *secM* mutations all result in stop codons early in the protein coding region, a possible explanation for the defects we observed is that SecA levels were reduced. In fact, the faint-blue-mutant-colony phenotype of all three *secM* mutants was complemented by plasmid pBAD42-secA, carrying *secA* alone, but not CJ1, carrying only *secM*. However, Western blot analysis and pulse-chase–immunoprecipitation for both the wild type and the *secM73* mutant showed no observable differences in SecA levels between the two strains (results not shown).

The biotinylated fusion proteins in *ftsY* and *secM* mutants stay in the cytoplasm. In a mutant defective only in the kinetics of protein translocation, biotinylation of a MalE-PSBT fusion does not block its translocation into the periplasm (10, 25). We had assumed that, similarly, our SRP mutants might be defective only in the kinetics of membrane protein insertion; the membrane proteins would likely still be inserted into the cytoplasmic membrane, although at a lower rate than that for the wild-type (37). We tested this hypothesis directly by determining whether the biotinylated domain of the periplasmic MalF-PSBT fusion in our mutants was exposed to the periplasm. To do so, we added trypsin to the spheroplasts prepared from mutants expressing the fusion protein. A trypsin site in the MalF-PSBT fusion is located in the second periplasmic domain in MalF. If properly inserted into the membrane, a trypsin digest may generate a 25-kDa band, replacing the full-length MalF-PSBT fusion band that was seen prior to the addition of trypsin. If the protein fails to be inserted into the membrane or is inserted in a different topology such that the trypsin-sensitive site is not exposed to the periplasm, the full-length fusion band will remain.

In a wild-type background, when the spheroplasts expressing the fusion protein were treated with trypsin, a 25-kDa band replaced the full-length MalF-PSBT fusion band as we expected (top left panel of Fig. 4, lanes 6 and 9), indicating the proper membrane insertion of this fusion protein. In the *secM93, secM98*, and *ftsY70* mutant spheroplasts, although the same 25-kDa band replaced most of the full-length fusion signals when trypsin was added, there was still a full-length MalF-PSBT signal remaining (top left panel of Fig. 4, lanes 7, 8, and 10), indicating that a proportion of the fusion protein was insensitive to trypsin and, therefore, unlikely to be inserted properly in all three mutants. When the same membranes were incubated with streptavidin-HRP to reveal the biotinylated signal, the lanes corresponding to mutant samples treated or not treated with trypsin all revealed a biotinylated band running at the same position as that of the full-length MalF-PSBT protein and the intensities of the biotinylated bands remained the same under both conditions (top right panel of Fig. 4, lanes 2, 3, and 5, and lanes 7, 8, and 10). These results indicated that the biotinylated fusion proteins in all the mutants were protected from trypsin digest. When the spheroplasts from the mutants were treated with Triton X-100, first to disrupt the membrane and then with trypsin, all the full-length fusion proteins disappeared, indicating that the inaccessibility of trypsin to the fusion protein was due to the protection provided by the cytoplasmic membrane (Fig. 4, lower panel). We could also confirm that the spheroplasts used for our trypsin digest (without Triton X-100) were intact based on the presence of the biotinylated cytoplasmic carrier protein, which disappeared when Triton X-100 was added to dissolve the membrane prior to the addition of trypsin.

DISCUSSION

A genetic screen for membrane protein defects has yielded point mutations in the *E. coli* genes coding for 4.5S RNA, Ffh,

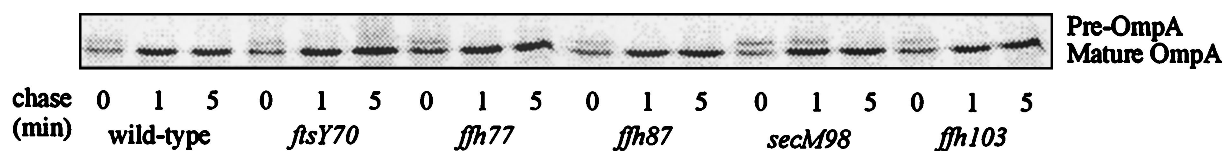

FIG. 3. Protein secretion is defective in cells containing mutations in *secM* but not in *ffh* or *ftsY*. Pulse-chase and immunoprecipitation of OmpA were carried out to examine the efficiency of OmpA translocation into the periplasm in HPT57 (wild type) and in HPT-UV98, HPT-UV77, HPT-UV87, HPT-UV103, and HPT-UV70, containing the *secM98*, *ffh-77*, *ffh-87*, *ffh-103*, and *ftsY70* mutations, respectively.

