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SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*

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The SecD protein is one of the components that has been suggested from genetic studies to be involved in the protein secretion across the cytoplasmic membrane of *Escherichia coli*. We examined the effect of anti-SecD IgG on protein secretion using spheroplasts. Inhibition of the secretion of OmpA and maltose-binding protein (MBP) by this IgG was observed with concomitant accumulation of their precursor and mature forms in spheroplasts. This effect was specific to anti-SecD IgG. Anti-SecE and anti-SecY IgGs, of which the epitopes are located at the periplasmic domains of SecE and SecY, respectively, did not interfere with the secretion. Time-course experiments investigating the processing of proMBP and the release of MBP from spheroplasts revealed that anti-SecD IgG interfered with the release of the translocated mature MBP. The mature form of MBP thus accumulated was sensitive to trypsin, which was externally added to spheroplasts, whereas MBP released into the medium was resistant to trypsin as the native MBP is. The precursor form of MBP accumulated in spheroplasts was also trypsin resistant. We conclude that SecD is directly involved in protein secretion and important for the release of proteins that have been translocated across the cytoplasmic membrane.

Key words: anti-SecD IgG/*Escherichia coli*/protein release/protein secretion/SecD protein

Introduction

Genetic studies have revealed that the following *sec* genes are involved in the protein translocation across the cytoplasmic membrane of *Escherichia coli*: *secA* (Oliver and Beckwith, 1981), *secB* (Kumamoto and Beckwith, 1983), *secE* (Riggs *et al.*, 1988), *secY* (Emr *et al.*, 1981; Ito *et al.*, 1983) and *secD* /*secF* (Gardel *et al.*, 1987, 1990). The Sec proteins encoded by these genes have been overproduced and purified (Kawasaki *et al.*, 1989; Cabelli *et al.*, 1988; Weiss *et al.*, 1988; Matsuyama *et al.*, 1990a, 1992b), and subjected to biochemical analyses. SecB was identified as a molecular chaperon, which interacts with presecretory proteins to maintain their translocation-competent conformation (Kumamoto, 1990). SecA, a peripheral membrane protein, was identified as translocation ATPase (Lill *et al.*, 1989), which plays a central role in the initial stage of the translocation by interacting with many of the components involved in the translocation reaction (Akita *et al.*, 1990; Lill *et al.*, 1990; Matsuyama *et al.*, 1990b; Shinkai *et al.*, 1991; Kimura *et al.*, 1991; Cabelli *et al.*, 1991; Hendrick and Wickner, 1991). Reconstitution studies demonstrated that SecY and SecE, integral membrane proteins, are essential components of the secretory machinery that is embedded in the membrane (Brundage *et al.*, 1990; Akimaru *et al.*, 1991; Nishiyama *et al.*, 1991; Tokuda *et al.*, 1991). The precise roles of these membrane proteins remain unknown, however. Although SecD and SecF were suggested to be integral membrane proteins as well, they did not enhance reconstituted translocation activity (Matsuyama *et al.*, 1992b). The number of SecD molecules in the cytoplasmic membrane of *E.coli* is estimated to be about the same as those of SecY, SecE and SecA, suggesting that these four proteins constitute the secretory machinery in the membrane (Matsuyama *et al.*, 1992b). The molecular number of SecF, on the other hand, was estimated to be far smaller (Matsuyama *et al.*, 1992b).

The deduced amino acid sequence of SecD suggests the presence of a large periplasmic domain in this membrane protein (Gardel *et al.*, 1990). Furthermore, *prl* mutations, which suppress signal peptide defects, have not been mapped to the *secD* gene (Stader *et al.*, 1989; Gardel *et al.*, 1990). This suggests that SecD may play a role in a later stage of translocation, which is highly difficult to demonstrate when protein translocation is assayed as the import into everted membrane vesicles or that into reconstituted proteoliposomes.

