SecF Stabilizes SecD and SecY, Components of the Protein Translocation Machinery of the *Escherichia coli* Cytoplasmic Membrane

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The effect of the overproduction of SecF encoded by the tac-secF gene on a plasmid on the synthesis of other Sec proteins was studied in *Escherichia coli*. SecF overproduction resulted in the simultaneous overproduction of SecD encoded by the tac-secD gene on a plasmid. Deletion of the orf6 gene, located downstream of the secF gene, had no effect on SecD overproduction. A pulse-chase experiment revealed that the overproduction was due to stabilization of SecD with SecF. SecF overproduction also resulted in the overproduction of SecY encoded by the tac-secF gene on a plasmid as well. SecF overproduction also enhanced the level of SecY expressed by the chromosomal secY gene. This SecF effect was not due to its effect on SecD or SecE, since SecF overproduction did not affect the levels of SecD and SecE expressed by the chromosomal secD and secE genes, respectively. SecE-dependent overproduction of SecY has already been demonstrated. It is suggested that SecF interacts with both SecD and SecY. SecE-SecY interaction has been demonstrated. It is likely, therefore, that all Sec proteins in the cytoplasmic membrane interact with each other.

A set of sec genes has been discovered to be involved in translocation of secretory proteins across the cytoplasmic membrane of *Escherichia coli*. These genes are secA (26), secB (17), secD (10), secE (27), secF (11), and secY (8, 13). All of the Sec proteins encoded by these genes have been overproduced and purified (1, 7, 14, 20, 22, 32, 33). Biochemical studies with everted membrane vesicles revealed that SecB is a membrane chaperon that forms a complex with presecretory proteins to maintain their exportable conformation (16), while SecA plays the central role in the early stage of translocation as a protein translocation ATPase (19).

The successful reconstitution of proteoliposomes from purified Sec proteins made it possible to perform biochemical studies on membrane-spanning Sec proteins. Thus, SecE and SecY were demonstrated to be essential membrane components of the translocation machinery (1, 6, 24, 32). A new 12-kDa membrane protein, p12, was also found to be involved in the translocation reaction by means of reconstitution analysis (25). Although SecD and SecF were genetically suggested to be membrane components of the machinery, neither studies with everted membrane vesicles nor reconstitution studies demonstrated their involvement in protein translocation (22).

A recent genetic study suggested that SecD and SecF functionally follow SecY (4). A biochemical study with *E. coli* spheroplasts also revealed that SecD is involved in a late step of the translocation reaction, most likely in the release of translocated proteins from the cytoplasmic membrane, since this process was specifically inhibited by prior treatment of spheroplasts with anti-SecD immunoglobulin G (21). SecF exhibits a topological profile similar to that of SecD (11), suggesting that SecF may also be involved in a late step of the translocation reaction. However, a preliminary experiment with anti-SecF immunoglobulin G failed to demonstrate this (23a). Very recent genetic and biochemical studies with a ΔsecDF strain suggested that SecD and SecF are required for efficient protein translocation but not for cell growth (26a) and that these Sec proteins are important for the maintenance of an electrochemical potential in the cytoplasmic membrane (2a).

Here we report that the overproduction of SecD in *E. coli* cells carrying a secD gene-possessing plasmid was SecF dependent and that the overproduced SecD was stabilized in the presence of the overproduced amount of SecF. We also report here that SecF stabilized SecY as well. It is suggested that SecF interacts with both SecD and SecY. SecE-SecY interaction has been demonstrated. It is likely, therefore, that all Sec proteins in the cytoplasmic membrane interact with each other.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* W3110M25 (ompT) (31) and JP91 (MC1000 leu + ΔsecDF15::kan recA::cat pcnB::Tn10) (a gift from J. Beckwith) and plasmids pMAN828 (tac-secF) (22), pMAN832 (tac-secDF) (22), pDR720 (28), pKEN403 (23), pQN805 (15), pBR322 (5), pGAP1 (araBAD-secDF) (26a), pMAN480 (20), pMAN510 (tac-secY) (20), pMAN809 (tac-secE) (20), and pUS12 (29) were used.

**Materials.** Restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were obtained from Takara Shuzo. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Nacalai Tesque, and l-arabinose was from Wako Pure Industries. Tran35S-label, a mixture of 70% [35S]methionine (1,000 Ci/mol) and 20% [35S]cysteine, was obtained from ICN.

