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Membrane biogenesis in *Escherichia coli*: effects of a *secA* mutation

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In *Escherichia coli* K-12, temperature-sensitive mutations in the *secA* gene have been shown to interfere with protein export. Here we show that the effect of a *secA* mutation is strongly pleiotropic on membrane biogenesis. Freeze-fracture experiments as well as cryosections of the cells revealed the appearance of intracytoplasmic membranes upon induction of the SecA phenotype. The permeability barrier of the outer membrane to detergents was lost. Two alterations in the outer membrane may be responsible for this effect, namely the reduced amounts of outer membrane proteins, or the reduction of the length of the core oligosaccharide of the lipopolysaccharide, which was observed in phage-sensitivity experiments and by SDS-polyacrylamide gel electrophoresis. Phospholipid analysis of the *secA* mutant, grown under restrictive conditions, revealed a lower content of the negatively charged phospholipid cardiolipin and of 18:1 fatty acid compared to those of the parental strain grown under identical conditions. These results are in line with the hypothesis that protein export and lipid metabolism are coupled.

Introduction

The cell envelope of *E. coli* consists of two membranes, the inner membrane and the outer membrane, which are separated by the peptidoglycan-containing periplasm. After their synthesis in the cytoplasm, many proteins are exported to these extracytoplasmic compartments.

Protein export is mostly initiated by a signal sequence which is located at the N-terminus of exported proteins. After targeting to the inner membrane, the protein has to be translocated across the membrane. Both binding to the membrane and translocation could be accomplished by components of a proteinaceous export apparatus [1,2]. The proteins encoded by the *sec* genes, mutations which interfere with protein export, may be components of such an export apparatus (for a review see Ref. 3). In addition, biochemical experiments have led to the identification of a protein, called 'trigger factor', which directly interacts with the precursor of outer membrane protein OmpA and which stimulates the translocation of this protein across inner membrane vesicles in an in vitro translocation assay [4,5]. On the other hand, other models suggest that lipid components are directly or indirectly involved in these processes [6–10]. Thus, the positively charged N-terminus of the signal sequence may bind to negatively charged phospholipids in the inner membrane. Furthermore, a hydrophilic protein could pass the hydrophobic environment of the membrane if the protein stays in contact with the hydrophilic part of the lipid components by the formation of non-bilayer structures [11]. Co-transport of lipid components can, in this view, play an important role in facilitating protein export. In this respect, it has been noticed that conditions designed to maintain transbilayer movement of lipids are similar to those required for protein export [9]. It should be noted that this kind of model does not exclude a role of proteins in the export process. Nevertheless, if such models are correct, one would predict that changes in lipid metabolism could exert an effect on protein export and, on the other hand, blocking protein export could lead to pleiotropic effects on lipid metabolism. Indeed, it was recently demonstrated that a *pgsA* mutant of *E. coli* K-12, which has a reduced capability to synthesize anionic phospholipids, is pleiotropically defective in protein export [12]. Furthermore, the biosynthesis or assembly of some outer membrane proteins of *E. coli*

Abbreviation: LPS, lipopolysaccharide.

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was shown to be dependent on fatty acid and/or phospholipid synthesis [13]. Also, mutations in lipopolysaccharide (LPS) biosynthesis, resulting in deep rough mutants, drastically affect the amount of several outer membrane proteins [14]. In the present study, we have investigated the possibility that a blockage in protein export leads to disturbances in lipid metabolism or, in general, in membrane biogenesis. We show using biological, biochemical and electronmicroscopic analysis that a temperature-sensitive *secA* mutant [15] is severely pleiotropic in membrane biogenesis.

Materials and Methods

Bacterial strains and culture conditions

All strains used are *E. coli* K-12 derivatives. Strain MM52 is a derivative of strain MC4100 and carries a *secA51* mutation [15]. It exhibits export defects when the growth temperature is raised above the permissive temperature of 30°C. At 42°C, this defect is lethal to the cells, but is not so at 37°C. Strain pop3234 contains a *lamB-lacZ* fusion gene on the chromosome [16]. Strain MC1000 contains a *galE* mutations [17]. Strain CE1229 is a derivative of MC4100 containing heptose-deficient LPS (Overduin, P. and Tommassen, J., unpublished data).

To induce the Sec phenotype of MM52, overnight cultures grown at 30°C in L-broth [18] were diluted 1:100 in fresh medium. After growing for 1.5 h at 30°C, the cells were incubated at the non-permissive temperature (37°C or 42°C).