In the present work, we employed spheroplasts and examined the effect of an anti-SecD antibody on the protein export across the cytoplasmic membrane. Pretreatment of spheroplasts with anti-SecD IgG resulted in inhibition of the release of secretory proteins into the medium and the accumulation of both the precursor and mature forms of presecretory proteins.

Results

Anti-SecD IgG inhibits protein secretion from spheroplasts

SecD, a cytoplasmic membrane protein, is assumed to possess a large periplasmic domain (Gardel *et al.*, 1990). The role of SecD in protein translocation across the cytoplasmic membrane was examined by treating spheroplasts with anti-SecD IgG. The antiserum we used in the present work was raised against a SecA-SecD fusion protein (Matsuyama *et al.*, 1992a). Anti-SecA IgG was, therefore, quantitatively removed on a SecA-conjugated column as described (Matsuyama *et al.*, 1992a).

Spheroplasts suspended in a 0.25 M sucrose solution were treated or not treated with anti-SecD IgG thus obtained, labeled with Tran^{35}S-label, and then fractionated into spheroplasts and the medium. Spheroplasts not treated with the antibody secreted several protein species into the medium (Figure 1, lanes 1 and 4), as reported previously (Metcalfe

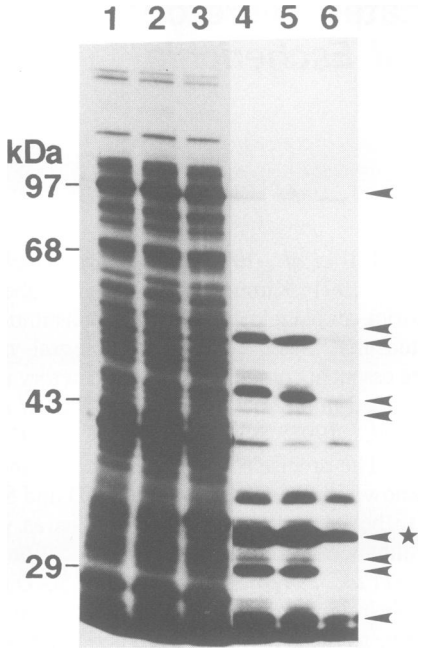

Fig. 1. Effect of anti-SecD IgG on protein secretion by spheroplasts. Spheroplasts (lanes 1 and 4) and spheroplasts treated with non-immune (lanes 2 and 5) or anti-SecD (lanes 3 and 6) IgG on ice for 3 min were incubated in a minimal medium for 6 min at 30°C, and then labeled with Tran^{35}S-label for 2 min, followed by a chase for 1 min with cold methionine and cysteine. The cultures were chilled in ice water, and then fractionated into spheroplasts (lanes 1−3) and the medium (lanes 4−6). Each fraction was analyzed by SDS−PAGE, followed by fluorography. The gel was exposed to X-ray films for 12 h (lanes 1−3) and 3 days (lanes 4−6). Arrowheads indicate proteins whose secretion into the medium was inhibited by anti-SecD IgG. ★ indicates the band which was identified as that of OmpA (see text). The migration positions of mol. wt markers are also indicated.

