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

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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
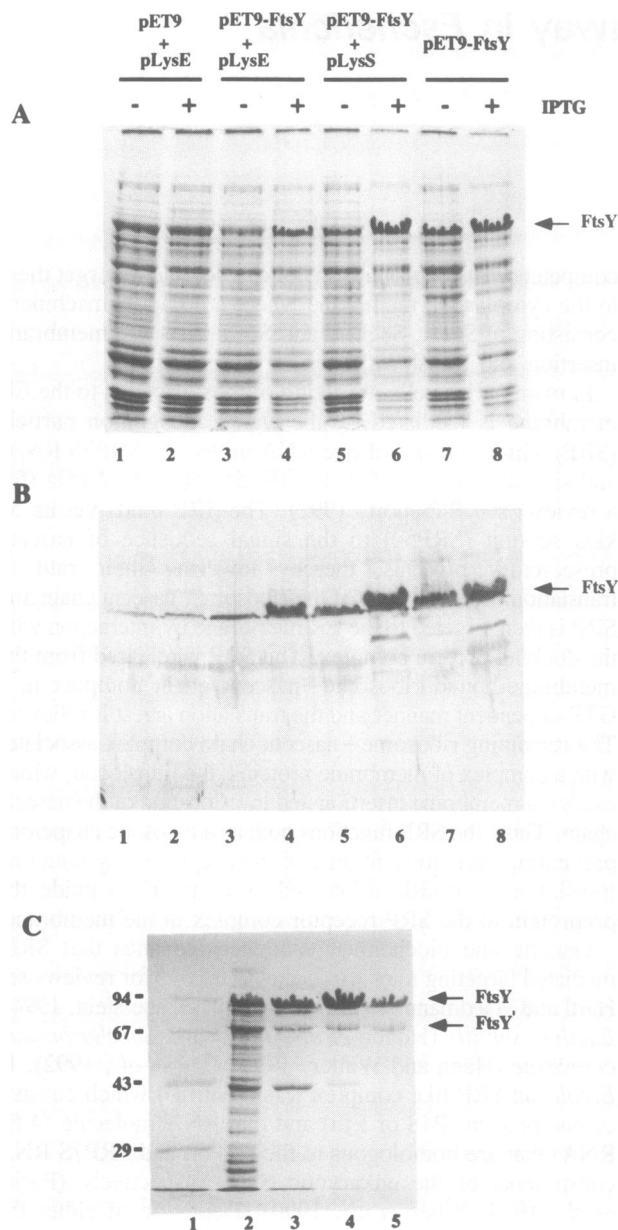
Fig. 1. Overexpression and purification of FtsY. BL21(DE3) cells carrying the indicated plasmids were grown for 1 h in YT with 0.4 mM IPTG or without induction as indicated. Proteins were identified by SDS–PAGE followed by Coomassie R-250 staining (A) and immunoblot analysis using antiserum against FtsY (B). (C) Purification of FtsY. Samples taken at different stages of the purification process were analyzed by SDS–PAGE and Coomassie R-250 staining. Lane 1, molecular weight markers; lane 2, total cell lysate after induction of FtsY; lane 3, peak fraction after the first run over MonoQ Sepharose; lane 4, peak fraction after the second run over MonoQ Sepharose; lane 5, final product, peak fraction after gel filtration using Superose-12.

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.

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
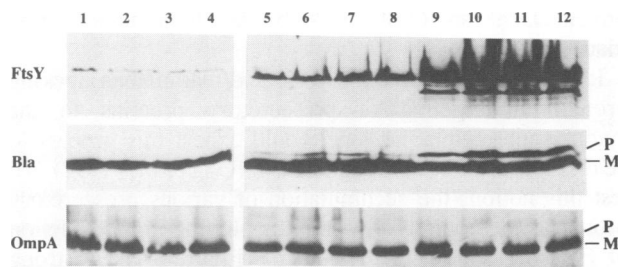
Fig. 2. Processing of presecretory proteins in cells overexpressing FtsY. Strain BL21(DE3) carrying either pACYC177ΔHaeII (lanes 1–4) or a combination of pACYC177ΔHaeII and pET9-FtsY (lanes 5–12) was grown in YT with 0.4 mM IPTG (lanes 1–4 and 9–12) or without induction (lanes 5–8). Samples were taken 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 antisera 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 *SecA*(Ts) strain grown at the non-permissive temperature (not shown).

