An alternative protein targeting pathway in *Escherichia coli*: studies on the role of FtsY

Joen Luirink¹, Corinne M.ten Hagen-Jongman, Coen C.van der Weijden, Bauke Oudega, Stephen High², Bernhard Dobberstein³ and Ron Kusters

¹Department of Microbiology, Institute of Molecular Biological Sciences, Biocentrum Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands, ²Department of Biochemistry, School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK and ³Zentrum für Molekulare Biologie Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, Germany

Communicated by B.Dobberstein

In *Escherichia coli*, a signal recognition particle (SRP) has been identified which binds specifically to the signal sequence of presecretory proteins and which appears to be essential for efficient translocation of a subset of proteins. In this study we have investigated the function of *E.coli* FtsY which shares sequence similarity with the α-subunit of the eukaryotic SRP receptor (‘docking protein’) in the membrane of the endoplasmic reticulum. A strain was constructed which allows the conditional expression of FtsY. Depletion of FtsY is shown to cause the accumulation of the precursor form of β-lactamase, OmpF and ribose binding protein in vivo, whereas the processing of various other presecretory proteins is unaffected. Furthermore, FtsY-depleted inverted cytoplasmic membrane vesicles are shown to be defective in the translocation of pre-β-lactamase using an in vitro import assay. Subcellular localization studies revealed that FtsY is located in part at the cytoplasmic membrane with which it seems peripherally associated. These observations suggest that FtsY is the functional *E.coli* homolog of the mammalian SRP receptor.

Key words: *Escherichia coli*/FtsY/protein targeting/signal recognition particle

Introduction

The targeting and translocation of proteins into and across the membranes of the endoplasmic reticulum (ER) in eukaryotes and the cytoplasmic membrane of prokaryotes occur by virtue of a hydrophobic N-terminal signal sequence. The structural and functional conservation of the signal sequence of eukaryotic and prokaryotic proteins suggests that the basic mechanisms of membrane targeting and translocation may be similar in both cases (Hartl and Wiedmann, 1993; High and Stirling, 1993; Luirink and Dobberstein, 1994).

In *Escherichia coli* several factors have been identified, by both genetic and biochemical means, which cooperate in the so-called general secretory pathway (for a review see Pugsley, 1993). In this pathway cytosolic molecular chaperones like SecB function to maintain the translocation competence of preproteins in the cytosol and to target them to the cytoplasmic membrane where a complex machinery consisting of SecA, SecD-F and SecY assists in membrane insertion and translocation.

In mammalian cells, targeting of most proteins to the ER membrane is mediated by the signal recognition particle (SRP) which consists of one RNA molecule (SRP7S RNA) and six polypeptides of 9, 14, 19, 54, 68 and 72 kDa (for a review, see Rapoport, 1992). The SRP binds via its 54 kDa subunit (SRP54) to the signal sequence of nascent presecretory proteins, thereby lowering their rate of translation. The complex of the ribosome, nascent chain and SRP is then targeted to the ER membrane by interaction with the docking protein complex. The SRP is released from the membrane-bound ribosome—nascent chain complex in a GTP-dependent manner and the translation arrest is relieved. The remaining ribosome—nascent chain complex associates with a complex of membrane proteins, the translocon, which catalyzes membrane insertion and translocation of the nascent chain. Thus, the SRP functions both as a cytosolic chaperone preventing premature folding of the preprotein by coupling translation to translocation and as a ‘pilot’ to guide the preprotein to the SRP receptor complex in the membrane.

Genetic and biochemical evidence indicates that SRP-mediated targeting may also occur in *E.coli* (for reviews see Hartl and Wiedmann, 1993; Luirink and Dobberstein, 1994), *Bacillus subtilis* (Honda et al., 1993) and *Saccharomyces cerevisiae* (Hann and Walter, 1991; Ogg et al., 1992). In *E.coli*, an SRP-like complex was identified which consists of one protein (P48 or Ffh) and one RNA molecule (4.5S RNA) that are homologous to the SRP54 and SRP7S RNA constituents of the eukaryotic SRP, respectively (Poritz et al., 1990; Ribes et al., 1990). Depletion of either the RNA or the protein component of the *E.coli* SRP affects the export of several secretory proteins (Ribes et al., 1990; Phillips and Silhavy, 1992). Moreover, P48 interacts specifically with the signal sequence of nascent presecretory proteins as was shown by photocross-linking in a crude *E.coli* cell lysate (Luirink et al., 1992) and with the use of a reconstituted chimeric SRP (Bernstein et al., 1993).

