# EXPRESSION OF OUTER MEMBRANE PROTEIN e OF *ESCHERICHIA COLI* K12 BY PHOSPHATE LIMITATION

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## 1. Introduction

*Escherichia coli* K12 contains several so-called major outer membrane proteins, the designation of which was different in various laboratories [1–4] but it is now agreed that the proteins will be named according to the designation of their structural gene [5]. The products of the *ompC* (min 48) and *ompF* (min 21) genes, formerly designated as c and b in our laboratory [1,6,7], are outer membrane proteins which function as aqueous pores through which many nutrients enter the cell via a diffusion-like process [8–13]. Mutants which lack both the *ompC* and *ompF* products are sensitive to 3% sodium dodecyl sulphate (SDS) [12,14]. They are often unstable and easily revert to SDS-resistant strains which have either regained one or both of the two porins or contain a a new major outer membrane protein. The most commonly found new protein is designated as e in our laboratory [14] or Ic [15] or E [16] by others. This new protein has the same electrophoretic mobility as protein a [14]. The isolation of bacteriophage TC45 which uses protein e as part of its receptor [17,18] has facilitated the localization of genes involved in the appearance of protein e. These genes, *nmpA* and *nmpB*, have been localized at min 82 [19,20] and min 8 [20], respectively. Like the *ompC* and *ompF* proteins, protein e also displays porin properties [14,16,20].

The presence of protein e in the wild type strain *E. coli* K12 has never been reported. We reasoned that this is probably due to the fact that the usual laboratory media do not force the cells to produce this protein. Several examples are known of outer membrane proteins which are only synthesized under special growth conditions. For example, the presence of maltose results in the appearance of the λ receptor protein in the outer membrane [21,22], whereas low [$Fe^{3+}$] result in the appearance of several outer membrane proteins which are involved in the uptake of these ions [23]. In an attempt to discover a function for the new protein we have grown *E. coli* K12 under a variety of conditions which the bacterium might meet in Nature. The results show that protein e is produced when the cells are grown under $P_i$ limitation.

## 2. Experimental

### 2.1. *Strains and growth conditions*

Only *E. coli* K12 strains were used. Strain PC0221 is $F^-$, prototrophic. Strain CE1200, which is $F^-$, *met, pro,* (*lam*), *rpoB*, was obtained from Dr J. Wouters [24]. Strain CE1108 is an *ompB, nmpA* derivative of strain CE1000. It lacks the *ompC* and *ompF* proteins and produces protein e constitutively [12,14].

Media containing fluid of human origin were prepared as follows. Sterile human blood plasma of a healthy volunteer was purchased from the Blood Bank, Utrecht. Feces fluid from a patient with a colonstoma was centrifuged and the supernatant fluid adjusted from pH 5 to 7.8. The fluid was sterilized by heating as filtration was not possible. Urine was given by healthy volunteers and sterilized by filtration through membrane filters (Millipore SA, Molsheim, pore size 0.45 μm, type HA). Solid media containing the fluids mentioned above as the growth medium were prepared by the addition of 20 vol. % of a sterilized 7.5% suspension of agar–agar. After inoculation of each plate with ~$5 \times 10^5$ cells strain PC0221 the plates were incubated anaerobically at 37°C using a GasPak anaerobic system (BBL, Div. Becton, Dickinson and Co., Cockeysville, MD). Cells were scraped from the plates

for analysis after incubation for 16 h except that those grown on urine were collected after 40 h.

To test the influence of carbon-, nitrogen-, sulphur- and phosphorus sources other than those present in the standard glucose minimal medium [25], one or more components of the latter medium were replaced. The following carbon- and energy-sources were tested instead of D-glucose: lactose; melibiose; D-fructose; L-fucose; D-galactose; D-mannitol; L-rhamnose, D-sorbitol; L-arabinose, D-ribose; D-xylose; glycerol; glycerol-1-phosphate; pyruvate; acetate; oleate; L-alanine; D-alanine. L-Alanine and D-alanine were tested as the only nitrogen source. D,L-methionine was tested as the only sulphur source. L-Alanine and D-alanine were tested as the only carbon plus nitrogen sources and glycerol-1-phosphate was tested as the only carbon plus phosphorus source.

