A mutant hunt for defects in membrane protein assembly yields mutations affecting the bacterial signal recognition particle and Sec machinery

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We describe an *Escherichia coli* genetic screen that yields mutations affecting two different cellular processes: disulfide bond formation and membrane protein assembly. The mutants defective in disulfide bond formation include additional classes of *dsbA* and *dsbB* mutations. The membrane protein assembly defective mutants contain a mutation in the *secA* operon and three mutations in the *ffs* gene, which encodes 4.5S RNA. These latter mutations are the only ones to be isolated in a gene encoding a component of the bacterial signal recognition particle by screening *in vivo* for defects in membrane protein insertion. A sensitive method for examining membrane protein localization shows that the *ffs* and *secA* locus mutations affect membrane assembly of the polytopic membrane protein, MalF. The *ffs* mutations also affect the membrane insertion of the FtsQ and the AcrB proteins. Although both the *ffs* and the *secA* locus mutations interfere with membrane protein assembly, only the latter also reduces export of a protein containing a cleavable signal sequence.

This is the story of a genetic selection designed to yield mutations affecting one cellular process that instead yielded mutations in another. And, how, years later, a modification of that selection gave us the mutations we originally sought.

Approximately 10 years ago we developed a genetic selection to isolate *Escherichia coli* mutants defective in membrane protein assembly. The strain we used expressed a fusion of β-galactosidase to a large periplasmic domain of the cytoplasmic membrane protein MalF (1) (Fig. 1). The resulting hybrid protein was incorporated into the membrane with a portion of the β-galactosidase exported to the periplasm, a portion in the membrane, and a third portion in the cytoplasm. Not surprisingly, this protein exhibited no β-galactosidase activity (2). We anticipated that mutations in genes required for membrane protein insertion would result in localization of the β-galactosidase to the cytoplasm where it would be enzymatically active and allow growth of cells on lactose. However, selecting for Lac$^+$ mutants of the *E. coli* strain expressing this fusion only yielded mutations disrupting disulfide bond formation (3). We speculated that in the wild-type background, disulfide bonds were formed in the exported portion of the β-galactosidase, thus stabilizing the transmembrane structure of the β-galactosidase and rendering it inactive. Inactivation of the disulfide bond formation pathway destabilized the transmembrane structure of β-galactosidase, allowing it to be retracted into the cytoplasm where it was enzymatically active (Fig. 1). This hypothesis was supported by the observation that adding DTT, a reductant able to break disulfide bonds, could restore β-galactosidase activity to this fusion protein (4).

With this Lac$^+$ genetic selection, we obtained mutations in two genes required for disulfide bond formation, *dsbA* and *dsbB* (3, 5). Extensive exploitation of this genetic selection repeatedly produced phenotypically null mutants that either introduced stop codons in the gene or eliminated one of the essential cysteine residues in the two proteins. It appeared that the Lac$^+$ selection demanded maximal levels of β-galactosidase activity, which in turn required full inactivation of the pathway for disulfide bond formation.

We were interested in carrying out a genetic structure-function analysis of DsbB and DsbA. In addition, we were seeking extragenic

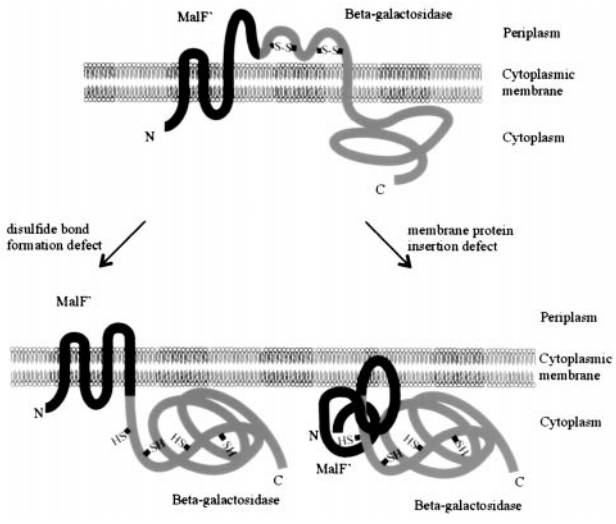

Fig. 1. The MalF-LacZ102 fusion protein and its topology in the wild type and the proposed localization of the fusion protein when cells are defective in the formation of disulfide bond or membrane protein insertion.

mutations in other genes that might cause less severe defects in disulfide bond formation. Because these goals did not seem to be achievable with the genetic selection we were using, we decided to modify our approach to find weaker mutants. Instead of selecting for Lac$^+$ colonies, we would screen for partial restoration of β-galactosidase activity to the fusion protein. Here, we describe the collection of mutants obtained with this screening procedure. We obtained additional weaker mutations in *dsbA* and *dsbB*. In addition, we identified mutations in genes that affect membrane protein insertion, the original goal of this project. These mutations are in *ffs*, encoding 4.5S RNA, the RNA component of the bacterial signal recognition particle (SRP), and in gene *X*, encoding a protein that is cotranslationally coupled with SecA. We report on mutations isolated in SRP in a screen or selection for mutants defective in membrane protein insertion. Using a sensitive assay for membrane protein insertion (6), we show that these mutations affect the assembly of several membrane proteins.