The role of the *E.coli* SRP in protein secretion is not known. Thus, the SRP may support co-translational translocation in a separate secretory pathway or may form part of the general secretory pathway. If the SRP functions in a separate targeting pathway, one would expect a membrane receptor for the SRP to exist. In this respect it is of interest that the C-terminal region of the *E.coli* protein FtsY displays striking sequence similarity with the α-subunit of the canine docking protein, leading to the hypothesis that FtsY may function as a membrane-bound receptor for the *E.coli* SRP (Bernstein et al., 1989; Römisch et al., 1989). Originally, FtsY has been implicated in cell division because its gene is located in an operon together with *ftsE* and *ftsX*, in which temperature sensitive mutations have been identified
Results

Overexpression and purification of FtsY

To examine the effects of overexpression of FtsY and to facilitate its purification, the gene encoding FtsY was subcloned into the expression vector pET9 under control of the T7 promoter. For expression, the resulting construct (pET9-FtsY) was transferred to E.coli BL21(DE3) which contains a chromosomal copy of the T7 polymerase gene under control of the lac promoter/operator. As shown in Figure 1A (lanes 7 and 8), cells harboring pET9-FtsY expressed a polypeptide which migrates during SDS–PAGE as a characteristically 'bulged' band at 92 kDa. Gill and Salmond have previously shown that FtsY migrates as a 92 kDa polypeptide although the molecular weight of FtsY as deduced from the DNA sequence is 54 kDa (Gill and Salmond, 1990). The band was positively identified as FtsY by means of immunoblotting using an antiserum raised against a synthetic C-terminal peptide of FtsY (Figure 1B, lanes 7 and 8).

pET9-FtsY was difficult to maintain stably in BL21(DE3), probably due to the detrimental effect of uninduced FtsY expression (Figure 1A and B, lane 7). To reduce the basal expression of FtsY, the compatible plasmids pLysE and pLysS were introduced which encode T7 lysozyme, an inhibitor of T7 RNA polymerase activity (Studier et al., 1990). Both pLysE and pLysS, which differ in the degree of T7 lysozyme expression, were able to stabilize pET9-FtsY in BL21(DE3) and reduce both induced and non-induced expression of FtsY (Figure 1A and B, lanes 3–6).

The FtsY protein was purified by anion exchange chromatography and gel filtration. At different stages in the purification procedure, samples were taken and analyzed by SDS–PAGE (Figure 1C). As shown in lane 5, a second protein co-purified with the FtsY protein. To identify this protein and to confirm the identity of FtsY, the N-terminal amino acid sequence of both proteins was determined (data not shown). The five N-terminal amino acid residues of the upper band were identical to the predicted sequence of FtsY. The lower band appeared to represent FtsY missing 14 N-terminal amino acid residues, probably as a result of proteolytic cleavage occurring during the purification. The overall purity of both bands together was estimated to be >95%. Remarkably, FtsY elutes from the gel filtration column as a single peak with an apparent molecular weight of >200 kDa. Gel filtration in the presence of 8 M urea did not change the elution profile, suggesting that the unexpected apparent molecular weight is not due to oligomerization. The reason for the aberrant mobility of FtsY in both gel filtration and SDS–PAGE is unknown.

Effects of FtsY overexpression

Induction of FtsY expression by growth of BL21(DE3) harboring pET9-FtsY and pLysE in the presence of IPTG had a negative effect on cell growth and caused cell filamentation only at late time points when cell growth was already affected. Strong overexpression of FtsY by growth of BL21(DE3) harboring pET9-FtsY in the presence of IPTG led to inclusion body formation (data not shown).

To examine the effects of FtsY overexpression on protein export, the accumulation of precursor forms of OmpA and β-lactamase was monitored in vivo by immunoblotting. IPTG was added to BL21(DE3) harboring pET9-FtsY and