Chemostat cultures of strain CE1200 were grown in a Bioflo C30 New Brunswick chemostat at pH 7.0 at 37°C under intensive stirring in the synthesis medium in [26]. Limitations for the sources of carbon, nitrogen and phosphorus were obtained by decreasing the standard concentrations of glucose, $NH_4Cl$ and $NaH_2PO_4$ 3–5-fold. Anti-foam was added at regular time intervals. Under limiting conditions cells were growing with a generation time of 3.5 h. Upon harvesting they were cooled immediately.

Cells of strain CE1108, serving as a source of protein e, were grown in yeast broth [25].

### 2.2. *Adsorption of phage TC45*

Irreversible phage adsorption to whole cells was measured as in [7].

### 2.3. *Membrane fractions*

After washing the cells cell envelopes were prepared as in [1,6]. A fraction containing peptidoglycan with proteins tightly but non-covalently attached to it (peptidoglycan–protein complexes) was obtained by incubation of cell envelopes at 60°C in a buffer containing 2% SDS, followed by ultracentrifugation [6,27,28].

### 2.4. *SDS–polyacrylamide gel electrophoresis*

Unless otherwise indicated our standard gel system [1] (system A) was used. Systems B–D refer to the systems used in [20] except that for systems B and C urea was also included in the stacking gel and in the electrode buffers.

## 3. Results

### 3.1. *Outer membrane protein patterns during growth in limiting substrate concentrations*

An extensive investigation for the presence of protein e after growth of strain PC0221 in 'natural media' (urine, feces fluid, plasma) and standard minimal medium with different carbon, nitrogen, sulphur and phosphorus sources did not show any protein e in cell envelope preparations analyzed by SDS–polyacrylamide gel electrophoresis (not shown).

Cell envelope protein patterns of strain CE1200 grown in limiting concentrations of nitrate ions as the only nitrogen source (fig.1.2.) of glucose as the only carbon plus energy source (fig.1.3) and of $P_i$ as the only phosphorus source (fig.1.4) were also examined. As a control cells were grown under the same conditions except that all components of the medium were present in standard concentrations (fig.1.1). Compared with the cell envelope protein pattern obtained after growth of strain CE1200 in complete Evans medium (fig.1.1), somewhat more protein a is produced under nitrate limitation (fig.1.2), whereas glucose limitation (fig.1.3) results in the production of strongly increased amounts of the *ompF* protein such that the ratio of porins over *ompA* protein, which usually is ~1 [24,29] has considerably increased. Phosphate limitation (fig.1.4) changes the pattern dramatically in that the amount of protein in the position of protein a is extremely high. As it will be shown later on that this increase is due to the production of another porin, it is clear that, as in the case of glucose limitation, also phosphate limitation results in a high ratio of porins over *ompA* protein. The experiment

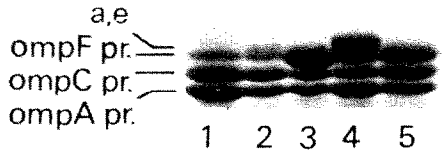


Fig.1. SDS–polyacrylamide gel electrophoresis patterns of cell envelope proteins of strain CE1200 after aerobic growth in the medium of [25] with excess of N,C and P-sources (1) and in medium with limiting amounts of nitrate (2), glucose (3) and $P_i$ (4) as the only sources of N, C and P. Slot 5 shows cell envelopes of cells first grown under phosphate limitation and subsequently grown for 20 h in the presence of excess phosphate. Only the relevant part of the gel is shown. System A was used.

with the phosphate limitation was repeated twice with the same result. In one of these experiments phosphate limitation was followed by growth in the presence of excess phosphate for 20 h, and this resulted in the disappearence of the band at the position of protein a (fig.1.5). These results showed that the extra amount of protein was specifically produced during growth under limiting phosphate concentrations and that the heavy band was not the result of a mutation. Since protein e has the same electrophoretic mobility as protein a in gel system A [14], we examined the possibility that the increase of the amount of protein in the position of protein a was due to the expression of protein e under phosphate limitation.