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 ColE1 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 *ftsYEX* operon and placing a complete *ftsY* copy under control of the *araB*
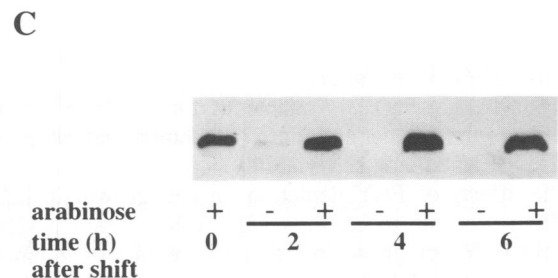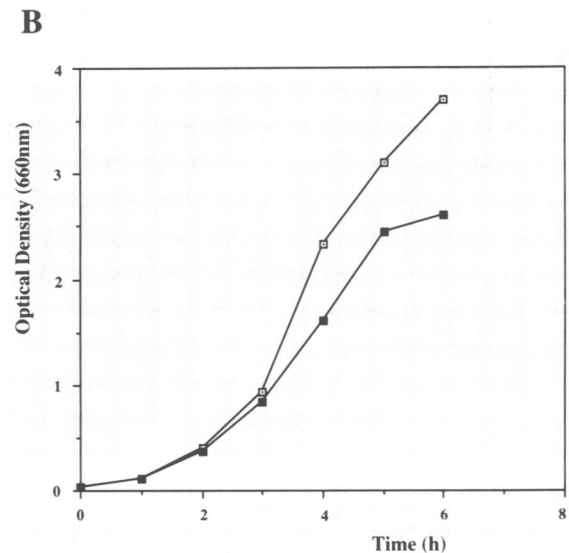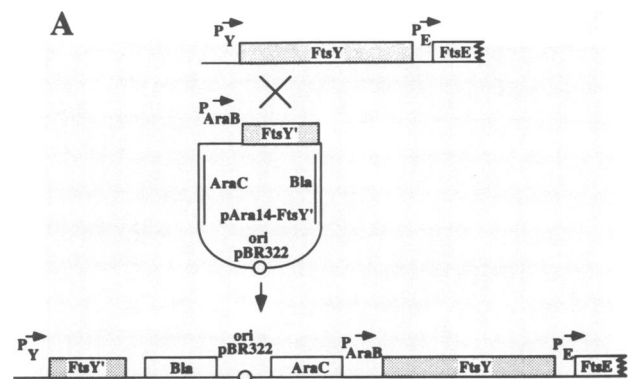

Fig. 3. Conditional expression of FtsY. (**A**) Construction of strain N4156::pAra14-FtsY' by integration of pAra14-FtsY' into the *ftsYEX* gene cluster of the *E.coli* N4156 chromosome resulting in P$_{AraB}$ controlled expression of the *ftsY* gene. P, promoter; Bla, gene encoding β-lactamase. (**B**) 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.

promoter/operator. Correct integration was confirmed by Southern blotting of *Hind*II- or *Xmn*I-digested chromosomal DNA extracted from integrants using the cloned 5' end of *ftsY* as a hybridization probe (data not shown).
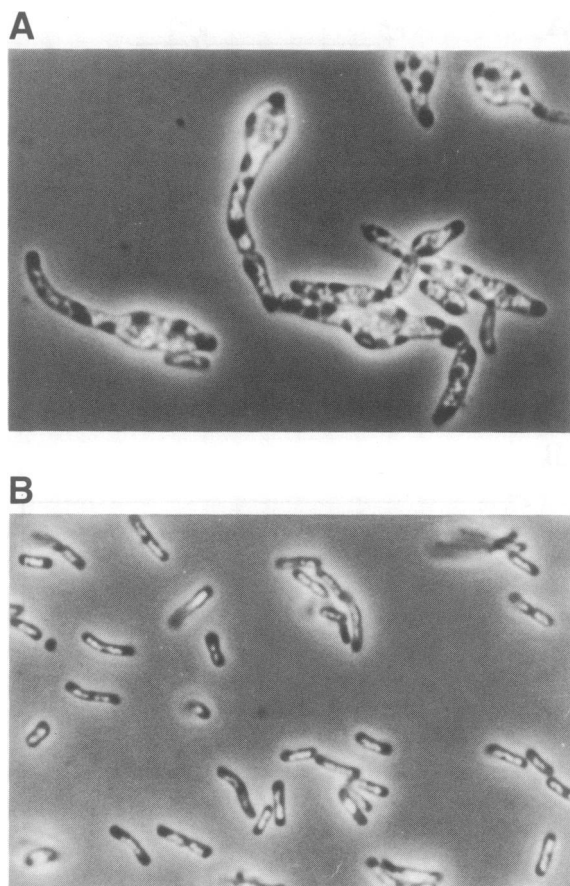
Fig. 4. Fluorescence micrographs (1600×) of FtsY-depleted (A) or 'wild-type' (B) cells. Strain N4156::pAra14-FtsY' was depleted of FtsY as described in the legend to Figure 3B. Micrographs were taken 5 h after the shift to medium supplemented with L-arabinose (B) or not supplemented (A).