that cause cell filamentation at the non-permissive temperature (Gill and Salmond, 1986). However, no fts mutations have been mapped in ftsY despite localized mutagenesis of the ftsYEX gene cluster (Gibbs et al., 1992). In this study, we demonstrate that FtsY is in part located at the cytoplasmic membrane and that depletion and overexpression of FtsY affects both cell morphology and protein export.
pACYC177ΔHaeII (encoding β-lactamase, see Materials and methods) at the early log phase of growth. Samples were taken at various time points after induction and analyzed by immunoblotting to determine the extent of FtsY induction. The level of FtsY was drastically increased 1 h after induction and remained very high throughout the induction period (Figure 2, upper panel, lanes 9–12). Intermediate and low (wild-type) expression was observed in the absence of inducer and in the absence of PET9-FtsY respectively (Figure 2, upper panel, lanes 1–8). In addition, OmpA and β-lactamase were identified in the samples by immunoblotting. A strong accumulation of pre-β-lactamase was observed from 1 h after induction of FtsY (Figure 2, middle panel, lanes 9–12). Even the intermediate uninduced FtsY expression resulted in the appearance of trace pre-β-lactamase (Figure 2, middle panel, lanes 5–8). In contrast, no pre-OmpA could be identified even after prolonged overexpression of FtsY (Figure 2, lower panel). These observations are indicative of a rather specific effect of FtsY overexpression on protein export resembling the effects of overexpression of P48 (Ribes et al., 1990) and depletion of 4.5S RNA (Poritz et al., 1990; Ribes et al., 1990), the constituents of the E. coli SRP.

Construction of a mutant with conditional FtsY expression

To study the role of FtsY in more detail, a mutant E. coli strain was constructed in which the expression of FtsY is under the regulation of the araB promoter and operator. The construction is shown schematically in Figure 3A and described in detail in Materials and methods. E. coli N4156, deficient in DNA polymerase I, was transformed to ampicillin resistance with pAra14-FtsY′ which contained the 5′ end of FtsY downstream from the araB promoter/operator region. Since pAra14-FtsY′ contains a ColEl type replicon which is unable to replicate in polA strains, transformants can only be obtained when pAra14-FtsY′ integrates into the chromosome. Integration will take place into the homologous chromosomal ftsY gene thereby disrupting the ftsY operon and placing a complete ftsY copy under control of the araB promoter/operator. Correct integration was confirmed by Southern blotting of HindII- or XmnI-digested chromosomal DNA extracted from integrants using the cloned 5′ end of ftsY as a hybridization probe (data not shown).

Fig. 2. Processing of presecretory proteins in cells overexpressing FtsY. Strain BL21(DE3) carrying either pACYC177ΔHaeII (lanes 1–4) or a combination of pACYC177ΔHaeII and PET9-FtsY (lanes 5–12) was grown in YT with 0.4 mM IPTG (lanes 1–4 and 9–12) or without induction (lanes 5–8). Samples were taken 1 h (lanes 1, 5 and 9), 2 h (lanes 2, 6 and 10), 3 h (lanes 3, 7 and 11) and 4 h (lanes 4, 8 and 12) after induction and analyzed by immunoblotting using antiserum against FtsY, β-lactamase (Bla) and OmpA as indicated at the left side of the blot panels. The positions of the precursor and mature forms of the secretory proteins are marked with 'P' and 'M' respectively, at the right side of the blot panels. The position of the precursor form of OmpA was identified in a SecY(Ts) strain grown at the non-permissive temperature (not shown).

Fig. 3. Conditional expression of FtsY. (A) Construction of strain N4156::pAra14-FtsY′ by integration of pAra14-FtsY′ into the ftsYEX gene cluster of the E. coli N4156 chromosome resulting in pAra156 controlled expression of the ftsY gene. P, promoter; Bla, gene encoding β-lactamase. (B) Growth curves of N4156::pAra14-FtsY′. Cells were grown overnight in YT supplemented with 0.4% fructose and 0.2% L-arabinose, collected by centrifugation, washed once in YT and used to inoculate YT containing 0.4% fructose only (■) or a combination of 0.4% fructose and 0.2% L-arabinose (□) at 0 h. (C) Extent of FtsY depletion. At the indicated time points after the shift to medium supplemented with or without L-arabinose (see under B), samples were taken and analyzed by immunoblotting using antiserum against FtsY.
proteins DnaK and GroEL was observed by immunoblotting (data not shown).

If FtsY functions like the yeast and mammalian docking protein in targeting of presecretory proteins to the cytoplasmic membrane, one would expect this process to be disrupted upon depletion of FtsY (Ogg et al., 1992). To test this notion, the accumulation of various presecretory proteins was monitored by immunoblotting upon depletion of FtsY in N4156::pAra14-FtsY' (Figure 5A). A strong accumulation of pre-β-lactamase is observed which is apparent as soon as 2 h after the shift to arabinose-free medium. In addition, precursor forms of OmpF and ribose binding protein (RBP) can be detected from 2 and 3 h after the start of depletion respectively. The weak accumulation of pre-OmpF (and pre-β-lactamase in longer exposures, not shown), observed in the arabinose-supplemented cells, might be due to a slight overproduction of FtsY (see also Figure 2). The processing of pre-OmpA, pre-OmpC and pre-MBP (maltose binding protein) seemed unaffected by depletion of FtsY.