Materials and Methods

Strains, Plasmids, Media, and Growth Conditions. Strains and plasmids are listed in Table 1. Strains were grown at 37°C or 30°C in

Abbreviations: SRP, signal recognition particle; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; PSBT, *Propionibacterium shermania* transcarboxylase.

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Table 1. Stains and plasmids

	Relevant characteristics	Reference or source
Strain		
CAG18480	MG1655 *nadB51*::Tn*10*	(36)
CAG599	F⁻ *zhg*::Tn*10 lacZ (am) trp (am) pho (am) supC (ts) mal (am) rpsL*	Harris D. Bernstein
DR388	DHB4 Δ(λattL − *lom*)::*bla trxC′*−′*lacZ argE*::Tn*10*	Daniel Ritz
EC294	MG1655 *leu*::Tn*10*	David S. Weiss
HPT57	MC1000 *phoA*+ *phoR*− *leu*+ λ102D1 (*malF-lacZ* 102, Amp^r)	This study
HPT129	HPT57 *zih*::Tn*10*	This study
HPT130	HPT57 *fadR*::Tn*10*	This study
HPT183	MC4100 *zba-3054*::Tn*10*	This study
HPT184	MC4100 *ffs29 zba-3054*::Tn*10*	This study
HPT185	MC4100 *ffs69 zba-3054*::Tn*10*	This study
HPT186	MC4100 *ffs91 zba-3054*::Tn*10*	This study
HPT242	JP313 *zba-3054*::Tn*10*	This study
HPT244	JP313 *ffs69 zba-3054*::Tn*10*	This study
HPT265	JP313 gene *X leu*::Tn*10*	This study
JP313	MC4100 Δ *ara*714	(37)
MC4100	F⁻ *araD*139 *relA*1 *thi rpsL*150 *flbB*5301 (*lacU*139) *deoC*7 *ptsF*25	Laboratory collection
RI89	MC1000 *phoA*+ *phoR*− *leu*+	(38)
RI90	RI89 *dsbA*::Kan	(38)
SG20253	MG1655 *zba*-3054::Tn*10*	(36)
Plasmid		
Litmus 28	Cloning vector with T7 promoter, Amp^r	New England Biolabs
pAcrB576-AP	pBR *acrR, acrA, -acrB576*-AP, Amp^r	(18)
pAJ1077	pBAD33-*acrA, acrB,* CM^r	Annick Jacq
pAM238	pSC101 origin, Spc^r	(39)
pBAD18	pBR origin with arabinose regulatory region, Amp^r	(40)
pBAD33-*ffs*	pBAD33-*ffs*, CM^r	Harris D. Bernstein
PBAD42-*secA*	PBAD42-*secA*, Spc^r	Jan-Willem De Gier
pCJ1	PACYC184-gene *X*, CM^r	Donald B. Oliver
pDHB5700	p_{tac}(Kan^r)	(41)
pGJ78 series (I, J, K, and L)	MalF-PSBT fusions at positions I, J, K, and L in pBR origin plasmid under IPTG-inducible promoter, Kan^r	(6)
pHP5	pAM238-*ffs*, Spc^r	This study
pHP33	pBAD18-*acrB′(Bsr*GI–*Hin*dIII fragment), Amp^r	This study
pHP38	pBAD18-AcrB′-PSBTHis$_6$ (PSBT His$_6$ fused at *Nco*I site), Amp^r	This study
pHP41	pBAD18-*ftsQ*, Amp^r	This study
pHP42	pBAD18-*ftsQ*-PSBT, Amp^r	This study
pHP44	pBR322-*acrR′acrA acrB576*-PSBT	This study
pLMG161	pBAD18-*ftsQ*, Amp^r	(42)
pMS421	placI^q, Spc^r	(43)
pNG102	pBR322 *malmalE*$^+$ *malF-lacZ* 102, Amp^r	(1)

NZY-rich medium (6) or M63 minimal medium supplemented with D-glucose or maltose (0.2%), B1 (1 μg/ml), and $MgSO_4$ (1 mM) (7). When needed, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) was added (20 μg/ml). For the screening, colonies were grown on M63 minimal maltose X-Gal plates for 2 days at 30°C. For the β-galactosidase assay (7), cells were grown at 30°C in NZY medium containing maltose (0.2%). For the induction of the FtsQ-*Propionibacterium shermania* transcarboxylase (PSBT) and MalF-PSBT fusions, 0.2% L-arabinose and 0.5 mM isopropylthio-galactopyranoside were added, respectively. Antibiotics were used at the following final concentrations unless otherwise specified: ampicillin, 200 μg/ml; kanamycin, 40 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 15 μg/ml; and chloramphenicol, 10 μg/ml.

