SecE-dependent overproduction of SecY in *Escherichia coli*

Evidence for interaction between two components of the secretory machinery

Shin-ichi Matsuyama, Jiro Akimaru and Shoji Mizushima

Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan

Received 15 June 1990

The *secY* and *secE* genes were individually cloned and placed under the control of the *tac* promoter on plasmids. Induction with isopropyl-β-D-thiogalactopyranoside resulted in the overproduction of SecE, but not that of SecY. The simultaneous induced expression of both genes in the same cells resulted in the overproduction of SecY together with that of SecE. SecY and SecE thus overproduced were localized in the cytoplasmic membrane as those expressed at the normal levels were. It is suggested that SecY and SecE interact with each other in the cytoplasmic membrane. The numbers of the SecY and SecE molecules per cell were estimated.

SecY protein; SecE protein; Protein secretion; SecY/SecE interaction; *Escherichia coli*

1. INTRODUCTION

Extensive genetic studies on the *Escherichia coli* cellular components required for the translocation of secretory proteins across the cytoplasmic membrane have revealed the existence of 5 genes, *secA*, *secB*, *secD*, *secE* and *secY* [1-6]. The roles of SecA [7] and SecB [8], coded for by the *secA* and *secB* genes, respectively, in protein translocation have been well studied in vitro using purified proteins. On the other hand, little is known as to the biochemical functions of SecY and SecE, both of which are transmembrane proteins [9,10]. This is most likely due to the difficulty in obtaining sufficient amounts of these proteins. SecY is a minor cytoplasmic membrane component. Its overproduction has been challenged using promoters which are highly efficient in transcription. Only slight overproduction has been achieved so far [11].

In this work, we succeeded in SecE-dependent overproduction of SecY. The overproduced SecY was localized in the cytoplasmic membrane together with SecE. These results suggest that SecY and SecE are closely related physically and probably functionally in the cytoplasmic membrane of *E. coli*.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

Escherichia coli JM83 [12], MC4100 [13] and PR520 (MC4100 *secEcsE501*) [4] were used.

Correspondence address: M. Matsuyama, Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate;

2.2. *Materials*

Restriction endonucleases and the T4 DNA ligase were obtained from Takara Shozo Co. IPTG was from Nacalai Tesque Inc. Synthetic oligonucleotide linkers ($^{5'}$d GATCCTAGGAGGTTTAAATTTATGGCTAAACAAC$^{3'}$, $^{3'}$GATCCTCCAAATTTAAATACCGATTTGTTGGC$^{5'}$; for *secY* manipulation) and ($^{5'}$GATCCTAGGAGGTTTAAATTTATGAGTGCGAATACCGAAG$^{3'}$, $^{3'}$GATCCTCCAAATTTAAATACTCACGCTTATGGCTTC$^{5'}$; for *secE* manipulation) were synthesized with an Applied Biosystems DNA synthesizer 380B.

2.3. *Construction of pMAN460 and pMAN480*

A 0.4 kb fragment carrying the T7 promoter was obtained by digestion of pET-3 [14] with *Hae*II. pKEN403 [15] was partially digested with *Hae*II, and the resulting 3.4 kb fragment containing the Km^r gene and the replicon from pSC101 [16] was ligated with the 0.4 kb *Hae*II fragment to construct pMAN460. A 0.4 kb fragment carrying the *tac* promoter was obtained by digestion of pUS12 [17] with *Eco*RI and *Bam*HI. pSY343 [18] was digested with *Eco*RI and *Bam*HI, and the resulting 8.5 kb fragment containing the Km^r gene and the runaway-replicon was ligated with the 0.4 kb *Eco*RI-*Bam*HI fragment to construct pMAN480.