If the accumulation of preproteins in FtsY-depleted cells is caused by a targeting defect one would expect them to remain untranslocated. To test this hypothesis, the accessibility of pre-β-lactamase for protease K was tested in N4156::pAra14-FtsY' cells which had been depleted for FtsY. The cells were treated with Mg^{2+}, EDTA or EDTA + Triton X-100, incubated in the presence or absence of protease K and subjected to SDS–PAGE and immunoblot analysis (Figure 5B). EDTA permeabilizes the outer membrane which allows access of the protease to the periplasm. Pre-β-lactamase was not degraded by protease K in cells treated with EDTA (Figure 5B, lane 4) consistent with the expectation that this form is inside the spheroplast. Under these conditions, OmpA (which has a protease-sensitive periplasmic domain) was degraded (Figure 5B, lane 8) leaving a protected outer membrane embedded domain of ~20 kDa as expected (Schweizer et al., 1978), indicating that protease K had access to the periplasm of the spheroplasted cells. In the lysed (Trition-treated) spheroplasts, pre-β-lactamase was degraded (Figure 5B, lane 6) confirming that this form is not intrinsically resistant to protease K digestion in contrast to mature β-lactamase (Minsky et al., 1986). In the absence of protease K some pre-β-lactamase was degraded in the lysed spheroplasts (Figure 5B, lane 5), possibly by endogenous E.coli proteases.

In order to obtain direct insight into the involvement of FtsY in protein targeting, an in vitro translocation assay was applied. Translation of pre-β-lactamase was carried out in an FtsY-depleted cell-free extract. Five minutes after the start of translation, inverted cytoplasmic membrane vesicles (IMVs) with either a wild-type level of FtsY (Figure 5C, lane 1) or with an undetectable level of FtsY (Figure 5C, lane 2–7) were added. Simultaneously, the reaction mixture was supplemented with purified FtsY to a final concentration ranging from 0 to 0.8 μM (Figure 5C, lanes 1–7). After protease K treatment, the amount of protected protein was determined. As can be seen in Figure 5C (lanes 2–7), translocation almost doubled upon addition of FtsY. Optimal translocation efficiency was reached at 0.2 μM FtsY. This concentration is within the range in which SecA is effective in stimulating in vitro translocation (Kusters et al., 1989). These results indicate that FtsY directly stimulates

**Effects of FtsY depletion**

N4156::pAra14-FtsY' is unable to form colonies in the absence of the inducer arabinose (not shown) indicating that FtsY is essential for cell viability.

The effects of FtsY depletion on cell growth in liquid medium are shown in Figure 3B. When strain N4156::pAra14-FtsY', pregrown in YT in the presence of arabinose, was shifted to YT lacking arabinose, the optical density of the culture lagged that of arabinose-supplemented cultures indicating impaired cell growth. In order to evaluate the degree of FtsY expression upon removal of the inducer arabinose, samples were taken at various times after the shift and analyzed by immunoblotting (Figure 3C). A strong reduction in the amount of FtsY was detected as soon as 2 h after the shift, indicating efficient depletion of the culture for FtsY.

The morphology of FtsY-depleted cells was examined by phase contrast and fluorescence microscopy after nucleoid staining of cells fixed with OsO₄. As shown in Figure 4, cells depleted for FtsY form short filaments of swollen cells with large spherical bulges unevenly distributed along the filaments. This characteristic phenotype is visible from 3 h after the shift to arabinose-free medium. In the bulging cells the nucleoids appear to spread out. Concomitant with the change in phenotype, a mild induction of the heat shock

![Fig. 4. Fluorescence micrographs (1600×) of FtsY-depleted (A) or 'wild-type' (B) cells. Strain N4156::pAra14-FtsY' was depleted of FtsY as described in the legend to Figure 3B. Micrographs were taken 5 h after the shift to medium supplemented with α-arabinose (B) or not supplemented (A).](image)
translocation. The decrease in translocation observed above the optimal FtsY concentration may be due to non-productive interactions of FtsY with the preprotein or components of the translocation apparatus, like the SRP. In vivo, overexpression of FtsY also provokes the specific accumulation of pre-β-lactamase (see above).

