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Subcellular localization of a PhoE-LacZ fusion protein in *E. coli* by protease accessibility experiments reveals an inner-membrane-spanning form of the protein

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Protease accessibility experiments were employed to localize a PhoE-LacZ hybrid protein, encompassing a large N-terminal fragment of the outer membrane PhoE protein of *E. coli*, fused to β-galactosidase, at the subcellular level. In previous studies, this protein was shown to co-fractionate with the outer membrane, whereas immunocytochemical methods suggested a cytoplasmic location. The present results confirm the latter localization. Moreover, it appears that a minor amount of hybrid protein spans the inner membrane, with the PhoE moiety in the periplasm and the β-galactosidase moiety in the cytoplasm. These membrane-spanning proteins might be responsible for the lethal jamming of the export machinery, observed upon induction of synthesis of the protein.

Outer membrane protein; Protein export; Hybrid protein; *(E. coli)*

1. INTRODUCTION

Gene fusion technology has been employed extensively to identify export signals in *E. coli* outer membrane proteins [1,2]. Thus, it has been reported that hybrid proteins consisting of an N-terminal portion of LamB protein and the cytoplasmic enzyme β-galactosidase co-fractionate with the outer membrane, provided that they contain a sufficiently large fragment of this outer membrane protein. However, these results must be interpreted with caution, since standard cell fractionation techniques may not be applicable to localize such hybrid proteins [3]. We have recently constructed a *phoE-lacZ* gene fusion, encoding a large N-terminal fragment (approx. 85%) of the P_i-limitation-inducible outer membrane protein PhoE, fused to β-galactosidase [4]. Expression of this hybrid gene is lethal to the cells and leads to the accumulation of precursors of other exported proteins. Cell fractionation experiments suggested that the hybrid protein is efficiently translocated to the outer membrane. On the other hand, the protein was shown by immunocytochemical labelling techniques to accumulate in the cytoplasm [4]. Here, we confirm the cytoplasmic accumulation of this hybrid protein by using protease accessibility experiments. Moreover, we demonstrate that a minor amount of the hybrid protein is located in the inner membrane, such that the PhoE moiety of the protein is attainable by trypsin from the periplasmic side of the membrane, whereas the β-galactosidase portion is still in the cytoplasm.

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2. EXPERIMENTAL

2.1. *Strain, plasmid and growth conditions*

E. coli K-12 strain MC4100 [5] and plasmid pJP102, which contains a *phoE-lacZ* fusion gene [4], have been described. Cells were grown in a synthetic medium in which the phosphate concentration can be varied [6]. This medium was supplemented with ampicillin (50 μg/ml) and with 660 μM K_2HPO_4 (high-phosphate medium) which is sufficient to repress the synthesis of PhoE protein. For derepression, an overnight culture in high-phosphate medium was diluted 1:12.5 in fresh medium without any phosphate added, and incubated further at 37°C. Under these conditions, derepression of the *pho* regulon occurs after approx. 2 h [4].

2.2. *Cell fractionation and SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was carried out as in [7], except that the polyacrylamide concentration was lowered to 9%. To analyse total cell protein patterns, cells were collected by centrifugation for 1 min in an Eppendorf centrifuge, resolved in sample buffer [7] and heated for 5 min at 95°C prior to application on a gel. Cell envelopes were isolated after ultrasonic disruption of the cells as described [7], except that the cell envelopes were collected by centrifugation for 30 min in an Eppendorf centrifuge. For identification of proteins on gels, the gel immuno-radioassay [8] was used.

2.3. *Protease accessibility experiments*

Cells were converted to spheroplasts as in [9]. To 1 ml of spheroplasts in 100 mM Tris-HCl, 0.25 mM sucrose, 0.25 mM EDTA (pH 8.0), 50 μg trypsin (Serva) was added. After 30 min incubation at 0°C, 20 mM $MgCl_2$ was added to stabilize the spheroplasts, and 200 μl of a solution of 0.1 M diisopropyl fluorophosphate in isopropanol to inactivate trypsin. Spheroplasts were collected by centrifugation for 1 min in an Eppendorf centrifuge and resuspended in hot (95°C) sample buffer for SDS-polyacrylamide gel electrophoresis.

In an alternative procedure, whole cells were incubated in the presence of either 10 mM $MgCl_2$ or 5 mM EDTA as in [10]. The presence of EDTA makes the outer membrane leaky, thus allowing the entrance of trypsin into the periplasm.