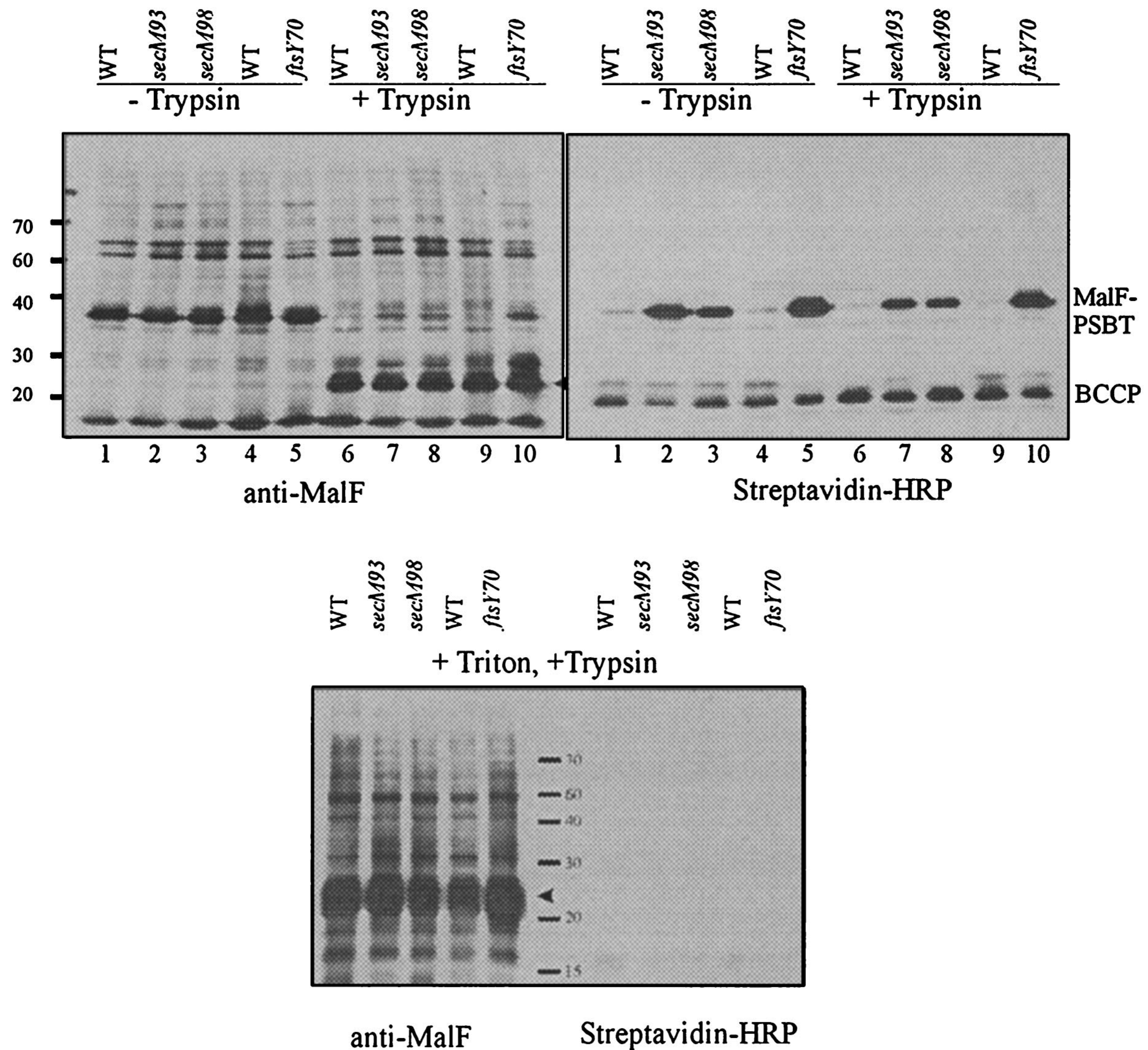

FIG. 4. The biotinylated PSBT domain is located in the cytoplasm. Spheroplasts were prepared from HPT264 (wild-type *secM*), HPT301 (*secM93*), HPT302 (*secM98*), HPT299 (wild-type *ftsY*), and HPT300 (*ftsY70*), each of which expresses the MalF-PSBT periplasmic fusion J from plasmid pGJ78-J. (Upper panels) Trypsin was added to the indicated samples. (Lower panel) Triton X-100 was added before trypsin. Anti-MalF PhoA J antibody was used to visualize the expressed fusion proteins, and streptavidin-HRP was used to visualize the biotinylated fusion protein. Arrowheads point to the 25-kDa band of the trypsin-digested product. WT, wild type.

and FtsY; in all components of the *E. coli* SRP and SRP receptors and in the *secM* gene, a regulator of SecA. Using the biotinylation assay, we demonstrated that all mutations lead to a defect in the proper insertion of the cytoplasmic membrane proteins MalF, AcrB, and FtsQ. SecM mutations, however, also caused slowed translocation of the outer membrane protein OmpA and the periplasmic protein MBP (37).

The *ffs* mutations. We suspect that the *ffs-23* and *ffs-46* mutations affect the 4.5S RNA differently from the way the *ffs-29* and *ffs-69* mutations described previously affect 4.5S RNA (37). *ffs-23*, which alters the base composition in the region corresponding to the start site of the mature 4.5S RNA, may cause a defect in the proper 5′-end processing of the precursor RNA. *ffs-46* alters two bases in the most internal stem region, and this region has been shown to be important for the interaction between 4.5S RNA and Ffh in vitro (47). Although the bases altered by the mutations do not directly contact Ffh according to the crystal structure of the Ffh M domain-4.5S RNA complex (2), they disrupt base pairing in this region, which can subsequently alter the conserved symmetric and asymmetric loops surrounding it and lead to the disruption of 4.5S RNA Ffh binding.

Ffh mutations in the N and G domains. The three *ffh* mutations each altered a different structural domain of Ffh: *ffh-77* resulted in an A37P change in the amino-terminal N domain; *ffh-89* resulted in a K119E change in the GTPase G domain; and *ffh-87* and *ffh-103* resulted in the same S382T change in the methionine-rich M domain. The A37P and S382T mutations did not alter the stability of the protein and, therefore, must affect some aspect of Ffh function. Alanine 37, which was