**Construction of plasmids.** The structures of the secD-secF region in various plasmids used in this study are shown in Fig. 1. pMAN832 carrying the entire secD-secF operon under the control of the tac promoter and the orf6 gene was used as the starting material, and plasmids were constructed as follows.

(i) **pKIS202.** pMAN832 was digested with *Eco*T22I and *Sna*BI, and the resulting large fragment was treated with T4 DNA polymerase. The fragment was then self-ligated to construct pKIS202.

(ii) **pKIS203.** pMAN832 was digested with *Eco*T22I and
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Salli, and the larger fragment obtained was treated with T4 DNA polymerase and then self-ligated.

(iii) pKIS204 and pKIS207. NruI-Salli large fragments derived from pKIS202 and pMAN832, respectively, were treated with T4 DNA polymerase and then self-ligated.

(iv) pKIS209. Plasmid pKIS209 carries the secF gene under the control of the trp promoter. A 4.8-kb fragment containing the kan gene and the replication origin of pSC101 was obtained from pKEN403 by digestion with EcoRI and HincII. The fragment was ligated with a 72-bp EcoRI-SmaI fragment from pDR720, containing the trp promoter, to construct pKIS208. Separately, pMAN828 was digested with NruI and Salli to remove the orf6 gene, treated with T4 DNA polymerase, and then self-ligated. The resulting plasmid, pKIS206, was digested with BglII, treated with T4 DNA polymerase, and then digested with BamHI to obtain a 1.1-kb fragment containing the secF gene. This fragment was inserted into the BamHI-blunted BglII site on pKIS208 to construct pKIS209.

(v) pKIS215. Plasmid pKIS215 carries the secF gene under the control of the araBAD promoter. A 1.8-kb DNA fragment carrying the araBAD promoter was prepared from pQN805 by digestion with SalI, treatment with T4 DNA polymerase, and then further digestion with Salli. This fragment was inserted into the Salli-blunted EcoRI site on pKIS209 to construct pKIS213. To substitute the tet gene for the kan gene on pKIS213, a fragment carrying the tet gene was obtained by digestion of pBR322 with Avai, followed by treatment with T4 DNA polymerase and digestion with EcoRI. This fragment was cloned into the EcoRI-SmaI site on pKIS213 to construct pKIS215.


Pulse-chase experiments. E. coli W3110M25 carrying pKIS204 and pKIS215 was grown to 4 × 10^9 cells per ml at 37°C in M63 minimal medium supplemented with 10 μg of thiamine per ml, 50 μg of yeast extract per ml, 2% glycerol, 20 μg of ampicillin per ml, 5 μg of tetracycline per ml, and 20 μg of each of all amino acids except methionine and cysteine per ml. Synthesis of SecF was induced with 1% L-arabinose. After 30 min, IPTG was added to a final concentration of 1 mM to induce SecF expression. After further incubation for 10 min, 1 min of pulse-labeling was initiated by the addition of 80 μCi of Tran35S-label to 8 ml of culture, followed by a chase in the presence of 30 μM nonradioactive methionine and cysteine. Aliquots (1 ml) were removed at various times, immediately chilled in ice water, and then subjected to trichloroacetic acid precipitation.

Immunoprecipitation. The trichloroacetic acid precipitate obtained after the pulse-chase was washed with acetone and ether and then solubilized in 100 μl of 50 mM Tris-HCl (pH 7.5)–1% sodium dodecyl sulfate (SDS)–1 mM EDTA. Aliquots (25 μl) were mixed with 1 ml of 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–2% Triton X-100–1 mM EDTA–1 mM (p-amidino-phenyl)ethanesulfonyl fluoride hydrochloride, and debris was removed by centrifugation at 16,000 × g for 5 min. To the resultant supernatant was added 10 μl of an anti-SecD antisera, and then the mixture was maintained for 12 h at 4°C. Fifty milliliters of 10% (wt/vol) IgG sorb was added to each sample, and then the mixture was kept at 4°C for 30 min. After centrifugation at 10,000 × g for 5 min, the pellet was washed twice with 0.5 ml of 50 mM Tris-HCl (pH 7.5)–0.5 M NaCl–0.05% SDS. The washed pellet was suspended in 30 μl of the
FIG. 3. SecF-dependent overproduction of SecD. W3110M25ΔsecDF harboring pKIS204 and pKIS215 was grown in the presence of 1 mM IPTG and the indicated concentrations of L-arabinose for 2 h. The membrane fraction was prepared and analyzed by SDS-PAGE, followed by staining with Coomassie brilliant blue (A) or Western blot analysis with anti-SecD (upper) and anti-SecF (lower) antisera (B). The migration positions of molecular mass markers and those of SecD and SecF are indicated. (C) The blots in panel B were densitometrically scanned, and the densities of the SecD and SecF bands were plotted against the concentrations of arabinose added to the cultures.

sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE) and then heated at 65°C for 5 min.

**SDS-PAGE and Western blot (immunoblot) analysis.** SDS-PAGE was carried out by the method of Laemmli (18). Only samples for pulse-chase experiments were boiled. Western blot analysis was carried out as described previously (35).

**Preparation of the membrane fraction.** Cells harvested by centrifugation were washed once with 20 mM Tris-HCl (pH 7.5), suspended in the same buffer, and then disrupted by sonication. After centrifugation at 3,000 × g for 3 min to remove unbroken cells, the insoluble fraction was recovered by centrifugation at 100,000 × g for 30 min and used as the membrane fraction.

**Measurement of OmpT protease activity.** The reaction mixture (100 µl) contained 3.6 µg of purified OmpT protease (24), 12 µg of purified SecF protein (22), and 100 µg of the cytoplasmic membrane from W3110 M25/pMAN510/pMAN809 (SecY/SecE overproducer) in 50 mM potassium phosphate (pH 7.5)–2.5% n-octyl-β-D-glucopyranoside–0.15% N-lauroylsarcosine sodium salt. Bovine serum albumin was used in place of SecF as a control. The SecY degradation by OmpT was initiated by adding the cytoplasmic membrane. The reaction was performed on ice. Aliquots (20 µl) were removed at 1, 3, 6, and 10 min and subjected to trichloroacetic acid precipitation. Samples were then analyzed by SDS-PAGE and Western blot analysis with an anti-SecY-1 antibody (24).

**RESULTS**

**Construction of plasmids lacking the secF or orf6 gene.** The secD and secF genes constitute an operon, which is followed by an open reading frame, orf6 (11). There is a transcriptional terminator between them. Plasmids carrying the tac promoter-controlled secD gene with or without the secF or orf6 gene were constructed (Fig. 1). Plasmid pKIS202 has a deletion near the 5' end of the secF gene, which generates a termination codon immediately after the deleted region; hence, only a small N-terminal portion of SecF can be synthesized. In contrast, pKIS207 possesses the secF gene but not the orf6 gene, and pKIS203 and pKIS204 have neither the secF gene nor the orf6 gene. The difference between the latter two plasmids is the presence and absence of the transcriptional terminator after the secD gene.

We also constructed a plasmid, pKIS215, which carries the araBAD promoter-controlled secF gene.

**Effects of deletion of the secF or orf6 gene on overproduction of SecD.** E. coli cells carrying one of these plasmids were cultured in LB, and the tac promoter-controlled genes were induced with 1 mM IPTG for 2 h. The cells were harvested, and the membrane fractions prepared from them were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining (Fig. 2A) or Western blotting (Fig. 2B). IPTG-induced overproduction of SecD was observed with the parental plasmid or pKIS207, which lacks the orf6 gene. On the other hand, the overproduction of SecD was appreciably suppressed when the plasmid lacked the secF gene, irrespective of the presence or absence of the orf6 gene on the plasmids. These results were confirmed by Western blot analysis (Fig. 2B). Furthermore, the latter analysis clearly demonstrated the overproduction of SecF by the secF gene-carrying plasmids, which is difficult to see in Fig. 2A because of the adjacent OmpA band.

Taken together, the results suggest that the overproduction of SecD was supported by the simultaneous overproduction of SecF. In contrast, SecF was overproduced alone, as demonstrated in lanes 6 in Fig. 2A and B. Overproduced SecD and SecF were recovered in the membrane fraction even when the fraction was treated with 6 M urea, suggesting that these
proteins exist as membrane-integrated, not aggregated, proteins.