3. RESULTS

3.1. *Solubilization of PhoE-LacZ hybrid protein*

In the course of these experiments, we noticed that the majority of PhoE-LacZ hybrid protein is produced in a form which cannot readily enter an SDS-polyacrylamide gel. When protein patterns of whole cells of strain MC4100 containing pJP102 after growth under P_i limitation were analysed by SDS-polyacrylamide gel electrophoresis, only a faint band of hybrid protein could be detected on the gel (fig.1b). However, when cell envelopes were prepared from a sample of the same culture, the intensity of this band as compared to other envelope proteins increased enormously (fig.1c). Apparently, the hybrid protein is present in whole cells as aggregates which are solubilized during cell envelope preparation. Also spheroplast formation led to solubilization of the hybrid protein (fig.1d).

3.2. *Trypsin treatment of spheroplasts*

To determine the subcellular localization of the PhoE-LacZ hybrid protein, cells of strain MC4100 containing pJP102 were grown under P_i limitation, converted to spheroplasts and the spheroplasts were treated with trypsin as described in section 2. The PhoE-LacZ hybrid protein appeared not to be affected under these conditions (fig.2a,b). In these experiments, OmpA protein can be considered as an internal control [10], since the C-terminus of this protein extends into the periplasm [11]. OmpA protein was degraded during this treatment (fig.2). Since the PhoE-LacZ protein was degraded when cell envelopes were treated with trypsin (fig.2c,d), the hybrid protein is not intrinsically resistant to the protease, but it is not accessible in the spheroplasts. Apparently, at least the majority of the hybrid protein is accumulated in the cytoplasm.

3.3. *Trypsin treatment of EDTA-permeabilized cells*

We investigated the possibility that the minor amounts of PhoE-LacZ protein which can enter a gel when whole cells are applied (fig.1b) form a separate pool of hybrid protein. This could be achieved by performing trypsin accessibility experiments under conditions where the majority of hybrid protein is not solubilized, namely by treatment of whole cells with trypsin in the presence of either Mg^{2+} or EDTA. In the presence of Mg^{2+},

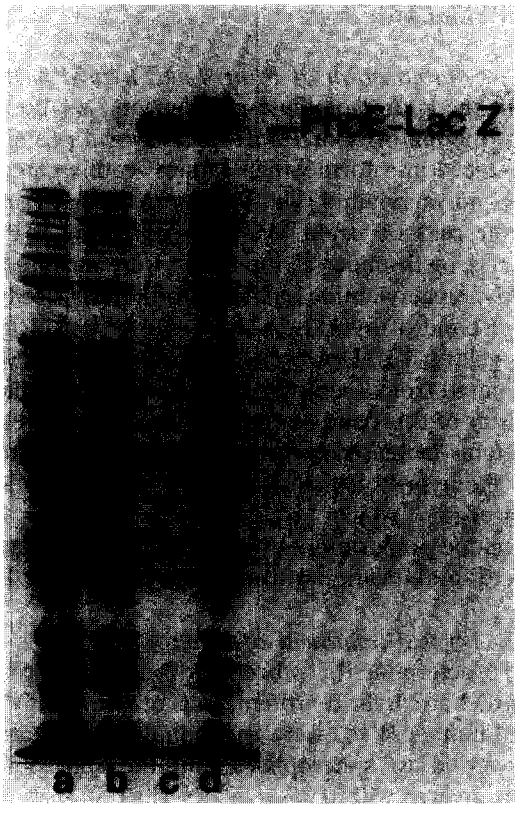

Fig.1. SDS-polyacrylamide gel electrophoresis patterns of total cell proteins of strain MC4100 (pJP102) after growth in high-phosphate medium (a) or after growth for 6 h under P_i limitation (b). Of the latter culture, the cell envelope protein patterns (c) and the protein patterns of spheroplasts (d) are also shown.