changed to proline as a result of the *ffh-77* mutation, is located in a stretch of amino acid residues in the N domain that are especially well conserved among distantly related species (ALLEADV in *E. coli*). The N domain of mammalian SRP54 was proposed to promote efficient signal sequence binding in an in vitro study that examined the binding by using SRP54 variants with mutations in this stretch of amino acid residues (18). Although A37 was not among the mutated residues, mutations altering LL and DV from the ALLEADV stretch all led to the decreased binding of signal sequences by SRP54. If the same function exists for the N domain of Ffh, we might expect the *ffh-77* mutation to affect its binding to the hydrophobic transmembrane segment of inner membrane proteins.

Lysine 119, which was converted to glutamate as a result of the *ffh-89* mutation, is completely conserved across bacterial species. It resides between the first and the second GTPase consensus elements (there are a total of four such elements in Ffh). It is not obvious from the current structural information how this mutation affects the function of Ffh.

The Ffh M domain S382T mutation likely affects Ffh-4.5S RNA interaction. The methionine-rich M domain, where the S382T mutations resulting from *ffh-87* and *ffh-103* reside, contains both the signal sequence recognition surface and the contact interface with 4.5S RNA (2). In the SRP core complex crystal, M382 forms hydrogen bonds with C62 from the symmetric loop and G48 from the asymmetric loop in conserved domain IV of the 4.5S RNA (2). When a plasmid carrying *ffs* (pHP5) was introduced into *ffh-87* and *ffh-103* mutants, the faint-blue-colony phenotype disappeared (results not shown). The same complementation was not observed when pHP5 was introduced into *ffh-77*. This implies that in the *ffh-87* and *ffh-103* mutants, because of the change of S382T, Ffh no longer interacts with the 4.5S RNA with 100% efficiency, and this results in fewer functional 4.5S RNA-Ffh complexes in the mutants than in the wild type. Introducing extra copies of the 4.5S RNA complements the defect by increasing its local concentration, thus promoting 4.5S RNA-Ffh complex formation. This complementation experiment also implies that the S382T mutant Ffh proteins remain intact in other functions such as FtsY binding.