Induction of the synthesis of the LamB-LacZ fusion protein in strain pop3234 with maltose was accomplished in a synthetic medium at 30°C as described [19]. Phage sensitivity was measured by plaque assays or by cross-streaking. SDS sensitivity was determined by streaking strains on SDS (Serva) containing yeast broth plates [20] from which sodium chloride was omitted.

LPS analysis

LPS analysis by SDS-polyacrylamide gel electrophoresis was performed as described [21].

Phospholipid analysis

To determine the headgroup composition phospholipids were isolated from 100 ml cultures [22] and separated by thin-layer chromatography (HPTLC, Merck Silica gel 60) in two dimensions, using chloroform/methanol/water (65:25:6) as solvent 1 and chloroform/methanol/acetic acid (65:25:10) as solvent 2. The phosphate content in different spots was determined [23]. Typically, 300 nmol phospholipid were analysed.

Fatty acid analysis

Phospholipids isolated from 250 ml cultures were separated on TLC in one dimension using chloroform/methanol/acetic acid (65:25:10) as the solvent and isolated from these plates. After hydrolysis for 2 h at 70°C in methanol/sulphuric acid (95:5 w/w) the fatty acid methyl esters were extracted with *n*-hexane (Lichrosolv, Merck) and washed with distilled water until neutralization. After overnight drying with sodium sulphate (anhydrous, PA, Merck), the extract was filtered and evaporated. Fatty acids were resuspended in iso-octane (Lichrosolv, Merck) and analysed by gas-liquid chromatography on a Packard model 419 Becker gas chromatograph with a 15% CP-TM-sil 84 column (Chrompack) at 185°C. The retention times of the fatty acids were compared to those of standard compounds. Percentages fatty acids were determined by calculating the peak areas. Typically, 1000 nmol phospholipid were analysed.

Electron microscopy

Cryo-ultramicrotomy was performed as described [24]. Sections were observed in a Philips EM-301 electron microscope at 60 kV. For quantification of the results, at least 200 sectioned cells of each sample were examined. Freeze-fracture procedure was performed according to standard procedures [25].

Results

Intracytoplasmic membranes in secA mutant MM52

To investigate whether induction of the Sec phenotype in *secA* mutant strain MM52 affects the membranes morphologically, ultrathin cryosections of this strain and of its parental strain MC4100 were analysed by electron microscopy. After induction of the Sec phenotype by growth at 42°C, membrane-like structures were observed within the cytoplasm of MM52 cells (Fig. 1). Such structures were not observed in the parental strain MC4100 and hardly in MM52 cells grown at 30°C. The number of MM52 cells, containing these structures and the extent of the structures rapidly increased upon the shift to 42°C. This increase was already observed after 30 min at 42°C. After 2 h induction, the structures were observed in 23% of the cell sections and this value increased to 48% after 6 h at 42°C.

The appearance of morphologically similar intracytoplasmic membrane-like structures was previously observed in strains containing *lamB-lacZ* fusion genes [19]. These fusion genes encode hybrid proteins, consisting of an N-terminal fragment, including the signal sequence, of outer membrane protein LamB, fused to the cytoplasmic enzyme β-galactosidase. Induction of the synthesis of these hybrid proteins is lethal to the cells and blocks the export of other proteins, resulting in the accumulation of unprocessed precursors [16]. In ultrathin cryosections of the cells, we observed that over 50% of the cells contained intracytoplasmic membrane-