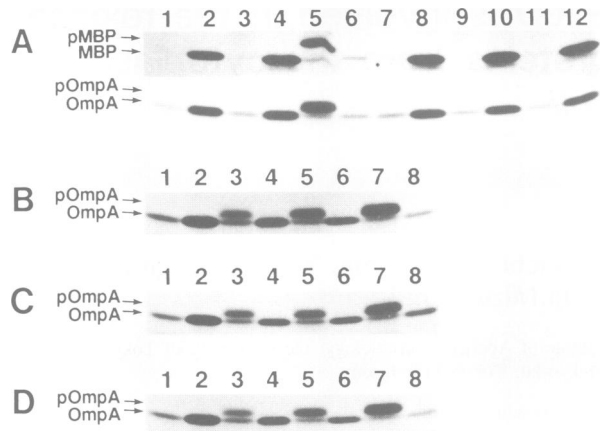

Fig. 2. Inhibition of the export of MBP and OmpA by anti-SecD IgG, and accumulation of their precursors. (**A**) Spheroplasts were treated on ice for 3 min with nothing (lanes 1 and 2), non-immune IgG (lanes 3 and 4), anti-SecD IgG (lanes 5 and 6), anti-SecA IgG (lanes 7 and 8), anti-SecE IgG (lanes 9 and 10) and anti-SecY IgG (lanes 11 and 12). (**B**) The anti-SecD IgG was further purified on a SecA−SecD fusion protein-conjugated column. Spheroplasts were treated on ice for 3 min with the pass-through fraction from the column (24 μM IgG) (lanes 1 and 2) or the following concentrations of purified anti-SecD IgG: 0.3 μM (lanes 3 and 4), 1 μM (lanes 5 and 6) and 3 μM (lanes 7 and 8). (**C**) Spheroplasts were treated on ice for 3 min with 150 μM non-immune Fab (lanes 1 and 2), or 17 μM (lanes 3 and 4), 50 μM (lanes 5 and 6) or 150 μM (lanes 7 and 8) anti-SecD Fab. (**D**) Spheroplasts were treated on ice for 3 min with 24 μM non-immune IgG (lanes 1 and 2), and 2.7 μM (lanes 3 and 4), 8μM (lanes 5 and 6) and 24 μM (lanes 7 and 8) anti-SecD IgG. In each experiment, the spheroplasts were labeled with Tran^{35}S-label as described in the legend to Figure 1, and then fractionated into spheroplasts (odd-numbered lanes) and the medium (even-numbered lanes). Both fractions were subjected to immunoprecipitation with anti-MBP (upper panel of A) and anti-OmpA (lower panel of A, and B, C and D) antisera, as described in Materials and methods, and then analyzed by SDS−PAGE and fluorography. The precursor and mature forms of MBP and OmpA are indicated.

and Holland, 1980; Sen and Nikaido, 1990). When spheroplasts were treated with anti-SecD IgG before labeling, the amounts of proteins secreted into the medium were remarkably decreased, despite that the treatment did not inhibit protein synthesis (Figure 1, lanes 3 and 6). A control IgG had no effect on protein export (Figure 1, lanes 2 and 5). The amounts of some protein species in the medium were not affected by the treatment with anti-SecD IgG. It is unclear whether these proteins were cytosolic ones or those secreted in an anti-SecD IgG independent manner.

To confirm that the proteins whose export was inhibited by anti-SecD IgG are authentic secretory ones, immunoprecipitation was carried out with antisera against maltose-binding protein (MBP) and OmpA, which are periplasmic and outer membrane proteins respectively. MBP and OmpA synthesized by control spheroplasts were secreted into the medium as mature forms (Figure 2A, lanes 1−4). When anti-SecD IgG-treated spheroplasts were used, their secretion was inhibited appreciably (Figure 2A, lane 6). Furthermore, treatment with anti-SecD IgG resulted in the accumulation of their precursors in spheroplasts (Figure 2A, lane 5). IgG species that did not bind to SecD protein did not exert inhibitory effects (Figure 2B, lanes 1 and 2). The IgG fraction eluted with acetic acid from the SecA−SecD-conjugated column also inhibited OmpA secretion (Figure 2B, lanes 3−8). From these results, we conclude that anti-SecD IgG inhibits the export of proteins from spheroplasts. From gel electrophoretic analysis, the band with an asterisk in Figure 1 was found to be that of OmpA. The band of MBP was not identified in Figure 1, probably because the amount was minute.