2.4. *Cloning of the secE gene*

The *secE* gene has unique *Xma*III and *Kpn*I sites in its upstream and downstream regions, respectively [19]. A 0.79 kb DNA fraction containing the *Xma*III-*Kpn*I fragment was isolated from the chromosomal DNA of MC4100 by agarose gel electrophoresis and then cloned into pMAN802 in which the *Pvu*II site had been converted to a *Kpn*I site on pBR322 [20] (Fig. 1A). The clones were used to transform PR520 (*secEcsE501*) and transformants that grew at 20°C were obtained. A 0.79 kb *Xma*III-*Kpn*I fragment from pMAN803, a plasmid carried by one of these transformants, was sequenced. The result was consistent with the sequence of the *secE* gene determined by Downing et al. [19]. From these results, we concluded that pMAN803 carries the entire *secE* gene.

2.5. *Preparation of site-specific antibodies against SecE and SecY*

Peptides corresponding to the Met1-Arg22 and Ser426-Arg443 regions of SecY were synthesized with an Applied Biosystems peptide synthesizer 430A, and antisera against the peptides, Y-1 and Y-5, respectively, were raised in rabbits as described (Tokuda, H.,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

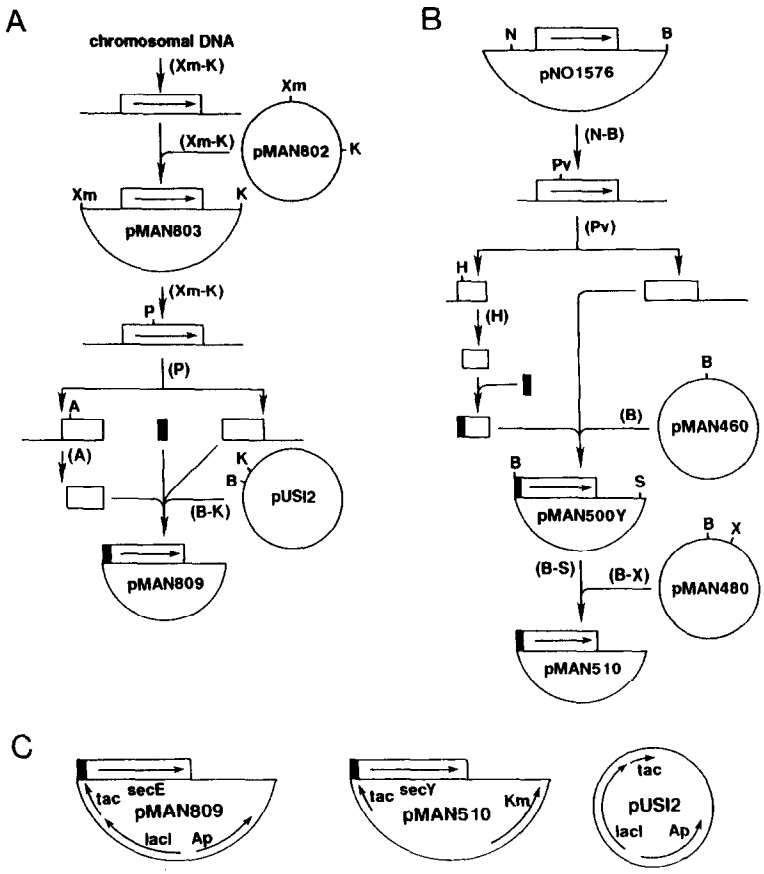

Fig. 1. Construction (A and B) and structure (C) of plasmids used. pNO1576 [26] carrying the *secY* gene was used as a starting plasmid for the *secY* manipulation. The open box denotes the coding region of the *secE* (A) or *secY* (B) genes. The arrow in the open box shows the direction of transcription. The closed box denotes the synthetic oligonucleotide linker. Restriction endonucleases used are shown in parentheses with the following abbreviations: N, *Nru*I; B, *Bam*HI; Pv, *Pvu*II; H, *Hpa*II; S, *Sal*I; X, *Xho*I; Xm, *Xma*III; K, *Kpn*I; P, *Pst*I; A, *Alu*I.

