ELECTROPHORETIC RESOLUTION OF THE 'MAJOR OUTER MEMBRANE PROTEIN' OF *ESCHERICHIA COLI* K12 INTO FOUR BANDS

Ben LUGTENBERG, Jos MEIJERS, Roel PETERS, Peter van der HOEK and Loek van ALPHEN

Department of Microbiology and Institute for Molecular Biology, State University, Transitorium 3, Padualaan 8, Utrecht, The Netherlands

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1. Introduction

The cell envelope of Enterobacteriaceae consists of two membranes separated by a peptidoglycan layer. Methods have been developed to separate the cytoplasmic membrane from the outer membrane [1,2]. The outer membrane of *Escherichia coli* contains 60% of the envelope protein [1]. Using polyacrylamide gelelectrophoresis, Schnaitman found six protein bands in the outer membrane fraction, of which one band (mol. wt 44 000) accounted for 70% of the total outer membrane protein [1]. This protein was referred to as the 'major outer membrane protein'. Schnaitman recently reported that this major outer membrane protein of *E. coli* strain 0 111-B4 could be resolved into three distinct bands, designated as protein bands 1, 2 and 3. Only bands 1 and 3 were found in *E. coli* K12 [3]. Henning et al. [4] too found that the major protein of outer membrane preparations of *E. coli* K12 could be resolved into two bands, for which they reported mol. wts of approx. 40 000. Ames has applied the gelelectrophoresis system of Laemmli [5] very successfully for the separation of the major envelope protein of *Salmonella typhimurium*. However, in cell envelopes of *E. coli* K12 strain HfrH she found only two major bands with mol. wts of 29 000 and 33 000 [6]. Also the system described by Neville [7] resolves the major outer membrane protein of *E. coli* K12 into two bands [8].

In this paper we describe a new gelelectrophoresis system which results in a better resolution of the major outer membrane protein of *E. coli* K12. The designation 'major outer membrane protein' is misleading as it excludes the lipoprotein (mol. wt 7000) described by Braun and Rehn [9], which certainly is a major outer membrane protein too. The designation 'major outer membrane protein' used in the present paper refers only to the 44 000 dalton protein described by Schnaitman [1]. Other authors calculated mol. wts between 29 000 [6] and 44 000 [1] for the same protein.

2. Experimental

2.1. *Strains and growth conditions*

The following *E. coli* K12 strains were used: strain PC 0008 is HfrH, *thi;* strain PC 0031 is HfrR4, *gal, tonA;* strain PC 0205 is F^-, *thr, leu, proA, purA, lacY, gal, xyl, mtl, mal, tsx, strA;* strain PC 0221 is F^-, prototrophic; strain PC 0612 is HfrKL16, thi and strain PC 1349 is F^-, *thr, leu, proA, argE, his, thi, recB21, recC22, sbcB, lacY, galK, xyl, mtl, ara, tsx, phx, strA, sup*-37 amber. The nomenclature is the one used by Taylor and Trotter [10]. Cells were grown under aeration at 37°C in yeast broth [11].

2.2. *Membrane fractions*

Cell envelopes were isolated at 0–4°C as follows: exponentially growing cells were harvested, washed with 0.9% NaCl and resuspended in 50 mM Tris–HCl pH 8.5 containing 2 mM EDTA. After sonic disruption for 4 times 15 sec under cooling, unbroken cells and large fragments were removed by centrifugation for 20 min at 1200 *g*. The supernatant fluid was centrifuged for 60 min at 225 000 *g*. The pellet obtained, con-

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taining the envelopes, was resuspended and washed once in the buffer described above and finally resuspended in 2 mM Tris–HCl, pH 7.8. Trypsin-treated cell envelopes were prepared by incubation of cell envelopes (10 mg protein per ml) in 20 mM Tris–HCl, pH 7.2, containing 100 μg trypsin per ml. After incubation for 15 min. at 37°C, the suspension was chilled at 0°C and centrifuged at 225 000 *g*. The resulting pellet was washed three times with Tris buffer and finally resuspended in 2 mM Tris–HCl, pH 7.8. Cytoplasmic and outer membranes were separated according to the procedure of Osborn et al. [2] with the modification that after the addition of EDTA the suspension was incubated for 15 min at 37°C to obtain good spheroplasts (> 98%).