Inhibition of protein secretion was specific to anti-SecD IgG

Similar experiments were carried out with anti-SecA, anti-SecE and anti-SecY IgGs (Figure 2A, lanes 7−12). The anti-SecY IgG used was raised against a synthetic peptide corresponding to a periplasmically exposed region of SecY (Gly198−His216). The anti-SecE and anti-SecA IgGs used were raised against whole molecules. To determine whether or not the anti-SecE IgG preparation recognizes the periplasmic domain of SecE, intact spheroplasts were treated with this anti-SecE IgG. After removal of free IgG by centrifugation, the spheroplasts recovered were solubilized with 1% Triton X-100 and then applied on a protein A−agarose column to remove IgG conjugates. Almost all the SecE was removed by this treatment, in the same way that anti-SecD IgG treatment removed SecD (Figure 3), indicating that the anti-SecE IgG preparation used reacts with the periplasmically exposed region of SecE. No removal of SecA on treatment with anti-SecA IgG was observed.

Despite the periplasmic localization of antigenic sites, the anti-SecE and anti-SecY IgGs did not prevent spheroplasts from exporting MBP and OmpA into the medium (Figure 2A, lanes 9−12). The inhibition of protein export

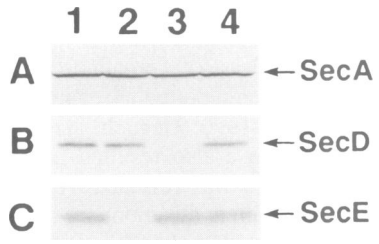

Fig. 3. Anti-SecE IgG reacted with the periplasmic epitope of SecE. Spheroplasts, which were treated with non-immune (lane 1), anti-SecE (lane 2), anti-SecD (lane 3) and anti-SecA (lane 4) IgGs on ice for 3 min, were recovered by centrifugation and then solubilized with 1% Triton X-100, followed by removal of IgG-conjugates on a protein A−agarose column. The IgG-depleted fractions were subjected to immunoblot analysis with anti-SecA (**A**), anti-SecD (**B**) or anti-SecE (**C**) antiserum. The positions of SecA, SecD and SecE are indicated.

by antibodies is thus specific to the anti-SecD antibody, supporting the view that the inhibition was not due to a secondary effect of the antibody binding to SecD on its adjacent components. This view was further supported by an experiment with a monovalent Fab prepared from anti-SecD IgG; the inhibition profile obtained with anti-SecD Fab was essentially the same as that with anti-SecD IgG (Figure 2C and D), though a higher concentration was required with the Fab. The results also suggest that the inhibition was not due to anti-SecD IgG-mediated aggregation of the translocation machinery, another possible indirect inhibitory effect of the IgG, which is divalent.

Finally, anti-SecD IgG used here did not react at all with SecF (Matsuyama *et al.*, 1992b), which also possesses a large periplasmic domain (Gardel *et al.*, 1990). This observation excludes the possible participation of SecF in the observed inhibitory effect.

Taking all the evidence together, we conclude that the SecD protein, especially its periplasmic domain, plays a role in the protein secretion across the cytoplasmic membrane.

Anti-SecD antibody interferes with the release of translocated mature proteins from spheroplasts

The results described so far suggest that the step of protein release from the membrane after translocation may be the target of anti-SecD IgG. The effect of anti-SecD IgG on the export of MBP into the medium was then examined more precisely (Figure 4). MBP was used as this is a periplasmic protein and hence should be easily discharged to the medium. With spheroplasts treated with a control IgG, radiolabeled MBP began to appear in the medium within 1 min after labeling. On the other hand, treatment with anti-SecD IgG resulted in a considerable delay in the appearance of MBP in the medium, with the concomitant accumulation of both MBP and proMBP in the spheroplast fraction.