3.2. *Protein e is induced by phosphate limitation*

The following 3 criteria were used to see whether the increased amount of protein at the electrophoretic position of protein a (fig.1.4) could be due to the presence of protein e.

(i) Like the *ompC* and *ompF* proteins [25,27,31] protein e is a peptidoglycan-associated protein [15,12,25,32]. Cell envelopes of cells of strain CE1108 grown in yeast broth and of strain CE1200 grown under phosphate limitation were heated in 2% SDS at 60°C and their peptidoglycan–protein complexes were isolated and analyzed by SDS gel electrophoresis in gel system A. The results showed that the increased amount of protein at the electrophoretic position of protein a is due to the synthesis of a new protein which, unlike protein a [25] but like protein e, is peptidoglycan-associated (fig.2.1, 2.2).

(ii) Several gel systems have been developed in which the major outer membrane proteins move to different positions relative to each other. The electrophoretic mobility of the new protein was indistinguishable from that of protein e in four different gel systems (fig.2.1–2.8).

(iii) Phage TC45 specifically recognizes outer membrane protein e as part of its receptor [18]. Like cells of strain CE1108 grown in yeast broth, cells of strain CE1200 grown under phosphate limitation inactivated this phage, whereas the TC45 adsorbing capacity was absent from the latter strain after growth in yeast broth (results not shown).

From these results we conclude that the new protein induced by phosphate limitation is identical to protein e.

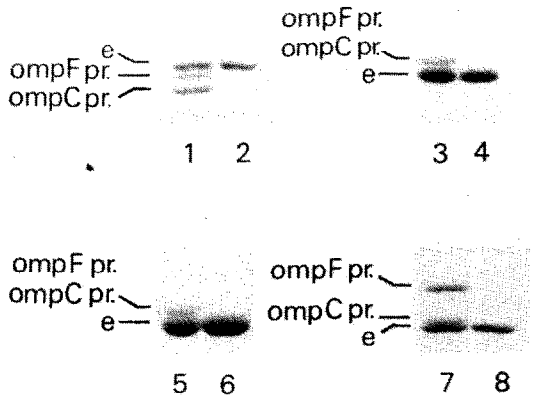


Fig.2. SDS–polyacrylamide gel electrophoresis patterns of peptidoglycan-associated proteins of cells of strains CE1200 (grown under phosphate limitation) (slots 1,3,5,7) and CE1108 (grown in yeast broth) (slots 2,4,6,8). Systems A (1,2), B (3,4), C (5,6) and D (7,8) were used. The *ompC* and *ompF* proteins were identified by using purified proteins [6,7,27] as a reference. Protein e is the only visible protein in the preparation of strain CE 1108. Only the relevant part of the gel is shown.

## 4. Discussion

During a search for growth conditions which might lead to the expression of protein e in wild-type *E. coli* cells we observed that phosphate limitation induced the expression of protein e. As it has been shown previously that protein e of strain CE1108 is able to function as a pore for several ions (including phosphate), amino acids, sugars, nucleoside monophosphates and for bis-(paranitrophenyl)-phosphate [11–13] it must be concluded that the e pore is not specific for $P_i$. Moreover, such a small ion should be able to enter the cell through *ompF* protein and *ompC* protein pores [8,11,12]. However, it seems reasonable to assume that protein e is synthesized in order to enable the cell to scavenge as many phosphate-containing nutrients as possible under conditions of limited phosphorous supply. We therefore suggest that the e pore is probably the most effective pore for $P_i$ and/or for phosphorous containing nutrients. As specificity towards phosphorous-containing compounds is hard to imagine for a diffusion pore, the e pore could be an effective pore for anionic compounds up to a certain size in general. These possibilities will be tested in the near future.

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