2.3. *Polyacrylamide gelelectrophoresis*

We used the slab gel apparatus described by Studier [12]. The slabs had a thickness of 1.5 mm. Routinely experiments were carried out with a running gel of 9 cm length; in some experiments longer gels (18 cm) were used. The gel was prepared following the experimental details described by Ames [6], except for the composition of the gel, which is a combination of those described by Laemmli [5] and Neville [7]. For the preparation of running and stacking gels the following solutions were used: stock solution I contained 44 g acrylamide plus 0.8 g methylene bisacrylamide while stock solution II contained 30 g plus 0.8 g respectively of these components. The volumes of both solutions were adjusted to 100 ml with deionized water. The solutions were kept at 4°C in the dark and could be used for at least 2 months. A fresh solution of ammonium persulphate (10 mg/ml) was prepared just before the preparation of the gel. Running gel solution contained: stock solution I, 6.25 ml; ammonium persulphate (10 mg/ml), 0.63 ml; 10% (w/v) SDS, 0.50 ml; 0.75 M Tris-HCl, pH 8.8, 12.50 ml, and, distilled water, 5.12 ml. Stacking gel solution contained: stock solution II, 0.50 ml; ammonium persulphate (10 mg/ml), 0.12 ml; 10% (w/v) SDS, 0.050 ml; 0.25 M Tris–HCl, pH 6.8, 2.50 ml, and, distilled water, 1.83 ml. Before pouring and polymerizing the gel, the solutions were deaerated. Polymerization was started by the addition of *N,N,N′,N′*-tetramethylenediamine in a final concentration of 0.2% (v/v). The gel was stored in a humid atmosphere and used after 16–22 h. Both electrode buffer contained 0.025 M Tris, 0.19 M glycine and 0.1% SDS. The pH of this solution was 8.3.

Protein samples were prepared in 0.0625 M Tris–HCl, pH 6.8, containing SDS (2%), glycerol (10%), bromophenol blue (0.001%), 2-mercaptoethanol (5%) and protein (0.5–1.0 mg/ml). The samples were boiled for 5 min. If necessary, the samples were stored at −20°C and boiled again before application. Samples containing a mixture standard proteins of known molecular weight were prepared as described previously and contained 1 μg of each protein per 20 μl of sample.

Routinely 20 μl of a sample were applied per slot. Electrophoresis was carried out at room temperature using a constant current of 25 or 30 mA per gel. The electrophoresis was stopped when the tracking dye was about one cm from the bottom of the gel, which happened after 3.5 or 4.5 h, depending on the current applied. Gels were stained overnight, under gentle shaking, in a solution of 0.1% Fast Green FCF in 50% methanol – 10% acetic acid. Gels were destained in 50% methanol – 10% acetic acid. Scanning of the band pattern was performed at a wave length of 536 nm with a Vitatron TLD 100 densitometer at a rate of 0.5 cm/min. In order to store the slabs, they were dried after soaking for at least 24 h in a solution of 50% methanol – 5% glycerol. The gel was spread on Whatman 3 MM chromatography paper and dried for 1.5 h at about 50°C in a glazing dryer. In order to release the gel from the metal plate of the glazing dryer, the plate was cooled for 1 h at 4°C.

2.4. *Molecular weights*

Protein bands are indicated by their molecular weights multiplied by 10^{-3} and followed by the letter K. The molecular weights of the standard proteins are indicated in the figures and refer to the following proteins: bovine serum albumin (67 K), catalase (60 K), ovoalbumin (45 K), lactate dehydrogenase (36 K), chymotrypsinogen A (25 K) and hen egg lysozyme (14 K). All standard proteins gave rise to one band except catalase which gave two bands very close to each other.

2.5. *Chemicals*

Acrylamide, methylenebisacrylamide and Triton-X 100 were obtained from Serva, Heidelberg, G.F.R.; Fast Green FCF from Sigma Chemical Co., St. Louis,

Mo., USA; sodium dodecylsulphate from BDH, Poole, England; glycine and hen egg lysozyme from Fluka, Buch, Switzerland. All other standard proteins of known molecular weight, used as standard and trypsin were obtained from Boehringer Mannheim, Amsterdam, The Netherlands.