The localization of proMBP and MBP in spheroplasts was then determined by means of trypsin treatment. ProMBP in the spheroplasts was entirely resistant to externally added trypsin, unless the spheroplasts were lysed with Triton X-100, whereas mature MBP in spheroplasts was highly sensitive to trypsin (Figure 5). It should be noted in this respect that mature MBP in the medium is as resistant to trypsin as intact MBP (Geller, 1990; Ueguchi and Ito, 1990) (Figure 5). It is strongly suggested, therefore, that anti-SecD IgG interferes with the release of translocated and processed MBP from the membrane, and hence results in the

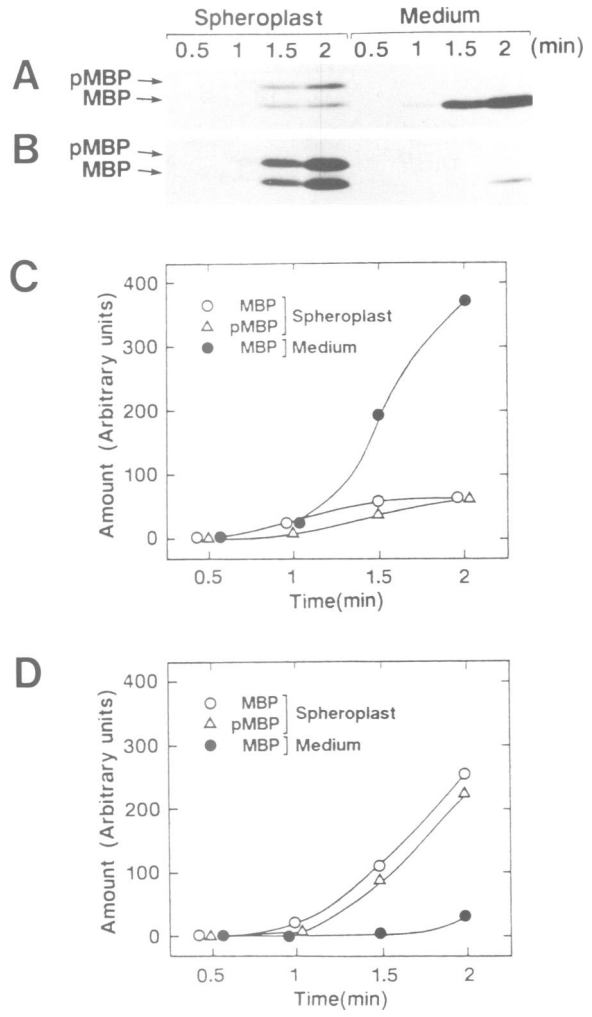

Fig. 4. The effect of anti-SecD IgG on the release of MBP into the medium. Spheroplasts were treated with non-immune (**A** and **C**) or anti-SecD (**B** and **D**) IgG on ice for 3 min. The minimal medium containing Tran^{35}S-label was added at zero time to initiate labeling at 30°C. Aliquots were removed at the indicated times, chilled in the presence of cold methionine and cysteine, and then fractionated into spheroplasts and the medium. Individual fractions were subjected to immunoprecipitation with anti-MBP antiserum, and then analyzed by SDS−PAGE and fluorography (A and B).The amounts of the precursor and mature forms of MBP on fluorograms were densitometrically determined and plotted (C and D). The numbers of methionine residues in proMBP (nine residues) and MBP (six residues) were taken into consideration.

accumulation of unfolded MBP on the surface of the spheroplasts.

Discussion

Recent reconstitution studies revealed that SecE and SecY, as well as SecA, are indispensable components of the protein translocation machinery of *E.coli* (Brundage *et al.*, 1990; Akimaru *et al.*, 1991). These studies, however, failed to demonstrate the involvement of SecD or SecF in the translocation reaction (Matsuyama *et al.*, 1992b). In the present work, we showed that SecD is directly involved in the protein translocation across the cytoplasmic membrane by functioning as a factor for the release of translocated proteins from the membrane. This view is consistent with the facts that SecD is suggested to possess a large periplasmic