Shiozuka, K. and Mizushima, S., manuscript submitted). A peptide corresponding to the Ser2-Lys18 region of SecE was also synthesized and an antiserum against it was raised by the same method.

2.6. *Immunoblotting*

Immunoblot analysis was carried out as described previously [21]. 5-Bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium were used for visualization.

2.7. *SDS-Polyacrylamide gel electrophoresis*

SDS-Polyacrylamide gel electrophoresis was carried out according to the method described by Laemmli [22]. The gel contained 13% acrylamide-0.35% *N,N'*-methylenebisacrylamide. All samples were applied to the gel without boiling.

3. RESULTS

3.1. *Construction of a plasmid carrying the tac-secY gene or tac-secE gene and expression of these genes*

Constructions of pMAN809 and pMAN510 were shown in Figs. 1A and 1B, respectively. pMAN809 carries the *tac-secE* gene and the *lacI* gene (Fig. 1C). pMAN510 carries *tac-secY* gene and the runaway replicon (Fig. 1C). For SecY synthesis, *E. coli* JM83 cells were transformed simultaneously with pMAN510 and pUSI2, a *lacI* gene-carrying plasmid (Fig. 1C), and cultured at 37°C for 2 h for runaway replication of pMAN510 followed by induction with IPTG. For SecE synthesis, pMAN809 was induced by the addition of IPTG.

The IPTG-induced cells were then subjected to SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue (Fig. 2A). In JM83 harboring pMAN809, the overproduction of a protein species corresponding to 16 kDa upon IPTG induction was observed (lanes 1–5). The molecular mass of SecE was estimated to be 13,600 Da from the deduced amino acid sequence [10,19]. On the other hand, in JM83 harboring pMAN510/pUSI2, such overproduction was not observed at the position where SecY was expected (lanes 6–10). Samples, which were gel electrophoresed in the same way, were also subjected to Western blotting with either anti-SecY antiserum Y-1 or anti-SecE antiserum (Figs. 2B and 2C). The overproduced protein species corresponding to 16 kDa reacted with the anti-SecE antiserum, indicating that it is SecE (Fig. 2C, lanes 1–5). The slower migration of SecE, compared to its molecular mass, is most likely due to its basic nature [10,19]. Although the amount of SecY increased slightly upon

the IPTG-induced expression of the *tac-secY* gene (Fig. 2B, lanes 6–10), the increase was far less significant than that in the case of SecE. It should be noted that the overproduction of SecE resulted in a simultaneous increase in the level of SecY to some extent (Fig. 2B, lanes 1–5). This suggested that the simultaneous synthesis of SecE may facilitate the overproduction of SecY.

3.2. *Simultaneous expression of the secY and secE genes permits overproduction of SecY*

Plasmids pMAN809 and pMAN510, carrying the *tac-secE/lacI* and *tac-secY* genes, respectively, were transferred into the same cells and then the genes were expressed simultaneously through the addition of IPTG. The IPTG-induced cells were subjected to SDS-polyacrylamide gel electrophoresis for analysis of total cellular proteins. Overproduction of a protein species corresponding to SecE was observed even on Coomassie Brilliant Blue staining (Fig. 2A, lanes 11–15). Although the result was not as clear as in the case of SecE, the IPTG-induced synthesis of a protein species corresponding to SecY was also observed. Western blotting analysis of these samples with anti-SecY antiserum Y-1 and anti-SecE antiserum proved that these proteins were SecY and SecE, respectively (Figs. 2B and 2C, lanes 11–15). Western blotting analysis revealed that the SecY band was reactive with not only antiserum Y-1, which reacts with the amino-terminus of SecY, but also antiserum Y-5, which reacts with its carboxyl-terminus (data not shown). This indicates that the protein thus overproduced constituted the entire SecY molecule.

Western blotting analysis was then performed for different amounts of cell samples, followed by densitometric quantification of SecY and SecE. More than 40-fold overproduction of SecY was achieved upon 300-fold overproduction of SecE (data not shown).