FtsY mutation. *ftsY70* has a single amino acid change of E414G in the GTPase domain of FtsY. This glutamate residue, however, is not among the most conserved residues across different species. Since the *ftsY70* mutation does not cause decreased stability of the protein, the E414G amino acid change likely affects some aspect of FtsY function.

SecM mutations. It is striking that all three mutations in the chromosomal region where *secA* resides were in the *secM* gene, resulting in two truncated versions of SecM protein. They each caused defects in membrane protein insertion as well as presecretory protein translocation. Since *secM* does not play a direct role in protein secretion (23), we initially speculated that the *secM* mutations caused the observed defects by reducing expression of the downstream *secA* gene. This inference was supported by the finding that a plasmid expressing *secA* alone, but not one expressing *secM* alone, complemented the defects in *secM*. However, Western blot analysis revealed that the SecA protein levels in the *secM* mutants and the wild-type cells were similar. Previously, an amber mutation in *secM* at amino acid position 132 resulted in the repression of SecA translation, possibly due to the blocking of *secA* translational initiation by base pairing of the *secA* Shine-Dalgarno sequence with the *secM* terminus (19, 31, 32). Normally, this base pairing is disrupted upon translation of *secM* into this region, thus allowing translation of SecA (11). The fact that we observed normal amounts of SecA protein in our *secM* early-termination mutants seems to contradict this earlier observation. It may be that there is a second translational start site later in *secM* which in these mutants initiates the translation of a protein internal to wild-type SecM, allowing downstream SecA translation. Alternatively, the normal SecA levels in the *secM* mutants could be a result of the derepression of *secA* expression. However, neither explanation allows a clear explanation for the phenotype of our *secM* mutants. Therefore, we leave open the possibility that *secM* may play a role in membrane protein insertion as well as in *secA* expression.

Biotinylated fusion proteins remain in the cytoplasm in the *ftsY70* and *secM* mutants. In the *ffh*, *ftsY*, and *secM* mutants, the biotinylated derivatives of the MalF-PSBT periplasmic fusion proteins were not accessible to trypsin added from the outside of the spheroplasts, indicating a cytoplasmic location for the biotinylated PSBT domain. We offer two possible explanations for our results. First, in the *ffs, ffh, ftsY*, and *secM* mutants, a portion of the MalF-PSBT protein failed to be inserted into the membrane and was thus biotinylated in the cytoplasm. Second, the mutations only slowed the kinetics of membrane protein insertion, allowing biotinylation of some of the MalF-PSBT, which then could no longer be inserted into the membrane. Previously, we observed that in a *sec* mutant defective in the kinetics of translocation, biotinylation of the PSBT fused to the last amino acid residue of MalE did not block its further translocation into the periplasm. Our results here may be explained by differences between the mechanisms by which secreted proteins are translocated into the periplasm and inner membrane proteins are assembled into the inner membrane.

Our results strengthen the conclusion that the SRP pathway plays an important role in the process of membrane protein insertion (7, 12, 15, 33, 39, 41). Our mutants provide an advantage for in vivo studies of membrane proteins in mutant strains, as our mutants exhibit relatively normal growth rates. Previous in vivo studies had either depleted the *ffh*, *ftsY*, and *ffs* gene products or used dominant lethal variants in which the basis of the dominant lethality was not well worked out. Although the majority of mutations in *ffs* affect membrane protein insertion as a result of the decreased amount of 4.5S RNA (37), the *ffh* and *ftsY* mutations likely affect some functional aspects of the Ffh and FtsY proteins. This genetic approach to the understanding of membrane protein insertion can be exploited further to yield mutations that help to dissect the individual steps of the pathway.

ACKNOWLEDGMENTS

We thank Harris D. Bernstein, Jan-Willem de Gier, Hiroshi Nikaido, and Don B. Oliver for providing strains, plasmids, and antibodies. We gratefully acknowledge advice given by members of the Beckwith Lab.

This work was supported by National Institutes of Health grant GM38922 to J.B. J.B. is an American Cancer Society research professor.

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