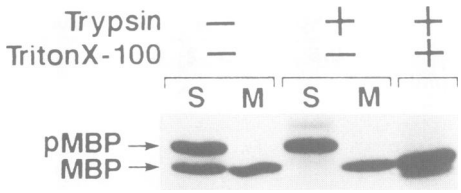

Fig. 5. Localization of the precursor and mature forms of MBP synthesized in anti-SecD IgG-treated spheroplasts. Spheroplasts treated with anti-SecD IgG were labeled as described in the legend to Figure 4. After labeling for 2 min, the culture was chilled in ice water and then treated with or without trypsin at 0°C for 30 min in the presence or absence of Triton X-100, as indicated. After inactivation of the trypsin with trypsin inhibitor, samples containing no Triton X-100 were fractionated into spheroplasts (S) and the medium (M). All fractions were subjected to immunoprecipitation with anti-MBP antiserum, followed by SDS−PAGE and fluorography. The positions of the precursor (pMBP) and mature (MBP) forms of MBP are indicated.

domain (Gardel *et al.*, 1990) and that the *secD* gene has not been identified as a gene responsible for *prl* mutations, which suppress signal peptide defects (Stader et *al.*, 1989; Gardel *et al.*, 1990). The second fact suggests that SecD participates in the translocation reaction after the signal peptide cleavage. Furthermore, Biekey-Brady and Silhavy (1992) very recently showed by means of the Sec titration assay that SecD functions after SecY in the secretory pathway. Although results presented here strongly suggest the involvement of SecD in the release of translocated proteins, the possibility that SecD is involved in the last step of the membrane translocation before the release has not been excluded yet.

An interesting observation in the present work was that the mature form of MBP that accumulated in anti-SecD IgG-treated spheroplasts was sensitive to externally added trypsin, whereas MBP released into the culture medium was resistant to trypsin, as native MBP is (Figure 5). This suggests that MBP thus accumulated exists on the spheroplast surface in an unfolded conformation. It is likely that SecD plays a role in the release of translocated proteins from the membrane, which in turn induces folding of the proteins to the native, trypsin-resistant form. Alternatively, SecD may play a role in the folding of translocated proteins, which in turn results in the release of the protein. The treatment of spheroplasts with anti-SecD IgG resulted in the accumulation of not only mature proteins but also precursor ones (Figures 2 and 4). It seems likely that precursor accumulation was the result of a traffic jam, which was caused by the interference of the release of translocated proteins. An experiment (Figure 2A) showed that the accumulation of ^{35}S-labeled MBP in spheroplasts was insignificant. This may be due to preaccumulation of non-labeled mature proteins, including MBP, since labeling was initiated after a 6 min preincubation.

Consistent with these present data, translocation intermediates of β-lactamase and alkaline phosphatase, which are mature in size and located on the periplasmic side of the cytoplasmic membrane, were more sensitive to trypsin than their active mature forms (Minsky *et al.*, 1986; Kamitani *et al.*, 1992). Recently, two groups independently found a periplasmic protein, called DsbA or PpfA, which catalyzes intramolecular disulfide bond formation in translocated protein molecules (Kamitani *et al.*, 1992; Bardwell *et al.*, 1991). A mutation in the gene encoding it prevented the release of translocated alkaline phosphatase into the periplasmic space (Kamitani *et al.*, 1992). These results indicate that folding, at least to a certain extent, is a prerequisite for the release of a protein from the membrane. The release step has been reported to require a proton motive force ($\Delta\bar{\mu}H^+$)(Geller, 1990; Ueguchi and Ito, 1990). The $\Delta\bar{\mu}H^+$-dependent release also resulted in a conformation change of MBP, which was revealed by the resistance to trypsin. We are uncertain at this moment, however, whether the SecD function is $\Delta\bar{\mu}H^+$-dependent or not.

The effect of anti-SecD IgG on the translocation of MBP was essentially the same as that on the OmpA translocation (Figure 2). Inhibition of secretion by anti-SecD IgG was also observed for other protein species (Figure 1). These findings suggest that SecD is a general releasing factor which plays a role in the translocation of both periplasmic and outer membrane proteins, which in turn suggests that OmpA may be translocated to the outer membrane via the periplasmic space, not the contact site of the two membranes, as suggested previously (Halegoua and Inouye, 1979).