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 OsO_4. 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

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 proteinase 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 proteinase 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 proteinase 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 proteinase K had access to the periplasm of the spheroplasted cells. In the lysed (Triton-treated) spheroplasts, pre-β-lactamase was degraded (Figure 5B, lane 6) confirming that this form is not intrinsically resistant to proteinase K digestion in contrast to mature β-lactamase (Minsky *et al.*, 1986). In the absence of proteinase 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 proteinase 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
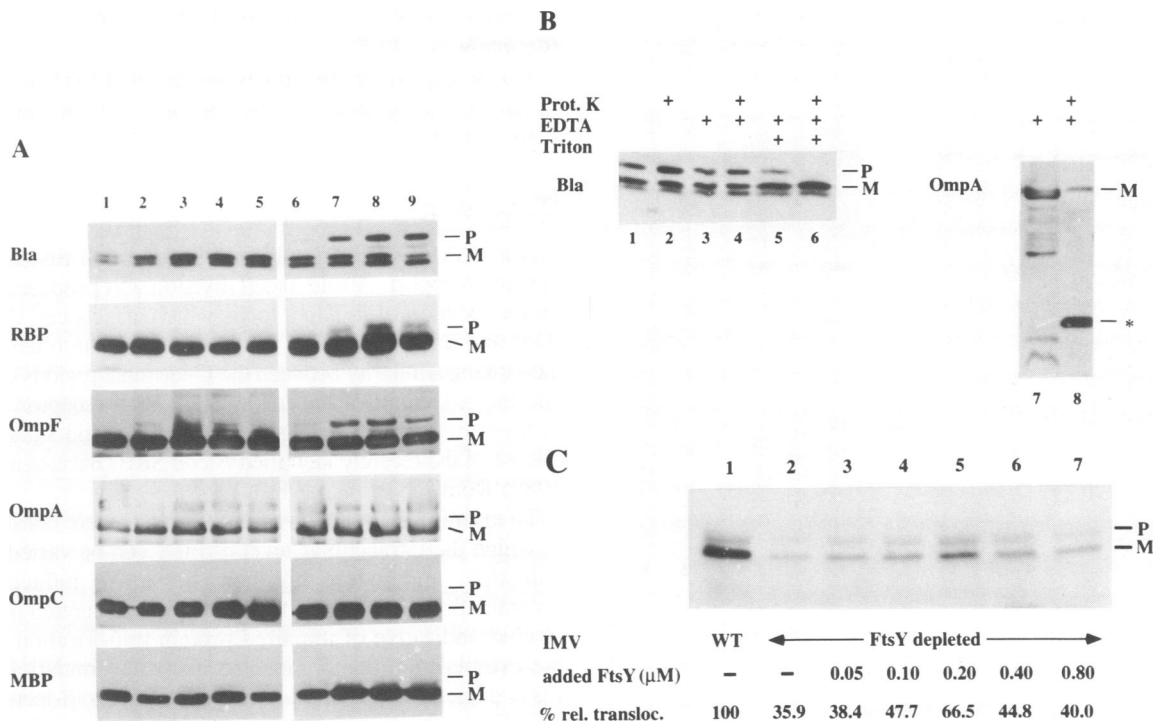
Fig 5. (A) *In vivo* processing of presecretory proteins upon depletion of FtsY. Cells of N4156::pAra14-FtsY' were depleted for FtsY as described in the legend of Figure 3B. Samples were taken at 0 h (lanes 1), 2 h (lanes 2 and 6), 3 h (lanes 3 and 7), 4 h (lanes 4 and 8) and 5 h (lanes 5 and 9) after the shift to medium supplemented with L-arabinose (lanes 2—5) or not supplemented (lanes 6—9) and analyzed by SDS—PAGE and immunoblotting using the indicated antisera. The positions of the precursor and mature forms of the secreted proteins are marked with 'P' and 'M', respectively, at the right side of the blot panels. The positions of the precursor forms were identified in a *secA*(Ts) strain grown at the non-permissive temperature (not shown). The bands migrating below the mature form of Bla and above the precursor form of OmpA represent crossreacting protein species of unknown origin (not shown). (B) Protease accessibility of pre-β-lactamase in FtsY-depleted spheroplasts. Cells of N4156::pAra14-FtsY' were depleted of FtsY for 4 h as described in the legend of Figure 3B. The cells were incubated in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of proteinase K in 100 mM Tris—HCl, 250 mM sucrose (pH 8.0) with either 10 mM MgCl$_2$ (lanes 1 and 2), 5 mM EDTA (lanes 3, 4, 7 and 8) or 5 mM EDTA + 1% Triton X-100 (lanes 5 and 6). Subsequently, the samples were TCA precipitated and analyzed as described in (A). The proteinase K-resistant fragment of OmpA is indicated at the right side of the blot panel (*). (C) *In vitro* translocation of pre-β-lactamase. ^{35}S-labeled pre-β-lactamase was synthesized by *in vitro* transcription and translation in the presence of an FtsY-depleted S-135 cell-free extract. Translocation across inverted cytoplasmic membrane vesicles (IMVs) derived from a wild-type (lane 1) or FtsY-depleted strain (lanes 2—7) was determined by proteolytic degradation of all non-translocated proteins. Purified FtsY was added to the reaction mixture at the indicated concentration. The translocation efficiency was expressed as a percentage of the translocation across wild-type (WT) IMVs (taken as 100%). This percentage is corrected for variation in the amount of protein synthesized. The positions of the precursor and mature forms of β-lactamase are marked with 'P' and 'M', respectively, at the right side of the gel panels.

