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Determinants of the Quantity of the Stable SecY Complex in the *Escherichia coli* Cell

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While SecY in wild-type *Escherichia coli* cells is stable and is complexed with other proteins within the membrane, moderately overexpressed and presumably uncomplexed SecY was degraded with a half-life of 2 min. The fact that the amount of stable SecY is strictly regulated by the degradation of excess SecY was demonstrated by competitive entry of the SecY$^+$ protein and a SecY-LacZα fusion protein into the stable pool. Simultaneous overexpression of SecE led to complete stabilization of excess SecY. Overproduced SecD and SecF did not affect the stability of SecY, but plasmids carrying *ORF12* located within the *secD-secF* operon partially stabilized this protein. In contrast, mutational reduction of the SecE content (but not the ORF12 content) led to the appearance of two populations of newly synthesized SecY molecules, one that was immediately degraded and one that was completely stable. Thus, the *E. coli* cell is equipped with a system that eliminates SecY unless it is complexed with SecE, a limiting partner of SecY. Our observations implied that in wild-type cells, SecY and SecE rapidly associate with each other and remain complexed.

Protein translocation across the cytoplasmic membrane of *Escherichia coli* is facilitated by a cytoplasmic chaperone (SecB in many cases), the peripheral membrane ATPase (SecA), and membrane-embedded factors (SecY, SecE, SecD, and SecF) (26, 35). Reconstitution studies have established that SecA, SecE, and SecY are essential components for the in vitro translocation reactions (1, 9).

SecY spans the membrane 10 times with both of its termini facing the cytoplasm (4). Genetic (7, 29) and biochemical (1, 9, 20) evidence indicates that SecY interacts closely with SecE, another integral membrane component, which spans the membrane three times (27). We have shown previously that high-level overexpression of *secY* is harmful to cells and that excess SecY in cells is unstable (2, 3). Matsuyama et al. (20) succeeded in overproducing and accumulating SecY in cells by overexpressing both the *secY* gene and the *secE* gene. These authors suggested that overproduced SecE stabilizes excessively synthesized SecY by forming a binary complex that resists proteolysis. Brundage et al. (8, 9) isolated a SecE-SecY-Band1 complex from a solubilized membrane preparation as the membrane-embedded component of the translocase.

This work was aimed at understanding the process(es) underlying the instability and stability of SecY in cells. Such knowledge not only should be fundamental for understanding dynamic aspects of protein translocation machinery but also should be useful for identifying components that interact with SecY. Our results indicate that SecE is the primary determinant of the stability of SecY. *ORF12* (12, 22), an open reading frame whose function within the *secD* operon was not known previously, was also found to affect the stability of overexpressed SecY.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* K-12 strain TW130 was used in most experiments in this study. This strain was a derivative of MC4100 (31) carrying *ompT*::*kan* (5) and *zhd-33*::Tn*10* (28). It was transformed with appropriate plasmids as indicated below. TW127 (MC4100 *secE501 argE*::Tn*10* *ompT*::*kan*) was constructed by cotransducing *secE501* and an adjacent *argE*::Tn*10* marker into AD202 (MC4100 *ompT*::*kan*) (5) by using P1 *vir* prepared from PR520 (23). For pulse-chase experiments, cells were grown in M9 medium (31) supplemented with 0.4% glycerol, 2 μg of thiamine per ml, and 20 μg of each amino acid except methionine and cysteine per ml. Ampicillin (50 μg/ml) and/or chloramphenicol (20 μg/ml) was added when plasmid-bearing cells were grown. To maximize expression of genes under *lac* promoter control, 1 mM cyclic AMP was added.

Plasmids. pKY318 was a pBR322-derived *secY* plasmid; the 2.8-kb *Pst*I fragment of the *spc* operon on pNO1573 (2) was cloned into pKY225, a *lac* promoter vector identical to pKY184 (33) except that the β-lactamase (*bla*) gene was derived from pUC119 (34). pKY248 was a pACYC184-derived *secY* plasmid; the *Hin*dIII-*Bam*HI fragment of pKY107 containing *secY* (29) was cloned into pKY238 (29), a *lac* promoter vector based on pACYC184. pKY258, carrying the *secY-lacZ*α fusion gene, was constructed as follows. The 1.5-kb *Hin*dIII fragment that contained *secY* from pKY3 (28) was cloned into pKY184. The *secY* and *lacZ*α reading frames were then fused by the method of Kunkel et al. (18) by using a synthetic 60-mer oligonucleotide that consisted of the last 10 codons of *secY* and codons 7 to 16 of *lacZ*α from pUC9 (with a regenerated *Hin*dIII site at the junction), yielding pKY234. Finally, the *Hin*dIII fragment of pKY234 was cloned into pKY238.