3.3. *Both SecY and SecE, which are overproduced, are localized in the cytoplasmic membrane*

SecY is localized in the cytoplasmic membrane [9]. The cytoplasmic membrane localization of SecE was

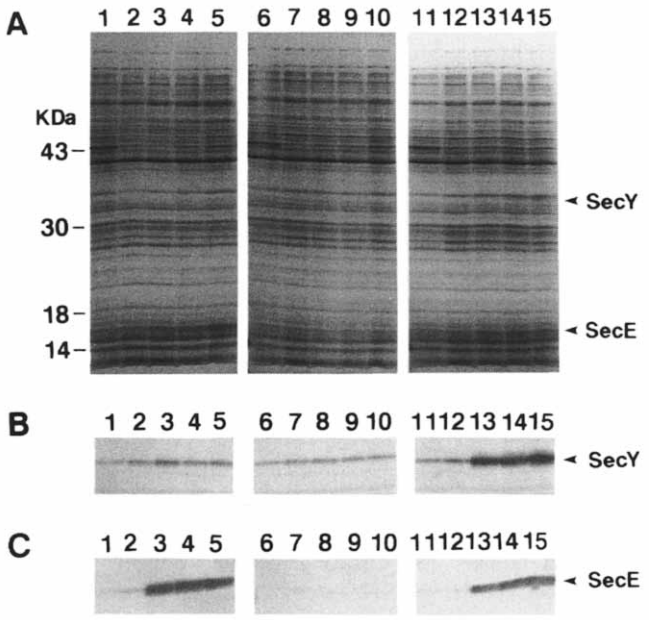

Fig. 2. Overproduction of SecE and SecY proteins. JM83 (lanes 1, 6 and 11), JM83/pMAN809 (lanes 2–5), JM83/pMAN510-pUSI2 (lanes 7–10) and JM83/pMAN510-pMAN809 (lanes 12–15) were grown in the presence of 1.5 mM IPTG. Aliquots were withdrawn at 0 (lanes 2, 7 and 12), 30 (lanes 3, 8 and 13), 60 (lanes 4, 9 and 14) and 120 min (lanes 1, 5, 6, 10, 11 and 15). Cell-equivalent amounts were analyzed on SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue (A) or Western blotting with anti-SecY antiserum Y-1 (B) and anti-SecE antiserum (C). The migration points of molecular weight markers and positions of SecE and SecY are indicated.