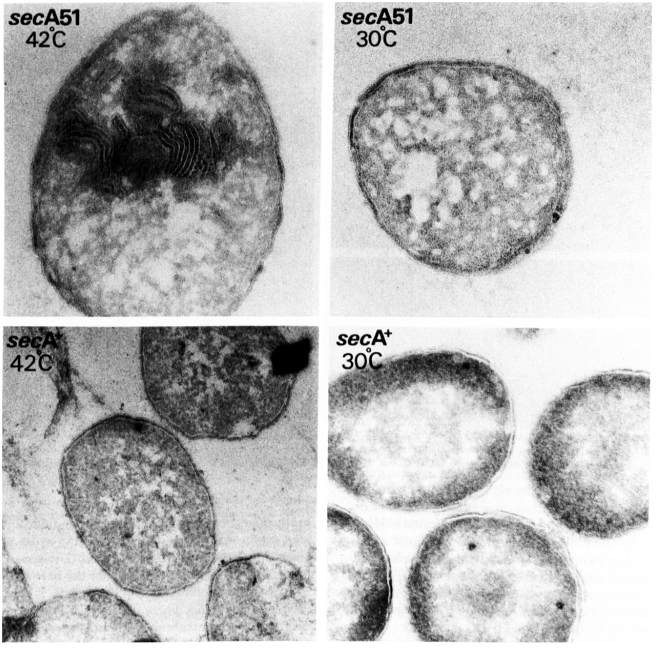

Fig. 1. Cryosections of *secA*51 mutant MM52 grown at 30 °C (*secA*51 30 °C), or for 6 h at 42 °C (*secA*51 42 °C) and of its parental strain MC4100 grown at 30 °C (*secA*$^+$ 30 °C) and 42 °C (*secA*$^+$ 42 °C). In MM52, grown at 42 °C, intracytoplasmic membranes are visible.

like structures upon induction of the synthesis of the fusion proteins [19].

To determine whether these membrane-like structures are actually membranes, freeze-fracture experiments were performed. Indeed, the MM52 cells contained membrane structures, located within the cytoplasm, with the fracture plane going through several membranes (Fig. 2). Particles were observed on the fracture face.

In addition, a substantial decrease in the amount of intramembranous particles in the outer-fracture plane of the outer membrane was found in cells of strain MM52 grown for 6 h at 42 °C, since particle-free areas were observed (Fig. 2). This was not found in control cells.

It has previously been reported that decreased amounts of major outer membrane proteins result in the loss of particles on the outer fracture face of the outer membrane [26]. By quenching from 0 °C, lateral lipid protein separation occurs which results in smooth, particle-free areas [25].

LPS alteration in the secA mutant strain

Whereas the reduced particle content in the outer membrane of the *secA* mutants can already be explained by a reduced outer membrane protein content, it has also been reported that mutations leading to an altered structure of the core moiety of the LPS result in a similar effect [25].

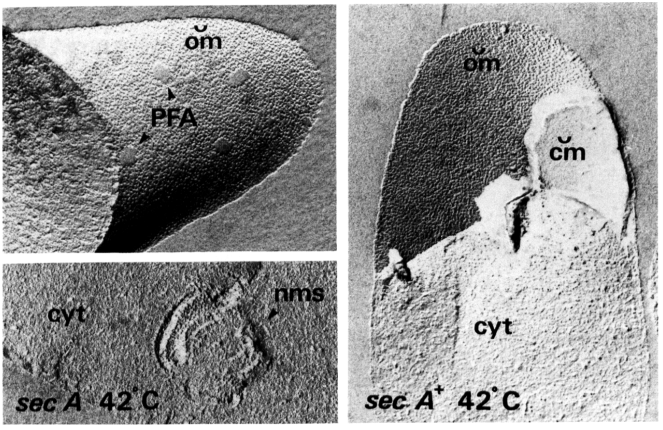

Fig. 2. Freeze fracture of *secA* mutant MM52 grown for 6 h at 42°C (*secA* 42°C) and its parental strain MC4100 (*secA*+ 42°C). MM52 contains a new membrane structure (nms) and several fracture faces can be seen. The concave (outer) fracture face of the outer membrane (om) is completely covered with particles in the case of MC4100, but in MM52 particle-free areas (PFA) are present. (cm, cytoplasmic membrane; cyt, cytoplasm).

Possible changes in LPS structure of strain MM52 were analysed with the help of phages which use LPS as (part of) their receptor. Like its parental strain MC4100, strain MM52 was sensitive to the phages T3, T7 and P1 at all growth temperatures tested. However, in contrast to strain MC4100, MM52 was sensitive to phage C21 at 37°C. At 30°C, strain MC4100 was resistant to this phage but MM52 revealed some residual sensitivity in a plaque assay.

C21 can infect only *E. coli* cells with galactose-deficient LPS [27]. Apparently, the LPS of MM52 contains a shortened core region upon induction of the Sec phenotype. This alteration in size was also detected by SDS-polyacrylamide gel electrophoresis (Fig. 3, lane 2). As could be visualized after extended silver staining of the gel, a portion of the LPS of MM52 grown at 42°C had an increased electrophoretic mobility, and was comparable to the LPS of a *galE* mutant (lane 5).