To overexpress other *sec* genes, we used a pBR322-based *lac* promoter vector, pKY224, which was identical to pNO1575 (17) except that its *Aat*II-*Alw*NI interval (*bla* region) and the multicloning site were replaced by the corresponding regions of pUC119. pKY285 was a SecE overproducer; a 0.6-kb *Kpn*I-*Mlu*I fragment containing *secE* was isolated from pSS105 (11), linked with a *Bam*HI linker, and cloned into pKY224. pKY296 carried *ORF12*, *secD*, and *secF*; pMAN813, a derivative of pCG169 (12) provided by S. Mizushima, was first digested with *Xba*I, blunted by T4 DNA polymerase, and then digested with *Sal*I. A resulting 4.5-kb fragment was cloned into pKY224 that had been digested with *Sal*I and *Sma*I. pKY287 carried *secF*; the blunt-ended 1.1-kb *Mlu*I-*Bcn*I fragment of pMAN813 was inserted into the *Sma*I site of pKY224. pKY311 overexpressed *secD* and *secF*. A 2.9-kb *Bsu*36I-*Bcn*I fragment

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of pMAN813 was first cloned into a vector similar to pKY225 and then excised by double digestion at the *Hin*dIII and *Sac*I sites flanking it; this was followed by cloning into the final vector. pKY313 carried *ORF12*; pKY296 was digested with *Bsu*36I and *Kpn*I, blunted, and ligated. DNA manipulations were performed by using standard procedures (24).

Pulse-chase experiments, immunoprecipitation of SecY, and SDS-polyacrylamide gel electrophoresis. Cells were pulse-labeled with [^{35}S]methionine (1,100 Ci/mmol; American Radiolabeled Chemicals) for different periods of time (see below). A chase experiment with unlabeled methionine was initiated by adding 200 μg of L-methionine per ml. At each time point, a portion of the labeled culture was mixed with an equal volume of 10% trichloroacetic acid. After the preparations were kept on ice for 15 min or longer, protein precipitates were collected by centrifugation at 4°C, washed with acetone, and then solubilized by incubation at 37°C for 5 min in buffer containing 1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.1), and 1 mM EDTA. SecY was immunoprecipitated after dilution of the SDS-solubilized samples with Lubrol buffer as described previously (15). Antigen-antibody complexes were dissociated in SDS sample buffer at 37°C and separated by SDS–15% polyacrylamide–0.12% *N,N'*-methylenebisacrylamide gel electrophoresis (13). After the gels were dried, the intensities of SecY radioactivity were determined with a model BAS2000 Bio-Image analyzer (Fuji Photo Film). Antiserum against the N-terminal synthetic peptide of SecY (19) was provided by W. Wickner.

Immunoblotting of SecY and SecE. Known amounts of total cell proteins (as determined by using a micro-bicinchonic acid protein assay kit purchased from Pierce) were separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a Zeta-Probe membrane filter (Bio-Rad). Equilibration of the gels with the blotting buffer was avoided (16). The filter was blocked with 5% skim milk dissolved in phosphate-buffered saline at 42°C for 1 h, incubated in the same buffer with appropriately diluted antiserum at room temperature for 1 h, washed, and treated with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad); the reaction was visualized on X-ray film by using an ECL Western blot (immunoblot) detection kit (Amersham), and values were determined with a laser-scanning densitometer (Biomed Instruments). Anti-SecE serum (20) was provided by S. Mizushima.