3. Results and discussion

3.1. *Improved resolution of the major outer membrane protein*

When membrane proteins of *E. coli* K12 strain PC 0008 were separated with the system according to Laemmli [5] the bands were very sharp but only two major outer membrane protein bands were visible. This observation is in agreement with a previous report in which envelope proteins of the same strain were described [6]. With the system described by Neville [7] three major outer membrane protein bands were visible but the pattern was rather diffuse. The system described in this paper, which can be considered as a combination of the two systems mentioned above, allows the resolution of the major envelope protein into four sharp bands designated **a**, **b**, **c** and **d** (fig.1A). Except for some outer membrane mutants, this result was found for all *E. coli* K12 strains tested so far.

Comparison of samples of envelopes, cytoplasmic and outer membranes showed that the polypeptides

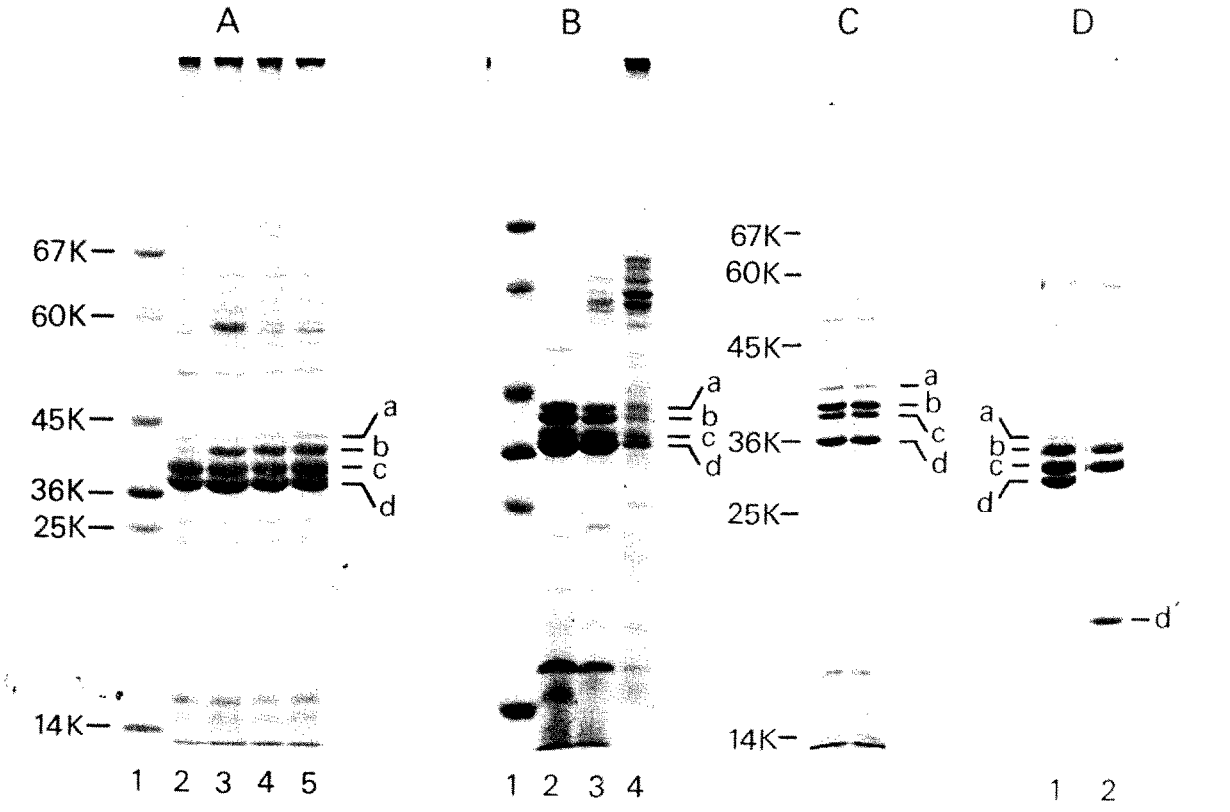

Fig.1. SDS-polyacrylamide gelelectrophoresis of membrane fractions of *E. coli* K12. All samples contained 20 μg of protein. The molecular weights of the standard proteins are indicated. A, 1: molecular weight standards; 2–5: cell envelope proteins of *E. coli* K12 strains PC 0221, PC 008, PC 0612 and PC 0031 respectively. B, 1: molecular weight standards; 2–4: outer membrane, cell envelopes and cytoplasmic membrane respectively of strain PC 1349. C: Longer gel (18 cm) with a cell envelope preparation of strain PC 1349. Pure SDS was used. D: Effect of incubation of cell envelopes of strain PC 0205 with trypsin. 1: cell envelopes; 2: cell envelopes after treatment with trypsin. The degradation product of **d**, band **d′**, is indicated.