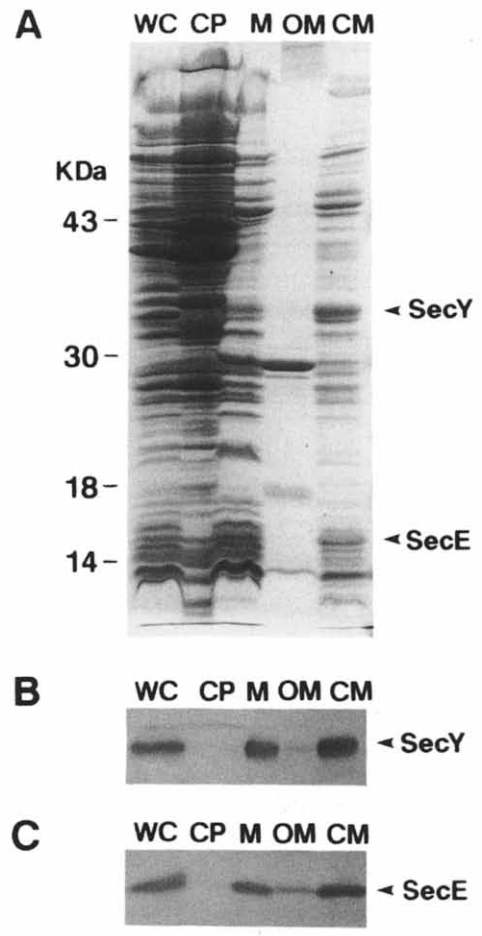

Fig. 3. Cellular localization of SecY and SecE proteins. JM83 harboring both pMAN809 and pMAN510 was grown for 2 h after addition of IPTG. Cell fractionation was carried out as described previously [27]. Cell-equivalent amounts of each fraction were analyzed on SDS-polyacrylamide gel electrophoresis and then stained (A) or subjected to Western blotting with anti-SecY antiserum Y-1 (B) or anti-SecE antiserum (C). Positions of SecE and SecY are indicated. Positions of migration of molecular weight markers are also indicated. WC, whole cell; CP, cytosol and periplasm fraction; M, membrane fraction; OM, outer membrane fraction; CM, cytoplasmic membrane fraction.

also suggested [10]. The IPTG induced cells containing overproduced amounts of SecY and SecE were subjected to cell fractionation followed by SDS-polyacrylamide gel electrophoresis and Western blotting (Figs. 3B and 3C). Both SecY and SecE were exclusively localized in the cytoplasmic membrane, suggesting that even the overproduced proteins were assembled properly into the cytoplasmic membrane. The localization in the cytoplasmic membrane of SecY and SecE was also clearly demonstrated on Coomassie Brilliant Blue staining of the gels (Figs. 3A and 4). SecY and SecE became major components of the cytoplasmic membrane after their overproduction. JM83 cells free from the plasmids were also subjected to the same cell fractionation. The cytoplasmic membrane localization of both proteins was again demonstrated (data not shown).

3.4. *Estimation of SecY and SecE contents in a cell*

Through densitometric tracing of the gel shown in Fig. 4, lane 3, SecY was estimated to amount to about 6.4% of the total cytoplasmic membrane protein of SecY-overproducing cells. Assuming that the cytoplasmic membrane protein accounts for 10% of the total cellular protein and one *E. coli* cell contains about 1.6×10^{-13} g protein [23], the number of SecY molecules in one SecY-overproducing cell was estimated to be about 1.2×10^4. Since a 40-fold increase in the SecY content was observed upon IPTG-induced overproduction, the number of SecY molecules in one plasmid-free *E. coli* cell was calculated to be about 300.

The numbers of SecE molecules in one cell were estimated in the same way. The values were 2.5×10^4 and 80 molecules for a SecE/SecY-overproducing cell and a plasmid-free cell, respectively.

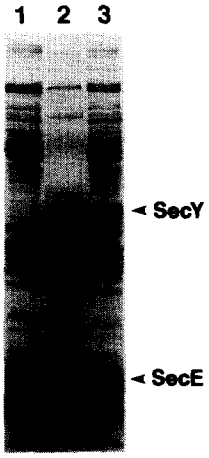

Fig. 4. Comparison of the protein composition of cytoplasmic membrane fractions prepared from JM83 (lane 1), JM83/pMAN809 (lane 2), and JM83/pMAN809-pMAN510 (lane 3). Cells were grown in the presence of 1.5 mM IPTG for 2 h. Cytoplasmic membrane fractions (40 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis and then stained. Positions of SecY and SecE are indicated.

4. DISCUSSION

In the present work we succeeded, for the first time, in overproducing SecY and SecE in large quantities. After the overproduction, both SecY and SecE became major constituents of the cytoplasmic membrane (Fig. 4). The SecE-dependent SecY overproduction thus achieved not only enables us to isolate a substantial amount of SecY as well as SecE from the cytoplasmic membrane, but also provided us with several pieces of interesting information. First of all, the results strongly suggest the existence of a firm interaction between these two membrane components involved in protein secretion. A recent genetic work also suggested the existence of such interaction [24].