RESULTS

Degradation of excess SecY. We used two *secY*-overexpressing plasmids with different copy numbers, pKY318 and pKY248; in both of these plasmids *secY* was under *lac* promoter control. Pulse-labeling and immunoprecipitation experiments showed that the differential rates of synthesis of SecY in pKY318-bearing and pKY248-bearing cells were about 20 and 4 times the rate in cells without any plasmid (the chromosomal SecY level), respectively. Figure 1 shows autoradiograms of SecY immunoprecipitates after pulse-chasing with [^{35}S]methionine and SDS-polyacrylamide gel electrophoresis. Whereas the level of chromosomally encoded SecY remained stable (no detectable degradation occurred) (Fig. 2 and 3), the SecY synthesized in the plasmid-bearing cells was unstable. SecY that was overexpressed about fourfold in the pKY248-bearing cells was particularly unstable, and its half-life was about 2 min (Fig. 1A and 2). Degradation ceased when the level of SecY reached the chromosomally encoded level (Fig. 1A). On the other hand, the SecY that was more extensively overexpressed in the pKY318-bearing cells was less unstable (Fig. 1B). The

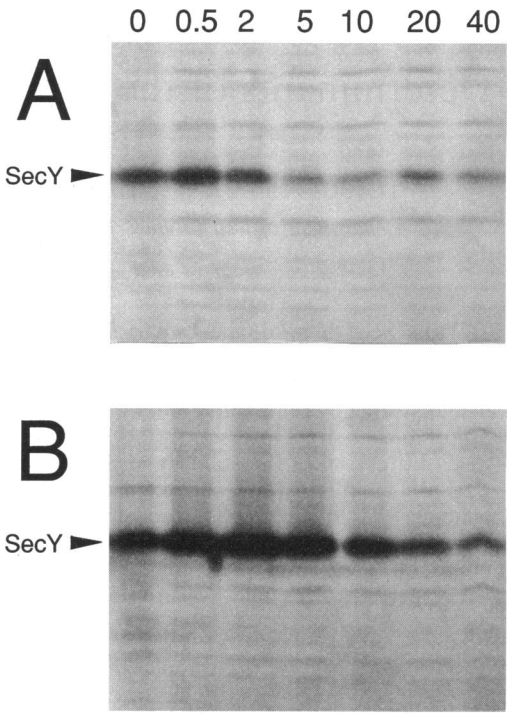

FIG. 1. Stability of oversynthesized SecY. Cells carrying pKY248 (A) or pKY318 (B), both of which carried *secY*$^+$, were pulse-labeled at 37°C with [^{35}S]methionine for 0.5 min and then chased with unlabeled L-methionine for different periods of time; the reactions were stopped by mixing the preparations with trichloroacetic acid. Approximately equal levels of radioactivity of SDS-solubilized total proteins were used, and SecY was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The numbers at the top indicate the lengths of the chase periods (in minutes).

half-life of SecY in the latter cells was somewhat variable from experiment to experiment, but it was at least about 10 min. A similar half-life was reported previously for SecY that was overexpressed even more than the SecY that was overexpressed from pKY318 (3). In these cases, either the proteolytic system responsible for SecY degradation was overloaded or SecY that exceeded a certain quantity in the cell may enter a pool different from the rapidly degrading pool of SecY. At any rate, we used the pKY248-bearing cells that exhibited rapid and reproducible SecY degradation for most of the experiments in this study.

Competitive entry of SecY and SecY-LacZα fusion protein into the stable pool. The difference in stability between the excessive and basal levels of SecY indicates that the intracellular SecY molecules can be in at least two different states and that some limiting factor determines the size of the stable pool. Thus, the increased rate of synthesis of SecY, as determined by increased pulse-labeling (Fig. 4A, lane 2), did not lead to a significant increase in steady-state level, as determined by immunoblotting (Fig. 4B, lanes 1 and 2). To demonstrate that the stable SecY pool in wild-type cells is fixed, we constructed a *secY-lacZ*α fusion gene in which the last codon of *secY* was immediately followed by codon 7 of *lacZ*α derived from pUC9. This *secY-lacZ*α fusion gene (*secY*α) was functional since the plasmid carrying *secY*α (pKY258) could complement several *secY* mutants (data not shown). Cells carrying this plasmid synthesized the SecYα protein at a rate that was severalfold higher than the rate of synthesis of chromosomally encoded