Strain Construction. HPT57 was constructed in several steps. First, we made a λ transducing phage (λ 102) containing the *malF-lacZ* 102 fusion under the promoter for the *malEFG* operon. We cloned the *Afl*III–*Dra*I fragment of pDHB5700 into λ *cI857 Sam7* (N6-methyladenine-free, New England Biolabs) cut with *Bsi*WI/*Xba*I to generate λ DBK733. This λ has homology to pBR322 near the replicative origin and in the C-terminal part of the *bla*. DNA from pNG102 was recombined onto λ DBK733 replacing the kanamycin-resistance gene with the *malE* promoter, *malE*, the *malF-LacZ* fusion 102 and a complete *bla* gene to generate λ 102. In a second step, strain RI 90 (*dsbA*$^-$) was lysogenized by λ 102 at 30°C as ampicillin-resistant strains that exhibited blue color on NZY plates containing X-Gal. The order of genetic markers in the lysogen is *gal-mod-attL-cIts-malF′-lacZ102-bla-attR*. In the third step, the prophage was stabilized to prevent loss in later manipulations by deleting one of the attachment sites. The lysogens were grown anaerobically at 42°C on NZY plates containing ampicillin (25 μg/ml), X-Gal, and potassium chlorate (0.2%), the latter selecting for inactivation of the *mod* gene. Under anaerobic conditions, nitrate reductase reduces chlorate to chlorite, lethal to the cells. A mutation/deletion in the *modABCD* operon causes resistance to chlorate because of a defect in the uptake of molybdate necessary for the assembly and function of nitrate reductase (8, 9). The medium and the high temperature incubation select for deletion events that remove the chromosomal region including *mod*, *attL*, and the ts *cI*, but leave intact *bla* and, therefore, also the *malF-lacZ* 102 fusion. Because the blue color caused by the cleavage of X-Gal by β-galactosidase does not develop under anaerobic conditions,

the plates subsequently were incubated aerobically at room temperature for 1 day.

Several blue colonies that answered this selection were tested for high temperature resistance, sensitivity to phage λ_{cI}, and resistance to low level of ampicillin (25 μg/ml) but not to high levels of ampicillin (the latter to verify the presence of a single copy of the *bla* gene on the chromosome). Colonies with the appropriate properties were grown on M63 minimal maltose X-Gal plates and examined under the light microscope for signs of instability (e.g., variation of the blue color within a colony). A P1 lysate made on a stable strain was used to transduce the *malF-lacZ* 102 fusion into a *dsbA*$^+$ strain (RI89) by its 100% linkage to the ampicillin-resistance marker. The newly constructed strain, HPT57, was used for the mutagenesis.

To move mutations to different strain backgrounds, we did P1 transductions as described (7). *ffs* alleles were moved to different strain backgrounds by using a transposon Tn*10* (originally in strain SG20253) 50% linked to the *ffs* gene. The gene *X* mutation was moved to other backgrounds by using a *leu*::Tn*10* (originally in strain EC294) 50% linked to the wild-type *secA* region.

Plasmid Construction. A 220-bp *Eco*RI–*Pst*I fragment of pBAD33-*ffs* was ligated to pAM238 cut with *Eco*RI–*Pst*I to generate pHP5. To make pHP42, we first constructed pHP41. Wild-type *ftsQ* was PCR amplified from pLMG161 by using primers FtsQ5′(*Eco*RI): 5′-AGCGAATTCTGGAACTGGCGGACCTA-3′ and FtsQ3′(*Xba*I): 5′-TGCTCTAGATTGTTGTTCTGCCTGTG-3′. The PCR product and pBAD18 both were cut with *Eco*RI–*Xba*I and ligated to generate pHP41. The biotinylatable domain PSBT coding region was PCR-amplified from pGJ78-J by using primers Biotin5′(*Xba*I): 5′-TGCTCTAGAGTCGCCGGTAAGGCCGGAGA-3′ and Biotin3′(*Sph*I): 5′-CGTGCATGCGTGCGTCAGCCGATCTTGATG-3′. This PCR product and pHP41 both were cut with *Xba*I–*Sph*I and ligated to generate pHP42. To construct plasmid pHP44, we first made pHP33 by cloning the 2,951-bp *Bsr*GI–*Hin*dIII fragment of pAJ1077 into the *Acc*65I/*Hin*dIII sites of pBAD18. PSBT fusion to amino acid 576 of AcrB then was constructed by first PCR-amplifying the biotinylatable domain from pGJ78-J by using primers Biotin5′(*Nco*I): 5′-AGGATCCATGGTTAGAGTCGCCGGTAAGGCCGGAGA-3′ and Biotin3′(His$_6$-*Hin*dIII): 5′-GCCCAAGCTTAGTGATGGTGATGGTGATGGCCGATCTTGATGAGACCCTG-3′. The PCR fragment was cut with *Nco*I and *Hin*dIII, and then ligated to the 6,068-bp *Nco*I–*Hin*dIII fragment of pHP33 to generate pHP38. The 770-bp *Xcm*I–*Hin*dIII fragment of pHP38 was ligated to the 6,329-bp *Xcm*I–*Hin*dIII fragment of pAcrB576-AP to produce the final product pHP44.