Due to the barrier function of the outer membrane for detergents, *E. coli* can grown in the presence of up to 5% SDS. Several mutations have been described which influence this barrier function including mutations in LPS core biosynthesis and in major outer membrane protein synthesis [28]. To determine whether the barrier function of the outer membrane of *secA* strain MM52 is intact, the strain was streaked on yeast broth plates, containing between 0% and 3% SDS. The *secA* strain was as resistant to 3% SDS as its parental strain MC4100 at 30°C, but at 37°C it was sensitive even to 0.5% SDS. This shows that the barrier function of the outer membrane for detergents is lost upon induction of the Sec phenotype, which may be due to a decreased outer membrane protein content as well as to the shortened LPS.

Phospholipid analysis of the secA mutant MM52

To investigate whether the phospholipid content of strain MM52 change upon induction of the Sec phenotype, cells were grown for 6 h at 30°C or at 42°C. Subsequently, phospholipids were isolated, separated two-dimensionally by thin-layer chromatography and the phosphate content of the different spots was determined (Table I). The phospholipid composition of MM52 grown at 30°C was similar to that found in strain MC4100. However, at 42°C, MM52 contains slightly but significantly reduced amounts of cardiolipin as compared to strain MC4100 grown under identical conditions. The phospholipid composition of strain pop3234 containing a *lamB-lacZ* fusion gene was also determined, since this strain reacted morphologically in a similar manner to strain MM52 upon induction of the synthesis of the hybrid protein. The phospholipid com-

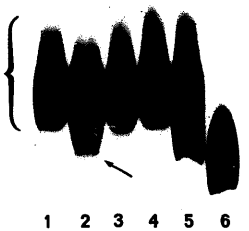

Fig. 3. Silver-stained SDS-polyacrylamide gel of proteinase K-treated cell envelopes, showing the LPS patterns of *secA* mutant MM52 grown at 30°C (lane 1) or for 6 h at 42°C (lane 2), and of MC4100 grown at 30°C (lane 3) or for 6 h at 42°C (lane 4). As a reference, the LPS patterns of *galE* mutant MC1000 grown at 37°C (lane 5) and of strain CE1229 with a heptose-deficient LPS (lane 6) are also shown. The bracket shows the position of wild-type LPS consisting of the lipid A moiety and the core sugars. The arrow indicates the faster migrating LPS species in strain MM52 after growth at 42°C.

TABLE I

Phospholipid headgroup composition

Headgroup composition of the different strains was determined by a phosphate analysis as described in Materials and Methods. Means were calculated after duplicate analysis of three independent cultures in the case of strains grown in L-broth and two cultures in the case of strains grown in synthetic medium (SM), (PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin).

Strain	Growth conditions	% Phospholipid		
		PE	PG	CL
MM52	30°C L-broth	78.3±1.8	20.1±2.1	1.6±0.6
MC4100	30°C L-broth	78.1±3.7	20.4±3.8	1.5±0.1
MM52	42°C L-broth	80.6±1.9	15.9±1.6	3.5±0.6
MC4100	42°C L-broth	77.4±1.9	17.7±2	5 ±0.6
pop3234	30°C SM+glucose	83.4±3.1	16.2±3.5	0.5±0.3
MC4100	30°C SM+glucose	81.5±3	17.7±3.3	0.8±0.3
pop3234	30°C SM+maltose	82.9±2.1	13.9±2.5	3.3±0.6
MC4100	30°C SM+maltose	84.4±3.4	14.6±3.4	1 ±0.1

TABLE II

Total fatty acid composition of MM52 and MC4100

Fatty acids were analysed as described in Materials and Methods from three independent cultures grown at 42°C (Cy, cyclopropane derivates; n.d., not detected).

Fatty acid	% Fatty acid in strain	
	MM52 42°C	MC4100 42°C
14:0	3 ±2.3	1.6±0.5
15:0-Cy	6.2±2.2	3 ±0.9
16:0	32.5±2.5	33.6±2.4
16:1	14.6±2.1	15.4±1.6
17:0-Cy and/or iso-18:0	21.9±0.9	19.4±3.2
18:0	n.d.	n.d.
18:1	11.2±2.8	18.3±2.2
19:0-Cy	8.2±1.8	5.9±1.5
Unidentified	2.6±0.7	2.9±0.5

position of pop3234 grown in glucose-containing synthetic medium was similar to that found in strain MC4100. However, cells producing the fusion protein by growth on maltose-containing medium contained strongly increased amounts of cardiolipin compared to the control strain MC4100 grown under the same condition (Table I).