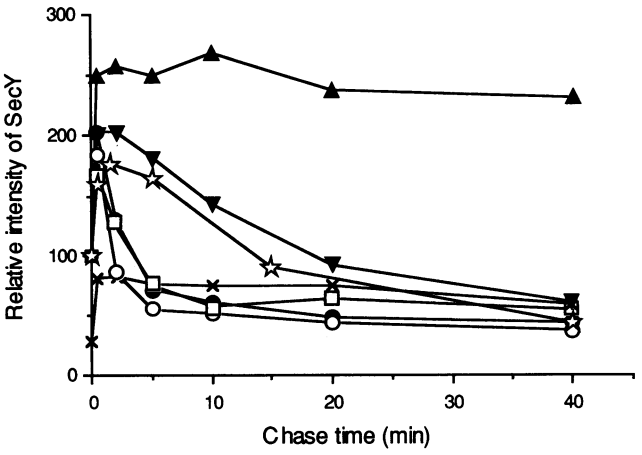

FIG. 2. Factors affecting SecY stability. Cells carrying pKY248 and additional plasmids were grown at 37°C and subjected to pulse-chase experiments as described in the legend to Fig. 1. The additional plasmids carried were pKY224 (vector) (○), pKY285 (*secE*) (▲), pKY296 (*ORF12-secD-secF*) (▼), pKY313 (*ORF12*) (☆), pKY311 (*secD-secF*) (●), and pKY287 (*secF*) (□). Anti-SecY precipitates were electrophoresed, and radioactive SecY bands were quantified. The zero-time value was defined as unity for each strain, and relative levels of radioactivity (expressed as percentages) were plotted. The results of a similar parallel experiment performed with strain TW130 (containing no plasmid) are also shown (×); the values were adjusted so that they reflect the differences in the differential rates of synthesis of SecY in the pKY248-carrying cells and the cells without any plasmid.

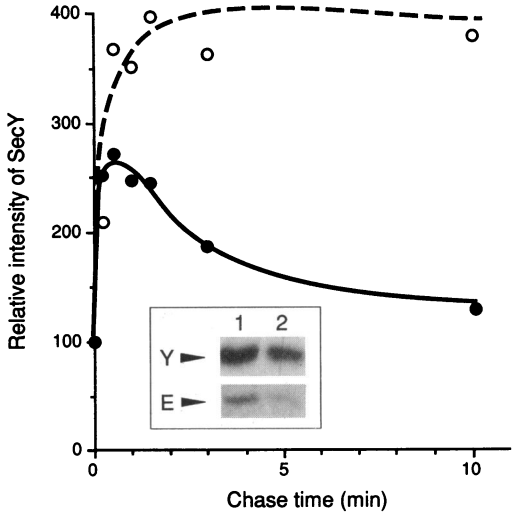

FIG. 3. Synthesis and degradation of SecY in *secE501* mutant cells. Strains TW130 (wild type) (○) and TW127 (*secE501*) (●) were grown at 37°C and pulse-labeled with [^{35}S]methionine for 15 s. After the cells were chased with unlabeled L-methionine for 0, 15, 30, 60, 90, 180, and 600 s, SecY was immunoprecipitated and separated by SDS-polyacrylamide gel electrophoresis. The levels of radioactivity associated with SecY were determined, and the values relative to the values at the zero-time chase point were plotted. The levels of SecY radioactivity before the chase and after normalization with total incorporation were essentially the same for both strains. (Inset) Steady-state accumulation of SecY (Y) and SecE (E) stained by their respective antibodies. Lane 1, strain TW130; lane 2, strain TW127.

$SecY^+$ (Fig. 4A, lane 3). Immunoblotting of the same samples showed that the level of $SecY^+$ was significantly lower in the *secY*α-overexpressing cells (Fig. 4B, lane 3). The sum of the $SecY^+$ level and the SecYα level was roughly equal to the level of $SecY^+$ in wild-type cells. Thus, newly synthesized SecY molecules should compete with each other in the formation of a stable complex.

SecE as the primary determinant of SecY stability. We introduced pBR322-derived plasmid pKY285 carrying the *secE* gene into the pKY248-bearing cells. The two plasmids can coexist because of different incompatibility properties. The introduction of the second plasmid did not appreciably affect the rate of synthesis of SecY from pKY248 (data not shown), but it markedly stabilized SecY. In the experiments shown in Fig. 2, cells carrying pKY248 and an additional plasmid were analyzed by using pulse-chase and immunoprecipitation experiments for the in vivo stability of the SecY protein. In the case of cells carrying the *secY* and *secE* plasmids, no significant degradation of the pulse-labeled SecY was observed up to the 40-min chase point (Fig. 2). This result is consistent with the immunoblotting data of Matsuyama et al. (20), who demonstrated that SecE-dependent accumulation of SecY occurred in cells.