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and used as recommended by the manufacturer.

Mutagenesis and Mapping of the Mutants. UV mutagenesis was carried out as described (7) with only minor modifications. Five milliliters of cells was irradiated by UV light (254 nm) for 80 sec. One milliliter of NZY medium then was added, and the entire culture was transferred to a red glass tube and grown at 37°C for 30 min. Appropriate amounts of cells then were plated on M63 minimal plates supplemented with maltose and X-Gal, grown at 30°C for 2 days, then stored at 4°C. Blue colonies were picked both after the 2-day 30°C incubation and after incubation at 4°C for several more days.

To map the mutations, we directly tested for linkage to candidate genes. Linkage to the *dsbA* and *dsbB* genes was determined by P1 transduction using a transposon Tn*10* in strain HPT129 90% linked to the wild-type *dsbA* and a transposon Tn*10* in strain HPT130 80% linked to the wild-type *dsbB*. Linkage to the *secA* and the *secE* genes was tested by P1 transduction using P1 lysates of strains EC294 and DR388 (with Tn*10*'s linked to the wild-type copy of the two genes). Linkage to the SRP components, *ffs* (4.5S RNA), *ffh* (Fifty-Four homolog), and *ftsY* (SRP receptor), was tested by P1 transduction using P1 lysates made on strains with Tn*10*'s linked to the wild-type genes: SG20253, CAG599, and CAG18480.

Pulse–Chase and Immunoprecipitation. Pulse–chase experiments were carried out as described (10). Cells were labeled with [^{35}S]methionine (ICN) for 20 sec, then chased for 0, 1, and 5 min. The subsequent immunoprecipitation was performed with polyclonal anti-OmpA antibody (laboratory collection). The precipitated proteins were separated on 12% SDS-polyacrylamide gel and visualized by using the Bio-Rad PhosphorImager.

Detection of the Fusion Proteins and the Biotinylated Proteins. A 37°C NZY overnight culture was diluted 1:100 in the same medium and grown under the same condition. The MalF-PSBT fusion was induced for about one generation with 0.5 mM isopropylthiogalactopyranoside. The FtsQ-PSBT fusion was induced with 0.2% L-arabinose at the point of dilution. The AcrB-PSBT fusion is under its own promoter and expressed constitutively; therefore no inducer was needed. Whole-cell proteins were precipitated after cells reach OD_{600} of 0.6 with 6% trichloroacetic acid and resuspended in SDS sample buffer containing 0.7 M β-mercaptoethanol. The resuspended protein sample was incubated at 37°C for at least 1 h, separated on 10% SDS-polyacrylamide gel, and transferred onto nitrocellulose membrane by using the semidry apparatus from Bio-Rad. To detect MalF-PSBT, FtsQ-PSBT, and AcrB576-PSBT, polyclonal anti-MalF-PhoA J, anti-FtsQ (laboratory collection), and anti-AcrB antibodies (Hiroshi Nikaido, University of California, San Francisco) were used. To detect biotinylated fusion proteins, the nitrocellulose was incubated with streptavidin-horseradish peroxidase (Amersham Pharmacia). The ECL system (Amersham Pharmacia) was used for the subsequent detection following the manufacturer's instruction.

Results

Screening for Increased β-Galactosidase Activity of the *malF-lacZ* Fusion Strain. We used a genetic screen that allows us to detect a range of β-galactosidase activities. After UV mutagenizing HPT57, a strain carrying the fusion in the chromosomal λatt site, we inspected colonies grown at 30°C on M63 minimal maltose plates (the fusion protein is induced by maltose) containing X-Gal for those that exhibited a less intense blue color than the Lac $^+$ derivatives (3). We also examined the plates after additional incubation at 4°C for several days, because this procedure allows development of blue color in mutant colonies that would not have been detected after the 30°C incubation. We screened more than one million UV-mutagenized colonies. Blue colonies appeared at a frequency of approximately 0.1%. Faint blue colonies were purified and they exhibited a range of blue color intensity.