Fatty acid analysis

To investigate possible changes in fatty acid content of MM52 the fatty acid methyl esters of the isolated phospholipids were analysed by gas-liquid chromatography. Duplicate determinations in each culture were fairly similar, but we found a rather large variation in fatty acid composition between different cultures of the same strain. Despite this variation, strain MM52 cultured at 42°C consistently contained reduced amounts of 18:1 fatty acid when compared to its parental strain MC4100 grown under identical conditions (Table II).

Discussion

In this study we show that blocking of protein export in *secA* mutant MM52 leads to several changes in membrane biogenesis: intracytoplasmic membranes appear, the LPS chemotype changes towards unsubstituted Rc or Rd and differences in phospholipid composition are found. It is not clear how these pleiotropic effects arise. Interestingly, we have observed morphologically similar intracytoplasmic membranes when protein export was blocked by the induction of the synthesis of LamB-LacZ hybrid protein [19], although the other pleiotropic effects of the *secA* mutant, such as reduced amounts of cardiolipin and 18:1 fatty acid, were not observed in these strains. In that paper, we postulated the possibility that the cells try to compensate the blocking effect of the hybrid proteins by

enlarging its export sites. Similarly, the cells may try to compensate the SecA defect by enlarging the export sites. However, it should be noted that in other *sec* mutants, i.e., *secY* and *secB*, such intracytoplasmic membranes were not observed (De Cock, H., et al., unpublished observation). One could argue that protein export involves multiple steps and that a blockage in each individual step leads to different pleiotropic effects. Then, the appearance of intracytoplasmic membranes in the LamB-LacZ fusion strains could be explained by assuming that the hybrid protein or precursors of other exported proteins which accumulate in these strains titrate out the SecA protein. In this respect, it should be noted that there are more parallels between the hybrid protein-producing strains and *secA* mutants. For instance, it has been reported that both the induction of the synthesis of a MalE-LacZ hybrid protein and induction of the SecA phenotype in *secA* mutants lead to the induction of the synthesis of SecA protein [29]. This effect was also observed in *secY* and *secD* mutants, but not in a *secB* mutant [30]. In addition, both the synthesis of LamB-LacZ fusion proteins [16] and *secA* mutations [15] lead to filamentation of the cells. This effect was not observed in *secY, secD* and secB mutants (De Cock, H., unpublished observations) and therefore seems to be more specific. Whereas a direct role of SecA protein in protein export has recently been confirmed by reconstitution of the protein in an in vitro translocation assay [31], it is possible that the protein fulfils an additional role in the cell in cell division. This suggestion is in agreement with the chromosomal location of *secA* in a cluster of genes involved in cell division [32].

The synthesis of the core region of the LPS occurs at the cytoplasmic side of the inner membrane. Although the glycosyl transferases can be isolated associated to the inner membrane, they are not integral membrane proteins and active enzyme can also be isolated from the soluble fraction [33]. Therefore, it seems unlikely that the glycosyl transferases require SecA for their assembly. Possibly, the change in LPS chemotype in the SecA mutant is a secondary effect of the appearance of the intracytoplasmic membranes. Thus, the glycosyl transferases may interact with these intracytoplasmic membranes, resulting in decreased enzyme activity at the inner membrane and translocation of LPS with an incomplete core region to the outer membrane. It should be noted that the change in LPS structure may provide an explanation for the observation that synthesis and export of different proteins are affected to different degrees in a *secA* mutant [34], since it has been reported that mutations which affect the LPS structure also affect the biosynthesis of various outer membrane proteins [14,35,36].

We observed in the *secA* mutant a slight decrease in cardiolipin content, whereas it was increased in the LamB-LacZ fusion strain. Although it is not clear how the blockages of protein export affect the cardiolipin content, these results suggest that cardiolipin may somehow be involved in protein export. This hypothesis is particularly important because cardiolipin has a strong tendency to form type II non-bilayer structures [11]. The induction of such structures upon interaction with amphipatic polypeptides is of particular interest [37]. Also, the decrease of 18 : 1 fatty acid observed in the *secA* mutant is of interest, since a role of this fatty acid in protein export has been postulated [9].

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