The SecF-dependent overproduction of SecD was further examined under more critical conditions. W3110M25 was transformed with both pKIS204, carrying the tac-secD gene, and pKIS215, carrying the araBAD-secF gene. Then the chromosomal secD-secF region of the strain was destroyed by transduction of the secD-secF::kan region from JP91/pGAP1. Thus, a strain possessing the tac-secD and araBAD-secF genes on plasmids but no secD-secF operon on the chromosome was constructed.

In the absence of arabinose, this strain overproduced neither SecF nor SecD, even in the presence of IPTG, which should have induced full expression of the secD gene (Fig. 3A and B). The addition of more than 0.01% arabinose to the culture medium resulted in the synthesis of SecF with concomitant overproduction of SecD. The overproduction of SecD roughly paralleled the amount of SecF synthesized (Fig. 3C). The synthesis of SecF was undetectable in the absence of arabinose, whereas SecD accumulated to some extent under these conditions, indicating that the SecD formation does not entirely depend on SecF.

SecF stabilizes SecD. The effects of SecF on the synthesis and degradation of SecD were examined by means of a pulse-chase experiment. A 1-min pulse experiment demonstrated that the rate of synthesis of SecD remained essentially the same, irrespective of the presence or absence of arabinose-induced SecF synthesis (Fig. 4A), whereas overproduction of SecD was suppressed in the absence of arabinose, as shown by data presented above. The results indicate that SecF did not affect either transcription or translation of the secD gene. The noninvolvement of SecF in secD transcription was indicated by the secF-dependent overproduction of SecD with tac-controlled secD genes (Fig. 3).

The pulse-labeled cells were then chased in the presence of cold methionine-cysteine. Rapid degradation of radiolabeled SecD with a half-life time of 30 min was observed when the araBAD-secF gene was not induced, whereas the degradation was appreciably suppressed in SecF-overproducing cells, the half-life time being 120 min (Fig. 4B and C). These results suggest that the overproduction of SecD was due to the increased stability of SecD in the SecF-overproducing cells.

Effects of SecF on the stability of other Sec proteins. SecA, SecB, and SecE can be overproduced alone (7, 14, 20, 33), whereas the overproduction of SecY largely depends on the simultaneous overproduction of SecE (20). The effect of pMAN928 (tac-secF)-dependent overproduction of SecF on the synthesis of SecY was examined in a strain harboring a plasmid carrying the tac-secY gene. A plasmid carrying and one not carrying the tac-secY gene were also used as controls. Consistent with previous observations (20), overproduction of SecE resulted in almost the same extent of overproduction of SecY (Fig. 5A and Table 1). The overproduction of SecF, which was about 5,000-fold, also resulted in the overproduction of SecY, although the extent of overproduction was not as high as that in the case of SecE (Fig. 5A and Table 1).

The effects of SecF overproduction on the levels of other Sec proteins, including SecD and SecY, expressed by the chromosomal genes were also examined (Fig. 5B). The level of SecY was enhanced upon the overproduction of SecF, whereas that of SecD was not. The levels of SecA and SecE did not change either. Since the chromosomal secF gene was functioning under the conditions used, it is likely that the chromosomal level of SecF is sufficient for stabilizing the chromosomal level of SecD but not that of SecY.

**FIG. 4.** Stability of SecD in the SecF-overproducing strain. (A) W3110M25secDF harboring pKIS204 (tac-secD) and pKIS215 (araBAD-secF) was grown in the presence of 1 mM IPTG and 1% arabinose (+) or 1 mM IPTG alone (−). The membrane fraction was prepared and analyzed by Western blot analysis with an anti-SecD antiserum (left). The same strain was grown in the presence (+) or absence (−) of 1% arabinose for 30 min. After induction of SecD expression with 1 mM IPTG for 10 min, Tran35S-label was added to the radioactivity level of 10 μCi/ml for 1 min pulse-labeling. The labeling was stopped by the addition of 10% trichloroacetic acid, and the trichloroacetic acid-insoluble fraction was subjected to immunoprecipitation with an anti-SecD antiserum, followed by SDS-PAGE and fluorography (right). The position of SecD is indicated. (B) W3110M25secDF harboring pKIS204 and pKIS215 was grown in the presence (+) or absence (−) of 1% arabinose for 30 min. SecD expression was then induced by the addition of 1 mM IPTG. After 10 min, Tran35S-label was added for 1 min pulse-labeling. Nonradioactive methionine and cysteine were then added (zero time), and aliquots were removed at the indicated times and subjected to trichloroacetic acid precipitation. Samples were subjected to immunoprecipitation with an anti-SecD antiserum and then analyzed by SDS-PAGE and fluorography. (C) The densities of the SecD protein band on the fluorograms in panel B were densitometrically determined and plotted as the amount of SecD.