We carried out P1 transduction to determine whether the blue color phenotype was caused by mutations in *dsbA* or *dsbB*. If unlinked, they then were tested for linkage to the *malF-lacZ* 102 fusion. P1 lysates were first made on each mutant, and the *malF-lacZ* fusion was transduced into the parental strain RI89 (lacking the fusion) by its 100% linkage to the ampicillin-resistance marker. Ampicillin-resistant blue transductants indicated that the mutations had altered the fusion.

One hundred and eight stable faint blue colonies were identified, and of these, 89 were studied extensively (Table 2). Among them, 27 were linked to *dsbA*, 20 to *dsbB*, and 14 to the *malF-lacZ* 102 fusion. We have characterized three missense mutations in *dsbA* altering amino acid residues other than the cysteines and two such mutations in *dsbB*. These mutant strains produced relatively stable proteins with altered activities. Studies on these mutants will be described elsewhere. For the remaining 28 strains, no defect in the

Table 2. Classification of the mutants

Class	Mutation	# of mutants identified	β-galactosidase activity
I	*dsbA* and *dsbB*	27 *dsbA* and 20 *dsbB*	5–140 u.*
II	λ102 (*malF-lacZ* 102)	14	ND
III	*ffs*	25	0.2–8 u.†
	Gene *X*	1	4–5 u.
	Unmapped	2	8–11 u.

ND, not determined.

*Miller units. For reference, the wild type has β-galactosidase activity ranges from 0.05 to 0.08 units, and the *dsbA* or *dsbB* null mutants have β-galactosidase activity ranges from 120 to 180 units.

†β-galactosidase activities were quite variable. Most *ffs* mutants gave β-galactosidase activities between 1 and 8 units. *ffs29, ffs69,* and *ffs91* used for subsequent studies repeatedly gave β-galactosidase activities above 1 unit.

formation of disulfide bonds (in OmpA) was found (data not shown).

Recalling the original goal of our genetic approach (to obtain membrane protein insertion mutants), we decided to analyze the mutations for their effects on this process. We first determined by P1 transduction linkage of mutations to *ffs*, *ffh*, and *ftsY,* coding for components of the bacterial SRP pathway, and *secA*, *E*, and *secY*, coding for components of the *E. coli* Sec pathway, all implicated to be involved in membrane protein insertion. (11–19).

Twenty-five of the 28 mutants had mutations linked to the *ffs* gene. Of the remaining three mutants, one had mutation/s linked to the *secA* region, and the last two remain unmapped.

The β-galactosidase phenotype of the mutation/s linked to the *secA* region was fully complemented by pBAD42-*secA* carrying only *secA* (data not shown). Sequencing revealed a single mutation in gene *X*, the first gene in the *secA* operon. This mutation introduces a stop codon that should truncate the gene *X* encoded protein after the 37th amino acid. We speculated that the mutation in gene *X* was affecting the expression of *secA*. First, the phenotypes are complemented by *secA* alone, but not by pCJ1 carrying gene *X* alone (data not shown). Second, effects on *secA* expression have been observed previously with a gene *X* mutation (20, 21). Finally, gene *X* does not appear to play a role in protein export (22), whereas the *X* mutation described here does interfere with export of a protein with a cleavable signal sequence (see below), presumably because of its effect on *secA*.

Although the *dsbA* and *dsbB* mutations resulted in β-galactosidase activities ranging in levels from low to near those of the *dsb* null, the *ffs*-linked mutations, the gene *X* mutation, and the two uncharacterized mutants all resulted in low levels of β-galactosidase activity (Table 2).

The *ffs*-Linked Mutations Are in the *ffs* Gene. The defect in β-galactosidase activity in all of the *ffs*-linked mutants was fully complemented by pHP5, carrying only the *ffs* gene (data not shown). We sequenced *ffs* in all 25 *ffs* mutants (Fig. 2). Twenty-three had a cytosine deletion from a stretch of five cytosines near the 3′ terminus of *ffs*, later referred to as *ffs29*. One *ffs* mutant (*ffs69*) had two cytosines changed to two thymines in the same stretch of five cytosines. The last *ffs* mutant (*ffs91*) changed two cytosines to two thymines in the DNA sequence corresponding to the 3′ end of the mature 4.5S RNA. According to the predicted secondary structure of 4.5S RNA (Fig. 2) (23), the *ffs29* and *ffs69* mutations should disrupt the pairing of bases.