In a previous reconstitution study, we failed to demonstrate the function of SecD (Matsuyama *et al.*, 1992b). We also failed to demonstrate the inhibition of *in vitro* protein translocation by anti-SecD IgG after entrapment of the IgG in everted membrane vesicles (Y.Fujita, S.Matsuyama and S.Mizushima, unpublished). We do not think that these results contradict the present ones. Firstly, the function of SecD can only be directly demonstrated as the release of translocated proteins from the membrane, which cannot be detected by the conventional assay procedure, which involves proteinase K resistance as a translocation criterion. It is difficult to demonstrate the release reaction with these experimental systems. Secondly, the accumulation of a precursor protein is seemingly the result of the stacking of the non-released mature protein. The accumulation may be observed, therefore, only when the secretory machinery is functioning catalytically. It is probable, however, that the translocation exhibited by everted membrane vesicles may not represent a catalytic process, since the number of the secretory machinery, which was estimated from its number per cell (Matsuyama *et al.*, 1992b), and that of presecretory proteins, which was estimated from the data given in (Schiebel *et al.*, 1991), in a conventional reaction mixture are roughly the same.

Materials and methods

Chemicals

Trypsin and soybean trypsin inhibitor were obtained from Funakoshi Co. Ltd. Protein A−agarose and immobilized papain were purchased from Pierce. IgGsorb was from Enzyme Center Inc. Tran^{35}S-label, a mixture of 70% [^{35}S]methionine and 20% [^{35}S]cysteine, 1000 Ci/mmol, was obtained from ICN.

Preparation of antibodies

The anti-SecD antiserum was raised in rabbits against the SecA-C95−SecD fusion protein as described by Matsuyama *et al.* (1992a). The anti-SecA and anti-SecE antisera were raised in rabbits against SecA and SecE intact molecules, respectively. The anti-SecY antiserum was raised against a synthetic peptide corresponding to the Gly198−His216 region of SecY (Nishiyama *et al.*, 1991). IgGs were isolated from these antisera on a protein A−agarose column.

The anti-SecD antiserum, which was raised against the SecA-C95−SecD fusion protein, contained both anti-SecA and anti-SecD IgGs (Matsuyama *et al.*, 1992a). Anti-SecA IgG was removed from the IgG fraction prepared from the antiserum on a SecA-C95-conjugated column as described (Matsuyama *et al.*, 1992a). This preparation, which did not react with SecA, was used as anti-SecD IgG.

Anti-SecD IgG was further purified by means of affinity chromatography on a column in which the SecA-C95−SecD fusion protein was covalently conjugated to activated CH-Sepharose 4B (Pharmacia LKB Biotechnology), as follows. Anti-SecD IgG (8 mg) was applied to a column (0.5 ml) that had been equilibrated with 50 mM potassium phosphate (pH 7.5), 150 mM NaCl. The column was washed with 10 ml of the same buffer. Anti-SecD IgG, which was bound to the column, was eluted with 0.1 M acetic acid. The fraction containing anti-SecD IgG was dialyzed against 50 mM potassium phosphate (pH 7.5) and purified anti-SecD IgG (0.5 mg) was obtained. Fab fragments were prepared on a protein A−agarose column from non-immune and anti-SecD IgGs, which had been digested with immobilized papain according to the method recommended by the manufacturer.

Protein secretion from spheroplasts

E.coli MC4100 (Casadaban, 1976) was grown to 5×10^8 cells/ml in M63 minimal medium (Miller, 1972) supplemented with 10 μg/ml thiamine, 2% glycerol and 20 μg/ml each of all amino acids except methionine and cysteine. Maltose (0.5%) was used to induce the synthesis of MBP. Cells from 10 ml of the culture were harvested by centrifugation and suspended in 10 mM Tris−HCl (pH 7.5)/0.75 M sucrose. After centrifugation, the cells were resuspended in the same buffer and then converted to spheroplasts as described by Osborn *et al.* (1972). The final volume of the spheroplast suspension was 3 ml. Almost all of the cells were converted to spheroplasts, as judged by phase-contrast microscopy.