Is SecE a determinant of the stability of SecY in normal cells without overproduction? To address this question, we used the *secE501* mutation (23), which reduced the level of expression of SecE about twofold. This conclusion was reached by Schatz et al. (25) from their measurements of the alkaline phosphatase activities of appropriate *secE-phoA* fusion constructions. Although the *secE501* mutant is phenotypically cold sensitive, reduction of *secE* expression is temperature independent. Immunoblotting experiments showed that the *secE* mutant cells contained reduced amounts of the SecY protein, as well as the SecE protein (about 50 to 65% of the levels in the wild-type strain; Fig. 3, inset). Figure 3 shows the results of pulse-chase experiments. In the wild-type cells, the intensities of SecY (compared with the 15-s pulse intensity) increased about 3.5-fold for the initial 30-s chase period and remained constant thereafter (Fig. 3). This initial increase reflected the completion of nascent chains of SecY. The initial incorporation of radioactivity into SecY was not changed by the *secE501* mutation. However, the amount of SecY pulse-labeled in the *secE501* mutant increased only 2.5-fold for the 15-s chase period. This was then followed by a rapid decrease; the half-life was again 2 to 3 min (Fig. 3). Completion of translation and degradation of the product should have been superimposed in the initial phase of the chase (Fig. 3). Roughly 50% of the maximal intensity remained after a 10-min chase. This result indicates that a fraction of newly synthesized SecY molecules are degraded rapidly when there is decreased availability of SecE. Thus, the amount of SecE not only determines the level of overproduced SecY but also determines the stability and final level of SecY expressed from the chromosomal wild-type gene.

Effects of the *secD* operon components on the stability of SecY. Among the plasmids carrying other *sec* genes, one carrying the *secD-secF* region of the chromosome (pKY296) significantly, although not completely, stabilized the SecY protein overexpressed from pKY248. The half-life was extended to about 10 min (Fig. 2). According to the corrected structure of the *secD-secF* region of the chromosome (22), this plasmid contained *ORF12*, *secD*, and *secF* as intact chromosomal genes (in that order of transcription). These three genes and probably two additional upstream genes, *tgt* and *queA*, constitute an operon. The promoter just in front of *ORF12* can also independently promote transcription of the three downstream genes (22). Although the results of in vitro translation

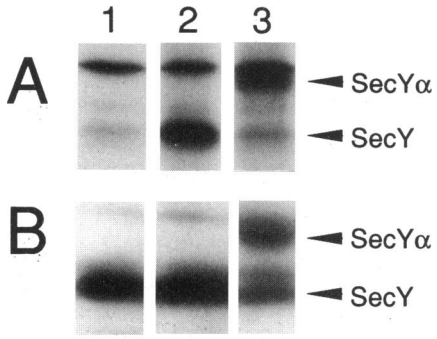

FIG. 4. Synthesis (A) and accumulation (B) of SecY and SecY-LacZα fusion protein. (A) Cells carrying pKY238 (vector) (lane 1), pKY248 (*secY*) (lane 2), or pKY258 (*secY-lacZα*) (lane 3) were pulse-labeled with [^{35}S]methionine at 37°C for 0.5 min. The SecY and SecYα proteins were immunoprecipitated with anti-SecY serum; this was followed by SDS-polyacrylamide gel electrophoresis and autoradiography. (B) Whole-cell proteins from the same cultures that were used in the experiment shown in panel A but without pulse-labeling were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nylon membrane filter. SecY and SecY-LacZα (SecYα) were detected immunologically by using anti-SecY serum.

experiments showed that *ORF12* is indeed translated (22), nothing is known about the function of this putative gene. ORF12 was called ORF3 by Gardel et al. (12).