**DISCUSSION**

The functional roles of all Sec proteins, except SecF, in protein translocation were elucidated previously. In the present study, we showed that SecF overproduction supports the simultaneous overproduction of SecD and SecY. We further showed that the overproduction of SecD was due to its stabilization. The results most likely suggest that SecF interacts with SecD and SecY. We previously estimated the number of molecules of SecF in one E. coli cell to be approximately 1/10 of that of other membrane Sec proteins, including SecY and SecD (22). One possible role of SecF in this connection is that it is involved in the process of the assembly of the presumed translocation machinery in the membrane. SecD or SecY...
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overproduced in the absence of a sufficient amount of SecF may not be properly integrated into the membrane and hence is unstable. An alternative possibility is that SecF directly participates in the translocation reaction. This does not necessarily contradict the existence of fewer SecF molecules. Analyses of the stoichiometry of components involved in reconstituted translocation activity supported the view that Sec proteins interact rather loosely with each other in the membrane (1, 25). It is likely, therefore, that they gather to form a complex only when a preprotein or a preprotein-SecA complex reaches them. The assembly of a translocation machinery on demand was also proposed on the basis of the results of genetic analysis (4). Thus, the smaller number of SecF molecules may well be able to stabilize SecD and SecY in the membrane. With the results presented in this study, we cannot determine which of these two possibilities is the case, however. One may also speculate that SecF is an inhibitor of a protease, which is somehow linked to the translocation process. The OmpT protease that specifically attacks SecY (2) or protease IV, a signal peptide peptidase (12), could be a candidate. We examined the effect of SecF on the degradation of SecY by OmpT as described in Materials and Methods. SecF did not inhibit OmpT activity at all, however (data not shown).

The following pairs of membrane proteins have been suggested to stabilize each other: SecE-SecY (20), MotA-MotB (34), and ExbB-TonB (9, 30). Since these proteins are constituents of the machineries for protein translocation, flagellum movement, and iron uptake, respectively, it is suggested that the stabilization is due to interaction between these proteins in the machineries. Although such mutual stabilization is not absolute proof for a molecular interaction, it is one of the useful markers for the existence of interaction. Very recently we discovered a novel membrane protein, p12, which is involved in the translocation reaction, and found that its overproduction induces SecY overproduction and that a reduction in the amount of SecE results in a reduction in the amount of p12 (25). Taking these facts together with those disclosed in the present study on SecF, SecD, and SecY, we have drawn a stabilization map (Fig. 6). Although this is not proof, it most likely represents interactions among membrane proteins involved in protein translocation. Supporting the stabilization map, interaction between SecE and SecY has been demonstrated both genetically and biochemically (3, 6).

Finally, it should be noted that a genetic study involving sec titration suggested the functional order of Sec proteins in the translocation reaction to be SecA-SecE-SecY-SecD (4), which is consistent with the stabilization map. In the present study, furthermore, the occurrence of interactions between SecD and SecF and between SecY and SecF were suggested. It is probable, therefore, that SecF connects early and late steps of the translocation reaction, which include SecE-SecY/p12 and SecD, respectively.

TABLE 1. SecF-dependent overproduction of SecY

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<tr>
<th>Protein</th>
<th>Overproduction (fold) of Sec proteins with indicated sec genes on plasmids</th>
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<tr>
<td></td>
<td>None</td>
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<td>SecY</td>
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<td>SecE</td>
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<td>SecF</td>
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* W3110M25 harboring pMAN480 and pUS12 (none), pMAN510 and pUS12 (tac-secY), pMAN510 and pMAN809 (tac-secY, tac-secE), or pMAN510 and pMAN828 (tac-secY, tac-secF) was grown under the conditions given in the legend to Fig. 5. The membrane fractions from these cells were diluted stepwise and then subjected to Western blot analysis. Fold overproduction was quantitated by densitometric scanning of the blots. The values for SecY, SecE, and SecF for W3110M25 harboring pMAN480 and pUS12 were taken as 1.0.

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REFERENCES