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 FstY 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 Na$_2$CO$_3$ 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
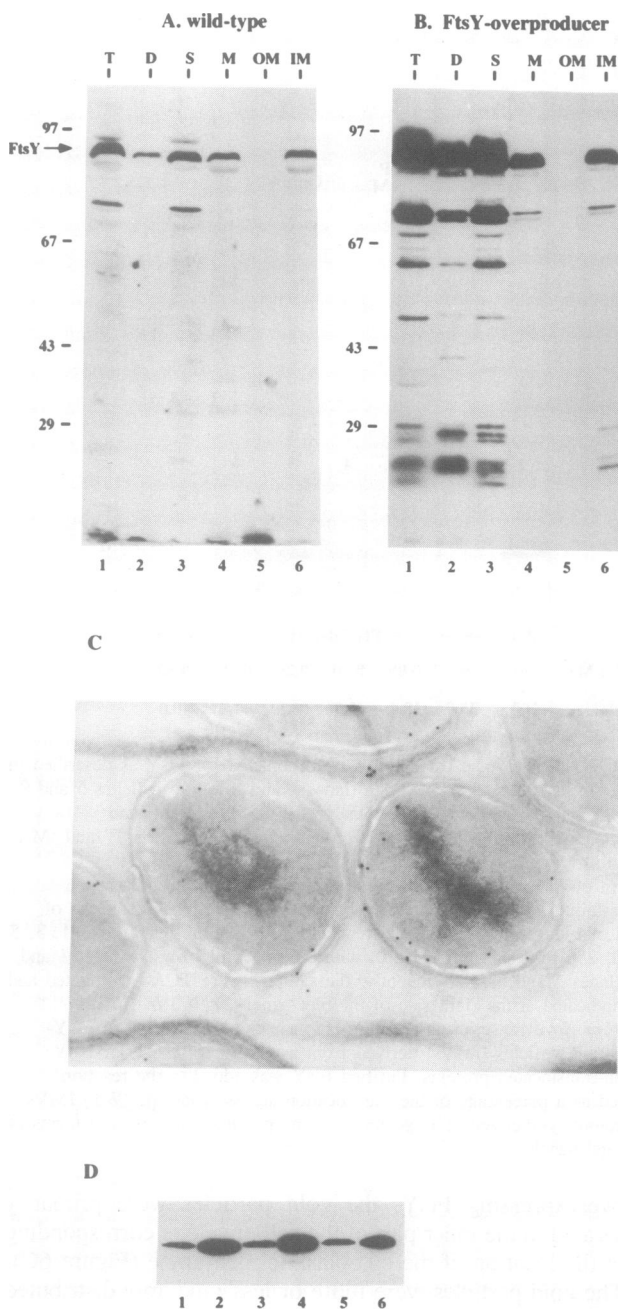
Fig. 6. Sucellular localization of FtsY. (**A**) Fractionation of strain BL21(DE3) carrying pET9 and pLysE (wild-type FtsY expression). (**B**) Fractionation of strain BL21(DE3) carrying pET9-FtsY and pLysE (FtsY overexpression). Cells were grown in YT to an absorbance at 660 nm of 0.3, induced with 0.4 mM IPTG and collected 2 h after induction. Fractions derived from 0.25 OD_{660} 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 pLysS 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 N4156 (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 Na_2CO_3 (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 *pbpA*(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 *ftsE*(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 Salmond (1987) using a maxicell expression system. Inspection of the FtsY sequence reveals no obvious membrane spanning segments (Gill and Salmond, 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* Rifʳ) and BL21 (F⁻ *hsdS gal*) (DE3) were used for initial subcloning and for expression of *ftsY* respectively (Studier *et al.*, 1990). Strain N4156 (*polA end thy gyrA*) was used for the construction of a strain with conditional expression of *ftsY* (Gellert *et al.*, 1977). Strain