We constructed plasmids pKY287, pKY311, and pKY313, which expressed *secF*, *secD* plus *secF*, and *ORF12*, respectively, under *lac* promoter control. Overproduction of SecD and/or SecF by pKY287 or pKY311 was confirmed by immunoblotting (data not shown). Of the three plasmid constructions, the one carrying *ORF12* (pKY313) exhibited a SecY-stabilizing ability (Fig. 2). ORF12-dependent accumulation of SecY was confirmed by immunoblotting experiments (data not shown). The fact that overproduced SecD and/or SecF did not appreciably stabilize the excess SecY may indicate that the stabilization effect of ORF12 is specific. However, we constructed a strain in which chromosomal *ORF12* was disrupted (32). In this strain, the chromosomally encoded SecY remained stable (data not shown).

DISCUSSION

E. coli cells appear to be equipped with a scavenging system that rapidly degrades SecY molecules that are not engaged in the translocation complex because of a lack of partner SecE molecules. Thus, the basal intracellular concentration of SecY is maintained even when excess SecY is synthesized. Matsuyama et al. (20) first reported that SecY can be overproduced in cells only when both *secY* and *secE* are overexpressed. Formally, the possibility that the SecE effect is at the level of transcription or translation had not been excluded. Our pulse-chase results show that the average half-life of moderately oversynthesized SecY is as short as about 2 min. SecY was completely stabilized by the simultaneous overexpression of *secE*. The fact that the SecE protein is a limiting component that determines the size of the stable SecY pool was demonstrated in the other direction as well; decreased SecE content due to a mutation in the translation initiation region of *secE* resulted in rapid degradation of a fraction of the SecY molecules, leading to a reduced SecY content in the cells. These results indicate that one of the intracellular roles played by SecE is to stabilize SecY in the membrane, although the results of the reconstitution studies indicate that SecE itself participates in translocation facilitation as well (1, 21). Truncated variants of SecE that contain only the most carboxy-terminal transmembrane segment and some surrounding regions can function in vivo and in vitro (21, 25). The interaction between SecY and SecE may involve transmembrane segment 3 of SecE.

Newly synthesized SecY molecules immediately enter the degradation pathway when insufficient SecE is available (Fig. 3). In other words, SecY and SecE should normally associate with each other immediately after they are synthesized or immediately after they are integrated into the membrane. The existence of distinct two populations of SecY, one that is rapidly degraded and one that is completely stable, in the *secE501* mutant suggests that the SecY-SecE complex persists once it is formed. This seems to disfavor the cyclic association-dissociation model of Bieker and Silhavy (7).

Our results indicate that the ORF12 product can stabilize excess SecY when it is overexpressed. As the deduced amino acid sequence of ORF12 contains an amino-terminal stretch enriched in hydrophobic amino acids (12, 22), it is possible that the ORF12 product, like SecE, interacts with SecY within the membrane. However, our gene disruption study showed that ORF12 is essential neither for protein export nor for stability of SecY, although a synthetic phenotype was observed between the disruption of *ORF12* and the *secY39* mutation (this combination caused growth impairment at 42°C [32]). We are in the process of dissecting the SecY complex genetically by using a dominant negative *secY* mutation, *secY^{-d}1*, and its suppressors (14, 29). In this system, overproduction of SecE corrects the export interference by the SecY^{-d}1 molecules that may sequester interacting components (29). As a similar multicopy overcomer of the dominant negative phenotype of *secY^{-d}1*, we also identified a new gene, *ydr* (14, 30). Interestingly, whereas overexpression of *ydr*, like overproduction of *ORF12*, stabilized the excess SecY (30), overexpression of *ORF12* improved protein export significantly in the presence of *secY^{-d}1* (32). These results raise the possibility that the ORF12 product, at least when it is overproduced, somehow interacts with SecY. Alternatively, the ORF12 product might merely inhibit a protease by saturating it.

Not all of the overproduced membrane proteins are degraded in *E. coli* cells. The stable class of membrane proteins includes the lactose permease (6) that is similar to SecY in being a multipath integral membrane protein (10). Although the proteolytic system that degrades excess SecY remains to be identified, it might well be membrane embedded. Such a system should recognize some specific features of the uncomplexed SecY protein within the membrane and conceivably be important for the overall functioning of the translocation machinery.

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ADDENDUM IN PROOF

K. J. Pogliano and J. Beckwith (personal communication) have proposed that *ORF12* and *ORF3* should now be designated *yajC*.

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