The molar ratio of SecY/SecE in the cytoplasmic membrane after simultaneous overproduction was estimated to be roughly the same, suggesting that the secretory machinery may contain equimolar amounts of SecY and SecE. The numbers of SecY and SecE molecules in one plasmid-free cell were, on the other hand, estimated to be 300 and 80, respectively. It is unclear whether or not these values mean different proportions of these two proteins in plasmid-free cells. Western blotting adopted for the estimation leads to more inaccuracy than direct estimation on the stained gel. The numbers of SecY and SecE molecules per non-overproducing cell were, in any event, roughly around 100–300. This suggests that the numbers of the putative secretory machinery per cell is around this figure. Contrary to this, based on the numbers of the MalE-LacZ molecules that became stuck to the cytoplasmic membrane, Ito et al. [25] estimated that an *E. coli* cell has roughly 2×10^4 sites for protein secretion.

Acknowledgements: This work was supported by a grant from the Ministry of Education, Science and Culture of Japan (61060001). We thank Iyoko Sugihara for her excellent secretarial support.

REFERENCES

[1] Oliver, D.B. and Beckwith, J. (1981) Cell 25, 2765–2772.
[2] Kumamoto, C. and Beckwith, J. (1983) J. Bacteriol. 154, 254–260.
[3] Gardel, C., Benson, S., Hunt, J., Michaelis, S. and Beckwith, J. (1987) J. Bacteriol. 169, 1286–1290.
[4] Riggs, P.D., Derman, A.I. and Beckwith, J. (1988) Genetics 118, 571–579.
[5] Emr, S.D., Hanley-Way, S. and Silhavy, T.J. (1981) Cell 23, 79–88.
[6] Shiba, K., Ito, K., Yura, T. and Cerretti, D.P. (1984) EMBO J. 3, 631–636.
[7] Tokuda, H. and Mizushima, S. (1990) J. Bioenerg. Biomembr. in press.
[8] Saier, M.H., Jr., Werner, P.K. and Müller, M. (1989) Microbiol. Rev. 53, 333–366.
[9] Akiyama, Y. and Ito, K. (1985) EMBO J. 4, 3351–3356.
[10] Schatz, P.J., Riggs, P.D., Jacq, A., Fath, M.J. and Beckwith, J. (1989) Genes Dev. 3, 1035–1044.
[11] Akiyama, Y. and Ito, K. (1986) Eur. J. Biochem. 159, 263–266.
[12] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
[13] Casadaban, M.J. (1976) J. Mol. Biol. 104, 541–555.

[14] Rosenberg, A.H., Lade, B.N., Chui, D., Lin, S., Dunn, J.J. and Studier, F.W. (1987) Gene 56, 125–135.
[15] Matsuyama, S. and Mizushima, S. (1985) J. Bacteriol. 162, 1196–1202.
[16] Cabello, F., Timmis, K. and Cohen, S.N. (1976) Nature 259, 285–290.
[17] Shibui, T., Uchida, M. and Teranishi, Y. (1988) Agric. Biol. Chem. 52, 983–988.
[18] Yasuda, S. and Takagi, T. (1983) J. Bacteriol. 154, 1153–1161.
[19] Downing, W.L., Sullivan, S.L., Gottesman, M.E. and Dennis, P.P. (1990) J. Bacteriol. 172, 1621–1627.
[20] Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Gene 2, 95–113.
[21] Yamada, H., Matsuyama, S., Tokuda, H. and Mizushima, S. (1989) J. Biol. Chem. 264, 18577–18581.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Maaløe, O. and Kjeldgaard, N.O. (1966) Control of Macromolecular Synthesis W.A. Benjamin, New York.
[24] Bieker, K.L. and Silhavy, T.J. (1990) Cell 61, 833–842.
[25] Ito, K., Bassford, P.J., Jr. and Beckwith, J. (1981) Cell 24, 707–717.
[26] Ito, K., Wittekind, W., Nomura, M., Shiba, K., Yura, T., Miura, A. and Nashimoto, H. (1983) Cell 32, 789–797.
[27] Yamada, H., Tokuda, H. and Mizushima, S. (1989) J. Biol. Chem. 264, 1723–1728.