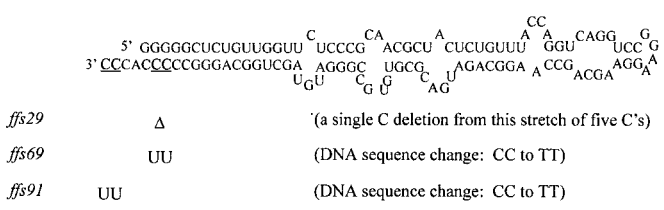

Fig. 2. The predicted 4.5S RNA structure. The three types of mutations are listed below the wild-type sequence. The wild-type sequences corresponding to the mutation sites are underlined.

We examined the steady-state 4.5S RNA levels in the *ffs* mutants by a RNase protection assay (result not shown). All mutants showed decreased steady-state levels of mature form 4.5S RNA: approximately 70%, 40%, and 30% relative to the wild-type amount for *ffs29*, *ffs91*, and *ffs69*, respectively. In addition, *ffs29* and *ffs69* mutants also produce a 4.5S RNA form that is larger than the mature form 114-nt size, possibly the precursor form of the 4.5S RNA. We suspect that the reduced amount of the mature form 4.5S RNA is responsible for the membrane insertion defect of the *ffs* mutants.

The *ffs* Mutants Are Defective in Membrane Protein Insertion of MalF. Given the nature of our genetic screening system and reports linking bacterial SRP to membrane protein insertion (11, 13, 16, 18), we suspected that the *ffs* mutations affected membrane insertion of the MalF-LacZ fusion protein (see Fig. 1). We expected that the defect in membrane protein assembly might be quite weak because of the low levels of β-galactosidase in the *ffs* mutants.

To compensate for the weak defect of the *ffs* mutants, we used a sensitive assay for membrane protein insertion defects developed previously in this laboratory (6). In this assay, a biotinylatable domain (80 aa in size) from the 1.3S subunit of PSBT is fused to a periplasmic domain of a membrane protein. This domain, if retained in the cytoplasm, is biotinylated by biotin ligase, an enzyme in *E. coli* cytoplasm. However, we had shown that, if efficiently exported, the domain is unbiotinylated. When cells had an export defect (e.g., a *secE* mutation), the periplasmic MalF-PSBT fusion proteins now were biotinylated because of the cytoplasmic localization of the fusion. This was true even in strains with only defects in the kinetics of export. In these strains, even though the PSBT domain ultimately is translocated across the membrane, it is resident in the cytoplasm long enough to allow biotinylation. In contrast to periplasmic fusions, when the PSBT domain is fused to a cytoplasmic domain of a membrane protein (cytoplasmic fusion), it is biotinylated under all conditions. Our earlier experiments used MalF; thus we had available a series of PSBT fusions to various positions in MalF (6). To test for membrane protein insertion defects, plasmid series pGJ78 I and J (periplasmic fusions), and K and L (cytoplasmic fusions) were expressed in the wild-type (HPT183) and the *ffs* mutant strains (HPT184–186) and the biotinylation state of the fusion proteins was examined.

The periplasmic fusions, which remained unbiotinylated in the wild-type background, all were biotinylated in the *ffs* mutants (Fig. 3). Because the *ffs* mutants do not have a secretion defect (see below), these experiments suggest that the MalF-PSBT periplasmic fusion proteins are incorporated into the membrane at a slower rate in the *ffs* mutants than in the wild type, thus allowing the attached PSBT domain to be biotinylated. In this assay, *ffs69* appears to elicit the strongest defect. The control cytoplasmic MalF-PSBT fusions were heavily biotinylated in all strain backgrounds.

We next wanted to compare a mutation affecting SRP and one affecting the Sec machinery in terms of their effects on protein translocation across the membrane and membrane protein insertion. We chose the *ffs69* mutation and the gene *X* mutation as they had comparable effects on the activity of the MalF-LacZ fusion protein (Table 2). We examined the effects of the two mutations on the translocation of a cell envelope protein with a signal sequence (OmpA) and on membrane protein insertion monitoring the biotinylation state of the MalF-PSBT fusions. The mutations had similar effects on the biotinylation of the MalF-PBST periplasmic fusions (data not shown). In contrast, the gene *X* mutant had a

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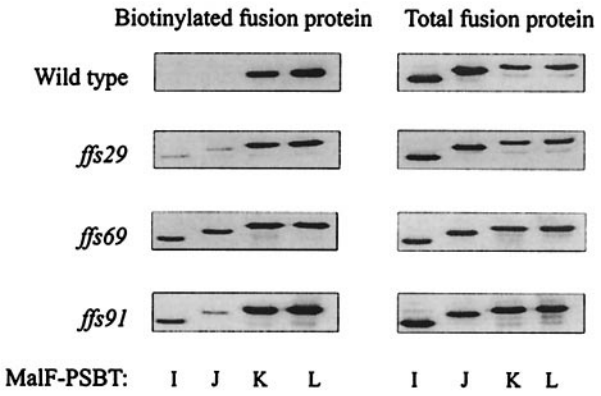

Fig. 3. Detection of the MalF-PSBT fusion proteins in wild type and the three *ffs* mutants. The MalF-PSBT periplasmic fusions I and J and cytoplasmic fusions K and L were expressed from the pGJ78 plasmid series (I through L) in either the wild type (HPT183) or the three *ffs* mutant strains (*ffs*29 in HPT184, *ffs*69 in HPT185, and *ffs*91 in HPT186).