Subcellular localization of FtsY
Strain BL21(DE3) expressing FtsY at wild-type or elevated levels was subjected to subcellular fractionation using immunoblotting to identify FtsY in the fractions. As shown in Figure 6A, FtsY expressed in wild-type amounts is found in both the soluble and cytoplasmic membrane fraction. In cells overexpressing FtsY (Figure 6B) relatively more FtsY is found in the soluble fraction which might suggest that the number of membrane binding sites for FtsY is limited.

The cellular distribution of FtsY was also examined by immunoelectron microscopy using affinity purified anti-FtsY antiserum and colloidal gold-labeled second antibody on ultrathin cryosections. Unfortunately, we were unable to detect FtsY expressed at wild-type levels. In cells overexpressing FtsY, the gold particles were primarily located in the inner part of the cell envelope corresponding to the location of the cytoplasmic membrane (Figure 6C). The gold particles were more or less randomly distributed both along the length and the poles of the cells without any visible concentration at constriction sites (not shown).

The apparent discrepancy between the localization of overproduced FtsY by fractionation versus immunoelectron microscopy might be caused by a release of membrane-associated FtsY into the soluble fraction during disruption of the cells prior to the fractionation procedure. Alternatively, cytoplasmic FtsY might have a conformation in ultrathin cryosections which is poorly recognized by the anti-FtsY antiserum.

To examine the nature of the association of FtsY with the cytoplasmic membrane, inverted cytoplasmic membrane vesicles derived from BL21(DE3) expressing FtsY at wild-type levels were extracted with 1 M NaCl, 4 M urea or 0.2 M Na₂CO₃ to remove peripherally associated proteins. These extraction procedures solubilized most but not all of the membrane-associated FtsY (Figure 6D). Under these conditions all SecY which is an integral inner membrane...
I and soluble immunoblotting 2294 purified anti-FtsY. pET9-FtsY extracted induction. Sections of microscopy D, immunoblotting using nm induction. Fractions overexpression). (FtsY carrying Fig. 6. Sucellular localization of FtsY. (A) Fractionation of strain BL21(DE3) carrying pET9 and pLysE (wild-type FtsY expression). (B) Fractionation of strain BL21(DE3) carrying pET9-FtsY and pLysE (FtsY overexpression). Cells were grown in YT to an absorbance at 660 nm of 0.3, induced with 0.4 mM IPTG and collected 2 h after induction. Fractions derived from 0.25 OD600 units were analyzed by immunoblotting using an antiserum against FtsY. T, total cell lysate; D, cellular debris (pellet of low speed centrifugation after cell lysis); S, soluble fraction; M, total membrane fraction; OM, outer membrane fraction; IM, cytoplasmic membrane fraction. (C) Immunoelectron microscopy of cells overexpressing FtsY. Strain BL21(DE3) carrying pET9-FtsY and pLysE was grown in YT to an absorbance at 660 nm of 0.3, induced with 0.4 mM IPTG and collected 30 min after induction. Sections of these cells were immunolabeled using affinity purified anti-FtsY. (D) Solubilization of peripheral inner membrane proteins. IMVs of strain N4136 (wild-type level of FtsY expression) were extracted with 1 M NaCl (lanes 1 and 2), 4 M urea (lanes 3 and 4) and 0.2 M Na2CO3 (lanes 5 and 6). Insoluble (lanes 1, 3 and 5) and soluble (lanes 2, 4 and 6) protein fractions were analyzed by immunoblotting using an antiserum against FtsY.

protein was recovered in the insoluble (membrane) fractions (not shown).

Taken together these studies suggest that FtsY is located in part at the cytoplasmic membrane to which most FtsY seems peripherally bound.

Discussion

E. coli FtsY has been implicated both in cell division and in protein export. A role in cell division was proposed based on the location of ftsY in a locus which affects cell division (Gill and Salmond, 1986; see below). On the other hand, the striking similarity between the C-terminal regions of FtsY and the α-subunit of the mammalian SRP receptor argues in favor of a role for FtsY in the docking and subsequent release of the recently identified E. coli SRP (Bernstein et al., 1989; Römisch et al., 1989).