LMC515 [*ftsE118*(Ts)*zhg-1*::Tn*10*] was used to study the effects of inactivating FtsE (Taschner *et al.*, 1988). Strain MM52, a derivative of MC4100 (F⁻ Δ*lacU*169 *araD* 136 *rpsL thi relA*) carrying a *secAts51* mutation (Oliver and Beckwith, 1981), was used for the identification of precursor forms of several 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 pDB1 (Gill and Salmond, 1990) were used for subcloning and controlled expression of *ftsY*. To study the effect of FtsY overexpression on the translocation of β-lactamase, pACYC177Δ*Hae*II was constructed by deletion of the 1.4 kb *Hae*II 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 (Laminet and Plückthun, 1989) was cut with *Alw*NI and treated with DNA polymerase I to create blunt ends. Subsequently, the plasmid was cut with *Nde*I and the resulting small *Nde*I−*Alw*NI fragment encompassing the complete β-lactamase gene was ligated into pET9a-adt treated with *Nde*I and *Stu*I 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 digoxygenin 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 *ftsYEX* operon. An *Nde*I restriction site was created at the starting ATG of *ftsY* by site directed mutagenesis in M13mp19 and the resulting *Nde*I 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-FtsY' which carries the 5' end of *ftsY* under control of the *araB* promoter−operator complex. In order to construct pAra14-FtsY', the first 495 bp of the *FtsY* coding sequence were amplified by PCR using pET9-FtsY as a template. Primers were designed to introduce *Nco*I and *Hind*III restriction sites at the 5' and 3' ends respectively. The fragment was sequenced and cloned between the *Nco*I and *Hind*III sites of pAra14 resulting in pAra14-FtsY'.

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 660 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 an FPLC MonoQ anion 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 translocation

The *in vitro* transcription, translation and translocation reactions were carried out basically as described (De Vrije *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

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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 excised 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→3 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 sarkosinate (Sarkosyl) (Chopra and Shales, 1980). Under these conditions marker proteins for the outer and cytoplasmic membrane fractions localized correctly.

Peripherally bound cytoplasmic membrane proteins were extracted from translocation competent cytoplasmic membrane vesicles with 1 M NaCl, 4 M urea and 0.2 M Na_2CO_3 as described (Cabelli *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

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Noted added in proof

Interaction of the *E.coli* SRP and FtsY has been recently reported [Miller *et al.* (1994) *Nature*, **367**, 657−659].