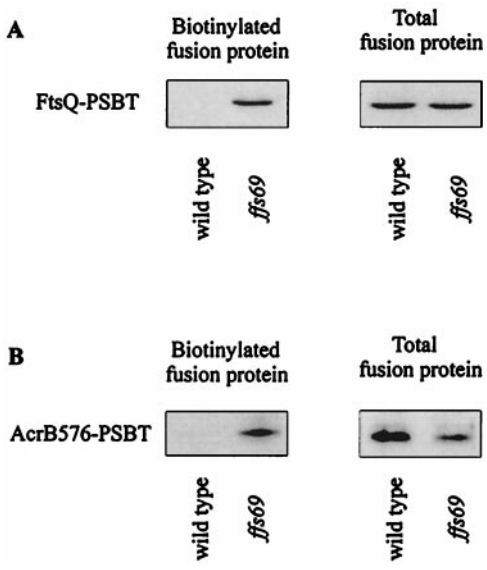

Fig. 5. *ffs69* mutation causes defects in the insertion of FtsQ and AcrB into the membrane. (*A*) Detection of the FtsQ-PSBT (expressed from pHP42) in the wild type (HPT242) and the strongest *ffs* mutant (*ffs*69 in HPT245). (*B*) Detection of the AcrB576-PSBT (expressed from pHP44) in the wild type (HPT183) and the strongest *ffs* mutant (*ffs*69 in HPT185).

significant defect in signal sequence cleavage of the OmpA protein, an indication of a translocation defect, whereas the *ffs* mutant had no measurable effect on this process (Fig. 4).

The *ffs* Mutants Are Defective in the Insertion of Membrane Proteins FtsQ and AcrB. Because the MalF-β-galactosidase fusion was used to detect the *ffs* mutations in the first place, it seemed possible that these mutations were affecting only the MalF. To show that the *ffs* mutations were affecting membrane protein insertion in general, we extended the use of the biotinylation assay to two other membrane proteins: FtsQ and AcrB.

FtsQ, a bitopic protein involved in cell division in *E. coli*, resides in the inner membrane with the amino terminus in the cytoplasm and the carboxyl terminus in the periplasm (24). The biotinylatable domain PSBT was fused after the last amino acid of FtsQ. We examined the extent of biotinylation of FtsQ-PSBT in wild-type HPT242 and the strongest *ffs* mutant HPT244 (*ffs69*) expressing this construct from pHP42. A similar biotinylation pattern to that of the MalF-PSBT fusions was seen. Although the FtsQ-PSBT remained unbiotinylated in the wild-type strain, the *ffs69* mutant accumulated biotinylated FtsQ-PSBT protein (Fig. 5*A*).

The biotinylatable domain PSBT also was fused to AcrB, an inner membrane protein with 12 transmembrane segments and a member of a membrane complex involved in multidrug resistance (25, 26). PSBT was fused after the 576th amino acid residue of AcrB to generate a periplasmic AcrB-PSBT fusion. Again, the strongest *ffs* mutant (*ffs69* in HPT185) was compared with the wild type (HPT183) for its effect on this polytopic membrane protein expressed from pHP44. As shown in Fig. 5*B*, whereas AcrB-PSBT remained unbiotinylated in the wild type, the *ffs* mutant accumulated biotinylated AcrB-PSBT.

In summary, we have demonstrated that the insertion of three topologically different membrane proteins is affected by the *ffs* mutations, isolated in an *in vivo* screen for the altered membrane localization of the MalF-LacZ fusion protein.

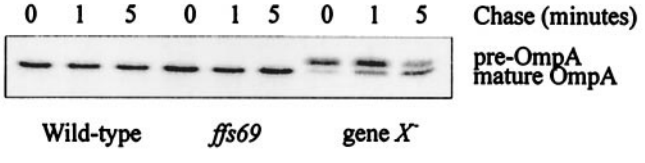

Fig. 4. Protein secretion is defective in the gene *X* mutant, but not in the *ffs* mutant. Pulse–chase and immunoprecipitation of OmpA were carried out as described. The wild type (JP313), the strongest *ffs* mutant (*ffs*69 in HPT244), and the gene *X* mutant (HPT265) were examined for the efficiency of the secretion pathway.