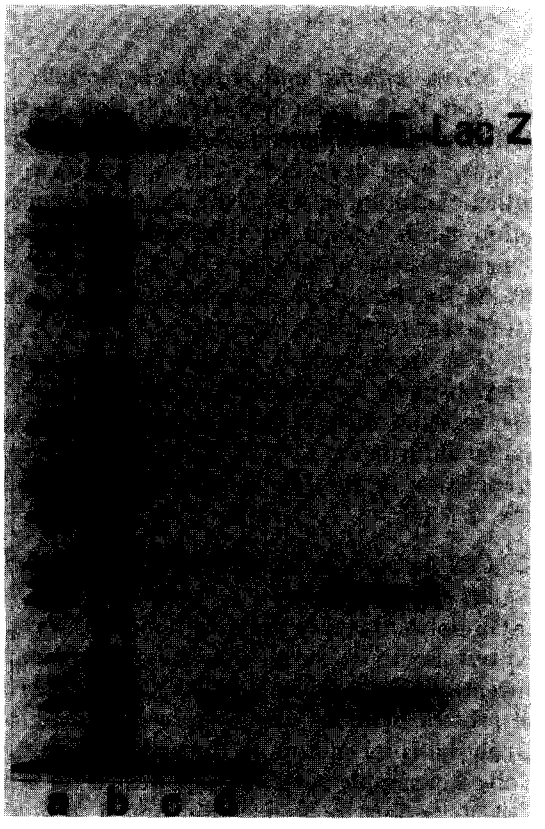

Fig.2. SDS-polyacrylamide gel electrophoresis protein patterns of spheroplasts and cell envelopes prepared from strain MC4100 (pJP102) after growth for 6 h under P_i limitation: (a) spheroplasts, (b) spheroplasts after trypsin treatment, (c) cell envelopes, (d) cell envelopes after trypsin treatment.

the hybrid protein was not degraded by trypsin, showing that the protein does not extend into the medium (not shown). However, the hybrid protein was degraded when EDTA was present during trypsin treatment to allow the entrance of the enzyme into the periplasm (fig.3). Two new bands appeared which might be degradation products of the hybrid protein, one with the same electrophoretic mobility as β-galactosidase and the other with an apparent molecular mass of 100 kDa. Using the gel immuno-radioassay (fig.4), it could be demonstrated that the former band, but not the latter, reacted with anti-β-galactosidase serum and therefore is a degradation product of the PhoE-LacZ hybrid protein. Also, a faint band with an apparent molecular mass of 79 kDa was detected. When antiserum directed against denatured PhoE protein was used in these assays, only the intact PhoE-LacZ hybrid protein reacted and no degradation products were observed (not shown). From these experiments it appears that a minor amount of hybrid protein spans the inner membrane, with the PhoE moiety being susceptible to proteolytic attack from the periplasm, but the β-galactosidase part being largely protected.

4. DISCUSSION

The subcellular localization of a PhoE-LacZ hybrid protein was investigated using protease accessibility experiments. The hybrid protein was found to accumulate in the cytoplasm, which is in

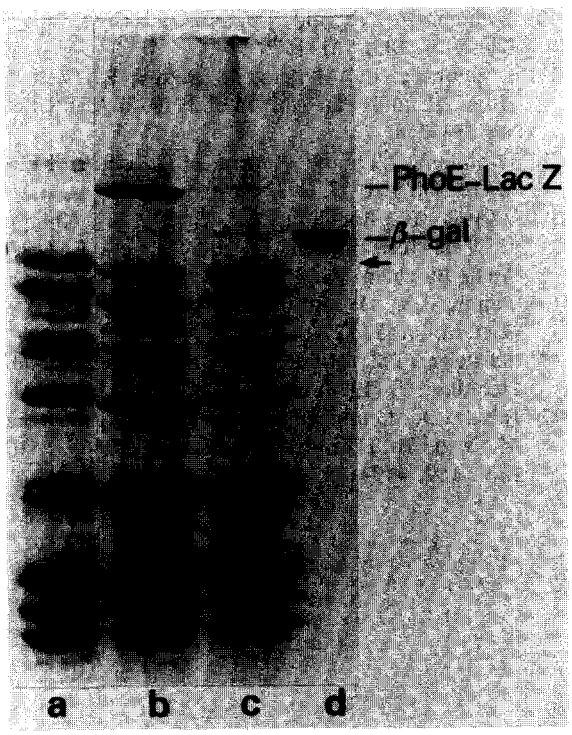

Fig.3. SDS-polyacrylamide gel electrophoresis patterns of total cell proteins of strain MC4100 (pJP102) after growth in high-phosphate medium (a) or after growth for 6 h under P_i limitation (b) and followed by EDTA-trypsin treatment (c). Lane d shows purified β-galactosidase as a reference. The arrow points to a band with an apparent molecular mass of 100 kDa which appears during trypsin treatment. Only the relevant part of the gel is shown.