To examine the function of FtsY, strains were constructed in which the intracellular level of FtsY can be varied. Both depletion and overexpression of FtsY induced the accumulation of precursor forms of several secreted proteins in vivo, indicative of impaired protein translocation across the cytoplasmic membrane. Precursor accumulation upon FtsY depletion, appeared to be specific for β-lactamase, OmpF and RBP and was already apparent after 2–3 h of depletion, suggesting that it is not an indirect effect of impaired cell growth or heat shock induction which are only apparent at later time points. Pre-β-lactamase also accumulated upon overproduction of FtsY which could be due to non-productive interactions between FtsY and the E. coli SRP. Furthermore, β-lactamase was translocated with reduced efficiency in an in vitro translocation assay in the absence of FtsY. Replenishment of cytoplasmic membrane vesicles depleted of FtsY with purified FtsY partially restored the translocation defect in a concentration dependent fashion, giving strong support to the notion that FtsY has a function in protein translocation.

Depletion of FtsY also affected cell morphology. The ‘bulging’ cell shape observed is similar to that reported for double mutants with defects in both cell division [ftsA(Ts), ftsQ(Ts) or ftsI(Ts)] and cell elongation systems [rodA(Ts) or phbA(Ts)] (Begg and Donachie, 1985). It is conceivable that depletion of FtsY indirectly affects cell shape by having an effect on the insertion of proteins involved in cell division and elongation into the cytoplasmic membrane.

In both ftsE and ftsX, which are located in the ftsYEX gene cluster, temperature sensitive mutations have been mapped which cause filamentation at the non-permissive temperature (Gill and Salmond, 1986; Gibbs et al., 1992). However, the classification of ftsE as a cell division gene is debatable since the ftsE(Ts) mutant forms filaments at the non-permissive temperature only in rich medium (Taschner et al., 1988). Furthermore, filamentation is not exclusively correlated with cell division defects and is also observed upon aberrant expression of factors involved in protein export like SecA (Oliver and Beckwith, 1981) and the constituents of the E. coli SRP, P48 (Phillips and Silhavy, 1992) and 4.5S RNA (Poritz et al., 1990; Ribes et al., 1990). Interestingly, FtsE was shown to be homologous with the ATP binding cassette family, a group of prokaryotic and eukaryotic nucleotide binding proteins that are involved in a variety of transport processes (Higgins et al., 1990). It is tempting to speculate that FtsY and FtsE (and perhaps FtsX), which are all
cytoplasmic membrane proteins, cooperate in the reception and insertion of a subset of proteins at the cytoplasmic membrane. However, we did not observe any accumulation of pre-β-lactamase in an ptsE(Ts) mutant grown at the non-permissive temperature (data not shown). Thus, elucidation of the function of FtsE awaits further analysis.

Another candidate for performing a role in the insertion of proteins into the cytoplasmic membrane is FtsH which is also a membrane-associated putative ATPase (Tomoyasu et al., 1993). Thermosensitive filamentation of a ftsH mutant was shown to be correlated with a decrease in the insertion of PBP3 into the cytoplasmic membrane (Ferreira et al., 1987). It would be interesting to gain knowledge about the effects of this mutation on the insertion and secretion of other proteins.

FtsY appeared to be located in part in the cytoplasmic membrane which is in agreement with localization studies by Gill and Salmon (1987) using a maxicell expression system. Inspection of the FtsY sequence reveals no obvious membrane spanning segments (Gill and Salmon, 1990). It is conceivable that FtsY interacts with other membrane components like FtsE, FtsX or perhaps an as yet unidentified E. coli homolog of the β-subunit of the mammalian SRP receptor. This might explain the limited number of association sites in the membrane to which most FtsY seems loosely bound. We cannot, however, exclude the possibility of a direct interaction of FtsY with phospholipids as was also observed for the overall negatively charged SecA protein (Breukink et al., 1992).

In conclusion, our observations support the hypothesis that FtsY is involved in an alternative pathway of protein targeting in E. coli, presumably as a cognate receptor for the SRP at the cytoplasmic membrane. The fact that the constituents of the SRP, P48 and 4.5S RNA, and FtsY are all essential in E. coli suggests that this pathway may be crucial for correct targeting of a subset of proteins. In this respect it is interesting that proteins that do not depend on SecB for efficient export, like β-lactamase and RBP, were most strongly affected by depletion of P48 (Phillips and Silhavy, 1992), suggesting different ways of chaperoning presecretory proteins in the cytoplasm. Depletion of FtsY seems to bring about a secretion defect of similar specificity.