Discussion

We set out to find new classes of mutations affecting disulfide bond formation. This goal was achieved as we detected additional classes of mutations in the *dsbA* and *dsbB.* We also had expected to find mutations affecting cellular components necessary for the reoxidation of DsbB. Recent results have shown that these components, quinones and cytochrome oxidases, are redundant, thereby explaining the failure to detect such mutations in the screen because mutations in multiple genes would be required to generate the phenotype (27, 28). However, an additional class of mutations was found, which, instead of altering disulfide bond formation, affected membrane protein assembly. This finding accomplishes the original purpose of the genetic approach initiated about 10 years ago. Thus, a single genetic screening procedure yields mutations in two distinct major cellular processes.

The isolation of *ffs* mutations described in this paper shows a genetic search designed to detect mutations affecting membrane protein assembly has yielded mutations involved in the process. These *ffs* mutant strains present an advantage over those used previously to assess the effects of disrupting SRP components. These earlier studies used either depletion strains or strains expressing dominant mutations so that membrane protein assembly was assayed under conditions where cells were severely affected in growth (11, 13, 16, 18). In such cases, it can be difficult to separate out secondary effects of depletion in cells that have been affected so strongly in their physiology. The *ffs* mutant strains described here grow either normally or with near-normal growth rates (*ffs69*). The defects in membrane protein insertion in the *ffs* mutants are probably weak, as indicated by the low levels of cytoplasmic localization of the MalF-LacZ hybrid. Nevertheless, we were able to overcome this weakness by using a sensitive assay. By attaching a biotinylatable domain to normally exported domains of membrane proteins, one can detect kinetic effects on incorporation of proteins into the membrane. Using this approach, we have shown that MalF, AcrB, and FtsQ at least partly depend on 4.5S RNA for

their assembly. *ffs69* caused the strongest defect, as represented by the highest amount of biotinylated fusion proteins. We show that the *ffs* mutants affect membrane proteins with multiple membrane spanning segments as well as with a single membrane spanning segment (FtsQ).

We have seen a strict distinction between the effects of the mutation affecting SecA (the gene *X* mutation) and the *ffs69* mutation on membrane protein assembly vs. protein secretion. Both classes of mutations caused comparable levels of biotinylation of MalF-PBST periplasmic fusions. In contrast, the gene *X* mutation resulted in a defect in OmpA export to the cell envelope, whereas the *ffs* mutations did not show such an effect. Furthermore, when PSBT was fused to the periplasmic MalE, the wild-type and the *ffs* mutants did not show any biotinylated protein whereas the gene *X* mutant did (data not shown). This experiment also rules out the possibility that *ffs* mutations affected only biotinylation, not membrane protein insertion. Although *secA* has been shown to be involved in the insertion of some inner membrane proteins but not others (12, 15, 17, 19, 29–31), our result suggests that SecA is at least partially required for the insertion of MalF.

We suspect that the clustering of *ffs* mutations in strings of cytosines near the 3′ is a result of the UV mutagenesis, which tends to affect adjacent pyrimidine-pyrimidine pairs (32). A pyrimidine-rich stretch such as that at the 3′ end of 4.5S RNA (see Fig. 2) may represent a mutational hot-spot for UV (33). The fact that we obtained 25 *ffs* mutations in this screen and only one in the *secA* region (gene *X* in this case), which presents a much larger target, is consistent with this interpretation, as is the absence of mutations in *ffh*, *ftsY*, and other *sec* genes. Characterization of a larger number of mutants or use of other mutagens may yield mutations in these other genes.

We hypothesize that the mutations in *ffs* cause membrane protein insertion defects by reducing the amount of mature 4.5S RNA. The degree of reduction of mature 4.5S RNA in the mutants correlated with the defect in membrane protein assembly as measured by the biotinylation assay. We assume that the unprocessed 4.5S RNA is nonfunctional or does not function as well as the processed form. Alternatively, the alterations of the 4.5S RNA may interfere with its function. For example, the mutated nucleotides could be involved in interaction with either the polypeptide to be targeted to the membrane or the Ffh protein itself. However, previous *in vitro* experiments had indicated that the region in 4.5S RNA interacting with the Ffh protein is the central stem-loop region, which is conserved across species (34). Recent crystal structure studies of the 4.5S RNA and Ffh complex also support this observation (35).

Finally, our results suggest why we were unable to obtain mutants affected in membrane protein assembly in the original selection. The selection for Lac^+ derivatives of the *malF-lacZ* fusion strain required high levels of restoration of the β-galactosidase activity. Mutations of such severity in membrane protein assembly probably would have resulted in membranes devoid of a substantial fraction of their proteins and would likely have been lethal. Indeed, our *ffs* mutants are found only among those with weak effects on the *malF-lacZ* fusion. Furthermore, among these weak mutants, the strongest *ffs69* mutant already shows a slight growth defect. We suspect that mutations with significantly stronger defects would not be viable.

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