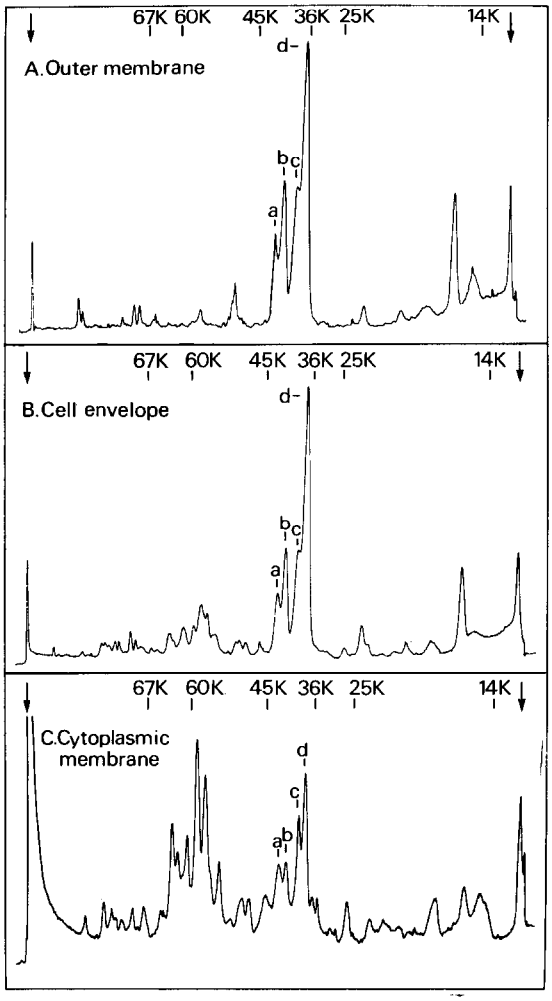

Fig.2. Scans of the three membrane preparations from the gel shown in fig.1B. The position of the molecular weights standard proteins are indicated. Arrows at the left-hand side indicate the top of the running gel, arrows at the right-hand side indicate the position of the tracking dye. It should be noted that the peak, corresponding with the fastest moving band is partly artificial, due to a turbid spot in that position (presumably lipid).

represented by these four bands, are all located in the outer membrane (fig.1B). Fig.2 shows scans of the protein patterns of the three membrane preparations of fig.1B. From these scans it is clear that **b**, **c** and **d** are major bands, while band **a** is rather weak. The results described so far were obtained with SDS (BDH, cat. no. 30175) that is contaminated with C10 and C14 derivatives. Recently it was observed that with more purified SDS (Serva, cat. no. 20760 or BDH cat. no. 30176) bands **a**, **b** and **c** are not separated from each other. However, when pure SDS was used in longer gels, bands **a**, **b** and **c** were also separated (fig.1C), showing that they are not artificial bands derived from one polypeptide. Consequently the new system resolves four outer membrane protein bands of *E. coli* K12 instead of the two bands described up till now [3,4,8]. From gels ran with pure SDS the following apparent molecular weights were calculated: **a** : 40 K; **b** : 38.5 K; **c** : 38 K; and **d** : 36 K.

The method described for drying of the slabs is extremely simple and can easily be used for at least nine gels at a time. Drying did not significantly influence the band pattern as was concluded from autoradiograms of gels containing labelled proteins (not shown).

3.2. *Comparison with other gel systems*

In order to compare the bands **a**, **b**, **c** and **d** with bands of other gel systems we tested preparations of a number of membrane fractions in various gel systems. The preparations included purified proteins **b** and **c** and cell envelopes of a strain that is deficient in polypeptide **d**. From the patterns obtained (fig.3) it was possible to indicate the positions of bands **b**, **c** and **d** in other gel systems, except in the one described by Henning et al. [4] as method II. The position of band **a** could be established in the systems described by

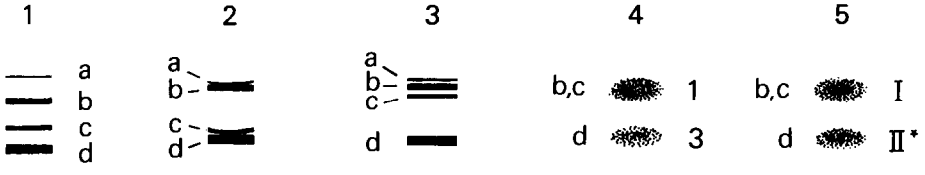

Fig.3. Schematic representation of patterns of the major outer membrane protein bands in various gel systems. The designation used by the authors is given at the right hand side. The position of bands **a**, **b**, **c** and **d** is given at the left hand side of each pattern. The gel systems are 1, the one described in this paper; 2, Laemmli [5]; 3, Neville [7]; 4, the Bragg-Hou system as used by Schnaitman [13]; 5, the system II described by Henning et al. [4]. For further explanation see text.