A notable exception is pre-OmpF which on the one hand binds SecB (Kumamoto and Francetic, 1993) but on the other hand accumulates in FtsY-depleted cells. However, the effect of depletion of P48 on the processing of pre-OmpF has not yet been reported (Phillips and Silhavy, 1992).

In analogy with the mammalian docking protein, FtsY may also play a role in the co-translational insertion of inner membrane proteins which would prevent the cytoplasmic exposure of hydrophobic regions in nascent polypeptides. We intend to investigate this possibility with special emphasis on inner membrane proteins involved in cell division. In addition, future studies will concentrate on the interaction of the SRP and FtsY and on the possible interplay between components of targeting pathways in E. coli.

Materials and methods

**Strains, plasmids and media**

*E. coli* HMS 174 (F- hsdR recA Rif') and BL21 (F- hsdS gal) (DE3) were used for initial subcloning and for expression of ftsY respectively (Studier et al., 1990). Strain N4156 (pOA end thy gyrA) was used for the construction of a strain with conditional expression of ftsY (Gellert et al., 1977). Strain LMC515 [ftsE18(Ts)zgh-1::Tn10] was used to study the effects of inactivating FtsE (Taschner et al., 1988). Strain MM542, a derivative of MC4100 (F- ΔlacU169 araD135 rpsL thi rrel) carrying a secA515 mutation (Oiler and Beckwith, 1981), was used for the identification of precursor forms of some presecretory proteins. Strain JM109 was used in routine cloning procedures (Sambrook et al., 1989).

Plasmids pET9a, pLysE, pLysS (Studier et al., 1990), pAra14 (Cagnon et al., 1991) and pDBI (Gill and Salmon, 1990) were used for subcloning and controlled expression of ftsY. To study the effect of FtsY overexpression on the translocation of β-lactamase, pACYC177HaelII was constructed by deletion of the 1.4 kb HaeII fragment from pACYC177 (Chang and Cohen, 1978) which is compatible with pET9a derivatives.

To facilitate in vitro expression, the gene encoding β-lactamase was subcloned in pET9a-adt, a derivative of pET9a which contains an extended multiple cloning site (MCS) downstream from the T7 promoter. Plasmid pAL2 carrying the β-lactamase gene (Lamminen and Plückthun, 1990) was cut with AlwNI and treated with DNA polymerase I to create blunt ends. Subsequently, the plasmid was cut with NdeI and the resulting small NdeI-AlwNI fragment encompassing the complete β-lactamase gene was ligated into pET9a-adt treated with NdeI and SalI which both cut in the MCS.

Cells were routinely grown in M9 or in YT medium supplemented with 0.4% glucose. N4156 derivatives were grown in M9 or YT medium supplemented with 0.4% fructose and 0.2% L-arabinose when indicated. If required, antibiotics were added to the culture medium (Sambrook et al., 1989).

**General methods**

Recombinant DNA techniques were carried out as described by Sambrook et al. (1989). A digoxigenin labeling and detection kit (Boehringer) was used to probe Southern blots. DNA sequencing was performed using the Taq Dye Primer Cycle Sequencing Kit and the 373A Automated DNA Sequencer of Applied Biosystems.

Protein was determined according to Bradford (1976) with bovine serum albumin as standard. SDS-PAGE and immunoblotting were carried out as described by Bollag and Edelstein (1991). Bound antibodies were visualized on immunoblots by enhanced chemiluminescence (Amersham).

**Overexpression and conditional expression of FtsY**

The T7 expression system was used for high level expression of FtsY (Studier et al., 1990). The ftsY gene was subcloned from pDB1, a pBR322 derivative which contains the complete ftsY.E. operon. An NdeI restriction site was created at the starting ATG of ftsY by site directed mutagenesis in M13mp19 and the resulting NdeI fragment encompassing the complete ftsY was cloned into the expression vector pET9a. The resulting plasmid was designated pET9-FtsY.

A strain which allows the conditional expression of ftsY was created by transforming N4156 to ampicillin resistance with pAra14-FsY" which carries the 5' end of ftsY under control of the araBAD promoter—operator complex. In order to construct pAra14-FsY" the first 495 bp of the FtsY coding sequence were amplified by PCR using pET9-FtsY as a template. Primers were designed to introduce Ncol and HindIII restriction sites at the 5' and 3' ends respectively. The fragment was sequenced and cloned between the Ncol and HindIII sites of pAra14 resulting in pAra14-FsY".