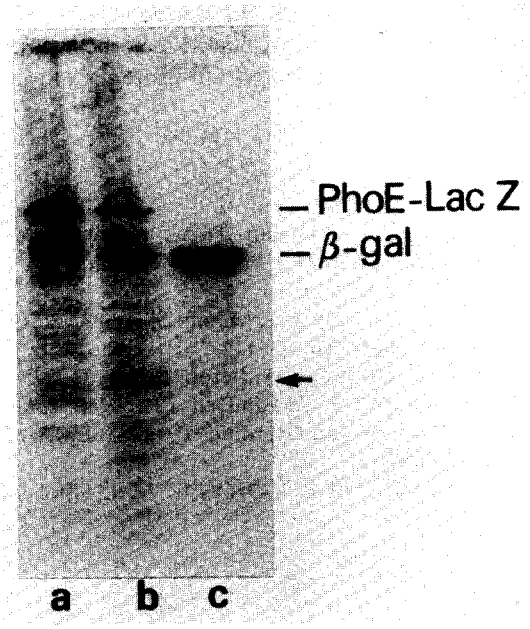

Fig.4. Gel immuno-radioassay applied on the samples shown in lanes b–d in fig.3. After SDS-polyacrylamide gel electrophoresis of the samples, the gel was sliced and a gel slice was successively incubated with anti-β-galactosidase serum and ^{125}I-labelled protein A. An autoradiogram of the gel slice is shown: (a) whole cells of induced MC4100 (pJP102), (b) cells after EDTA-trypsin treatment and (c) β-galactosidase. The arrow points to a band in lane b with an apparent molecular mass of 79 kDa.

agreement with results from immunocytochemical labelling techniques but in contrast to cell fractionation data [4]. Apparently, standard cell fractionation techniques are not reliable when used to localize this kind of hybrid protein and aggregate formation (see section 3.1) may be responsible for the fractionation artifacts. In the light of these results, data available for other hybrid proteins [1,2] also need to be re-valuated.

The C-terminal 50 amino acids of PhoE protein are lacking in the hybrid protein which was used in these studies. Since the protein apparently accumulates in the cytoplasm, this could mean that essential export information is located in this fragment. However, we have described a number of *phoE* genes with internal deletions, some of which lack the DNA corresponding to this C-terminal fragment [12]. The products of these mutant genes are efficiently transported to the periplasm. Therefore, it is more likely that the β-galactosidase moiety of the hybrid protein prevents export. Indeed, evidence for this supposition was obtained during these investigations. The PhoE moiety of a minor amount of hybrid protein appears to be transported through the inner membrane, since it is attainable by trypsin from the periplasmic side of the membrane. Export seems to be hampered at or within the β-galactosidase moiety, leaving protected fragments of the size of β-galactosidase and of 79 kDa (fig.4). Especially the former protected fragment suggests that the β-galactosidase moiety is (at least partially) folded in its normal configuration and that this folding prevents export. It seems also likely that these membrane-spanning hybrid

proteins are responsible for the reported jamming of the export machinery, resulting in the accumulation of precursors of exported proteins and in cell death [4].

REFERENCES

[1] Silhavy, T.J., Shuman, H.A., Beckwith, J. and Schwartz, M. (1977) Proc. Natl. Acad. Sci. USA 74, 5411–5415.
[2] Hall, M.N., Schwartz, M. and Silhavy, T.J. (1982) J. Mol. Biol. 156, 93–112.
[3] Tommassen, J. (1986) Microb. Pathog. 1, 225–228.
[4] Tommassen, J., Leunissen, J., Van Damme-Jongsten, M. and Overduin, P. (1985) EMBO J. 4, 1041–1047.
[5] Casadaban, M.J. (1976) J. Mol. Biol. 104, 541–555.
[6] Tommassen, J. and Lugtenberg, B. (1980) J. Bacteriol. 143, 151–157.
[7] Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P. and Van Alphen, L. (1975) FEBS Lett. 58, 254–258.
[8] Poolman, J.T. and Zanen, H.C. (1980) FEMS Microbiol. Lett. 7, 293–296.
[9] Witholt, B., Boekhout, M., Brock, M., Kingma, J., Van Heerikhuizen, H. and De Leij, L. (1976) Anal. Biochem. 74, 160–170.
[10] Tommassen, J. and Lugtenberg, B. (1984) J. Bacteriol. 157, 327–329.
[11] Schweizer, M., Hindennach, I., Garten, W. and Henning, U. (1978) Eur. J. Biochem. 82, 211–217.
[12] Bosch, D., Leunissen, J., Verbakel, J., De Jong, M., Van Erp, H. and Tommassen, J. (1986) J. Mol. Biol. 189, 449–455.