Laemmli [5] and Neville [7] because band **a** was partly separated from band **b**. This was not the case in the system used by Schnaitman [13]. With method II described by Henning et al. [4] we were unable to resolve the major outer membrane protein into more than one band. However, as both Henning's protein II* and protein **d** are sensitive to trypsin (fig.1D), heat-modifiable [14] and absent in *tolG* mutants [15], they must be identical. Henning's protein I consists of two polypeptides (references 16 and 17 and U. Henning, personal communication), one of which is the matrix protein isolated by Rosenbusch [18] from *E. coli* BE [17]. We isolated this protein from *E. coli* BE and found that it moved to the position of band **b**. We assume that the other polypeptide in band I is protein **c**. The position of polypeptide **a** in Henning's system remains unknown.

Schnaitman [13] reported that band 3 consists of two different polypeptides 3a and 3b. In *E. coli* K12 strain P 400 the amount of protein 3b is small and its bacteriophage K3 resistant mutant P 460 is missing protein 3a, while protein 3b is present. (P. Reeves, personal communication). Protein **d** was found to be absent from gel profiles of mutant P460, using our gel system. Bands **d** and II* are therefore most likely identical to band 3a and might contain small amounts of protein 3b.

Protein IV described by Henning et al. [4,16] corresponds with Braun's lipoprotein [4]. This is the only envelope protein that can be labelled under conditions of starvation of a histidine auxotroph for histidine [19]. It is also the only envelope protein that is soluble in 10% trichloroacetic acid after boiling of envelopes with SDS [20]. We found that only the fastest moving protein in our system (fig.1) has these properties (not shown). The amount of dye that is bound by this lipoprotein varies from gel to gel. It gives a visible band in fig.1A, B and C.

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References

[1] Schnaitman, C. A. (1970) J. Bacteriol. 104, 890–901.
[2] Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972.
[3] Schnaitman, C. A. (1974) J. Bacteriol. 118, 454–464.
[4] Henning, U., Höhn, B. and Sonntag, I. (1973) Eur. J. Biochem. 39, 27–36.
[5] Laemmli, U. K. (1970) Nature (London) 227, 680–685.
[6] Ames, G. F. (1973) J. Biol. Chem. 249, 634–644.
[7] Neville, D. M. (1971) J. Biol. Chem. 246, 6328–6334.
[8] Skurray, R. A., Hancock, R. E. W. and Reeves, P. (1974) J. Bacteriol. 119, 726–735.
[9] Braun, V. and Rehn, K. (1969) Eur. J. Biochem. 10, 426–438.
[10] Taylor, A. L. and Trotter, C. D. (1972) Bacteriol. Rev. 36, 504–524.
[11] Lugtenberg, E. J. J. and de Haan, P. G. (1971) Antonie van Leeuwenhoek J. Microbiol. Serol. 37, 537–552.
[12] Studier, F. W. (1973) J. Mol. Biol. 79, 237–248.
[13] Schnaitman, C. A. (1974) J. Bacteriol. 118, 442–453.
[14] Reithmeier, R. A. F. and Bragg, P. D., FEBS Lett. (1974) 41, 195–198.
[15] Chai, T. and Foulds, J. (1974) J. Mol. Biol. 85, 465–474.
[16] Garten, W. and Henning, U. (1974) Eur. J. Biochem. 47, 343–352.
[17] Henning, U. and Haller, I., FEBS Lett. in press.
[18] Rosenbusch, J. P. (1974) J. Biol. Chem. 249, 8019–8029.
[19] Hirashima, A. and Inouye, M. (1973) Nature (London) 242, 405–407.
[20] Hirashima, A., Wu, H. C., Venkateswaran, P. S. and Inouye, M. (1973) J. Biol. Chem. 248, 5654–5659.