**Purification of FtsY**

FtsY was purified from overproducing cells. Strain BL21(DE3) carrying pET9-FtsY and pLysE was grown in 1 l of YT medium to an optical density at 600 nm of 0.4 and induced for FtsY expression by the addition of 0.4 mM IPTG. After 2 h of induction, the cells were harvested, resuspended in 10 ml of 50 mM Tris—HCl (pH 7.5) and 10% glycerol (buffer A), frozen in liquid nitrogen and stored at −80°C. The cell suspension was thawed and passed twice through a French pressure cell at 8000 p.s.i. Cell debris and membranes were removed in two centrifugation steps (5 min at 15 000 g followed by 30 min at 165 000 g). The supernatant was applied twice to a FPLC MonoQ ion exchange column (Pharmacia) and eluted with a non-linear gradient of NaCl in buffer A. FtsY eluted at 390 mM NaCl and was further purified by gel filtration using a Superose-12 column (Pharmacia). The main peak was recovered and analyzed.

**In vitro translation**

The in vitro transcription, translation and translocation reactions were carried out basically as described (De Vriege et al., 1987). T7 polymerase (Boehringer) was used for transcription of plasmid pET9-PAL2. The S-135 extract used for translation was prepared from N4156:pAra14-FtsY" grown in the absence of arabinose for 3 h which reduced the amount of FtsY to undetectable levels. The same strain and conditions were used for the isolation of inverted cytoplasmic membrane vesicles (IMVs) depleted of FtsY. IMVs
with wild-type levels of FtsY were isolated from strain N4156 grown to the same optical density at 660 nm.

The translocation reaction was initiated 5 min after the start of translation by the addition of IMVs (~0.4 mg protein/ml). After 20 min of incubation at 37°C, proteinase K was added (200 μg/ml) to degrade all non-translocated proteins. This reaction was carried out for 15 min at 37°C and stopped by the addition of trichloroacetic acid to a final concentration of 20% (w/v). After precipitation, the samples were analyzed by SDS–PAGE and fluorography. Radiolabeled proteins were quantified by liquid scintillation counting of excited bands. The efficiency of translocation is defined as the amount of protected protein (precursor and mature β-lactamase) relative to the amount of synthesized protein (determined by omitting proteinase K treatment in part of each sample). The maximum value in each experiment was taken as 100%.

**Protease accessibility of pre-β-lactamase**

The accessibility of pre-β-lactamase for proteinase K was tested in untreated, spheroplasted or lysed cells essentially as described by Bosch et al. (1989).

**Antibodies**

The polyclonal anti-FtsY antiserum 790 was raised in rabbit against a peptide which consists of the 17 C-terminal amino acid residues of FtsY. Cross-reactivity was verified by immunoblotting in the presence or absence of competition from the peptide against which the antiserum was raised. For immunoelectron microscopy the antiserum was affinity purified on nitrocellulose-bound FtsY.

Anti-β-lactamase antiserum was obtained from 5 Prime Inc.

**Subcellular localization of FtsY**

Subcellular fractions of cells were prepared essentially as described (Lugtenberg et al., 1975). Cells were lysed by freezing and thawing combined with short ultrasonic treatment. The cell debris was removed from the lysate by sedimentation. Cell envelopes were separated from the soluble fraction (containing cytoplasmic and periplasmic proteins) by ultracentrifugation. Cytoplasmic membrane proteins were separated from outer membrane proteins by selective solubilization in sodium lauryl sarcosinate (Sarkosyl) (Chopra and Shales, 1980). Under these conditions marker proteins for the outer and cytoplasmic membrane fractions localized correctly.

Peripheral bound cytoplasmic membrane proteins were extracted from translocation competent cytoplasmic membrane vesicles with 1 M NaCl, 4 M urea and 0.2 M Na2CO3 as described complexes (Cabello et al., 1991).

**Immunocytochemical localization with gold complexes on ultrathin cryosections of E.coli cells was carried out with affinity purified anti-FtsY antiserum and 10 nm gold—protein A complexes essentially as described previously (Van Putten et al., 1988).**

**Microscopic techniques**

Cell fixation, nucleoid staining, phase contrast and fluorescence microscopy were carried out as described (Mulder and Woldringh, 1989).

**Acknowledgements**

We thank J.Voskuil and C.Woldringh for help in morphological studies, F.Steghuis for technical assistance, H.de Cock, M.Hoffmang and L.Randall for antisera and J.-M.van Dijl, G.Salmond and C.Cagnon for plasmids and strains.

**References**