Labeling with Tran^{35}S-label was carried out as follows. Three hundred microliters of the spheroplast suspension was treated with 1.2 mg of IgGs in 30 μl of 50 mM potassium phosphate (pH 7.5)/0.25 M sucrose on ice for 3 min, and then diluted with 750 μl of the 0.25 M sucrose-containing M63 medium described above. After incubation for 6 min at 30°C, Tran^{35}S-label was added to the radioactivity level of 10−30 μCi/ml for 2 min labeling, followed by a 1 min chase with 12 mM each of non-radioactive methionine and cysteine. This mixture was immediately chilled in ice water, and then centrifuged at 16 000 *g* for 2 min to obtain spheroplasts and the medium. Each fraction was subjected to trichloroacetic acid (TCA) precipitation, followed by SDS−PAGE or immunoprecipitation.

Immunoprecipitation

The TCA precipitate was solubilized in 50 μl of 50 mM Tris−HCl (pH 7.5), 1% SDS, 1 mM EDTA, and then boiled for 3 min. The solution was 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*-amidinophenyl)methanesulfonyl fluoride hydrochloride, and then debris was removed by centrifugation at 16 000 *g* for 5 min. Antiserum (2 μl) against MBP or OmpA was added to the resultant supernatant and the mixture was incubated overnight at 4°C. Fifty microliters of 10% (w/v) IgGsorb was added to each sample and then the mixture was kept at 4°C for 20 min. After centrifugation at 10 000 *g* for 5 min, the pellets were successively washed with 0.5 ml of 50 mM Tris−HCl (pH 7.5), 1 M NaCl, 1% Triton X-100, and 0.5 ml of 50 mM Tris−HCl (pH 7.5), 0.5 M NaCl, 0.05% SDS. The washed pellets were each resuspended in 30 μl of the sample buffer for SDS−PAGE and then boiled for 5 min.

SDS−PAGE

SDS−PAGE was carried out as described by Laemmli (1970). 10% acrylamide, 0.13% *N,N'*-methylenebisacrylamide gels were used for fluorography, and 12.5% acrylamide, 0.33 % *N,N'*-methylenebisacrylamide ones for immunoblot analysis.

Immunoblot analysis

Three hundred microliters of a spheroplast suspension was treated with 1.2 mg of anti-SecE, anti-SecD, anti-SecA or non-immune IgG on ice for 3 min, and then diluted with 750 μl of M63 medium containing 0.25 M sucrose. After incubation at 30°C for 2 min, the spheroplasts were collected by centrifugation at 16 000 *g* for 2 min, and then washed with M63 medium containing 0.25 M sucrose. The washed spheroplasts were solubilized in 50 mM Tris−HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and then passed through a protein A−agarose column. The pass-through fraction was subjected to TCA precipitation, followed by SDS−PAGE. Proteins on SDS gels were transferred to nitrocellulose sheets using a Semidry Transfer Cell (Bio-Rad). Blots were visualized with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as described by Yamada *et al.* (1989).

Trypsin digestion

One microliter of a ^{35}S-labeled culture was treated with 50 μg/ml trypsin in the presence or absence of 1% Triton X-100 at 0°C for 30 min. The soybean trypsin inhibitor was then added to 100 μg/ml, followed by incubation at 0°C for 5 min. Samples treated with trypsin in the presence of Triton X-100 were subjected to TCA precipitation. Samples treated similarly in the absence of Triton X-100 were centrifuged at 16 000 *g* for 2 min to obtain spheroplasts and the medium. Both fractions were treated with 10% TCA and the precipitates formed were subjected to immunoprecipitation as described in the preceding section. The trypsin inhibitor (100 μg/ml) was added together with the antiserum.

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