Major Outer Membrane Proteins of *Escherichia coli* Strains of Human Origin

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The major outer membrane protein patterns of 45 *Escherichia coli* strains of human origin were compared with that of *E. coli* K12 by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Preparations of the former strains contained between two and five major bands in the molecular weight range between 30000 and 42000. The patterns were very heterogeneous with respect to the numbers and electrophoretic mobilities of the major outer membrane protein bands. In all cases the fastest moving band was characterized as a protein similar to the *ompA* protein of strain K12 as it was partially degraded by trypsin and reacted specifically with antiserum against the purified *ompA* protein in a gel immuno-radioassay. All the other major outer membrane proteins are related to the *ompC* and *ompF* proteins (the porins) of strain K12 as they were peptidoglycan-associated and reacted with antisera against the purified *ompC* and/or *ompF* proteins.

INTRODUCTION

The cell envelope of the *Enterobacteriaceae* consists of three layers: a cytoplasmic membrane, a peptidoglycan layer and, covalently attached to it, an outer membrane (DiRienzo *et al.*, 1978). Analysis of the outer membrane protein pattern with sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis revealed several so-called ‘major’ proteins (Ames *et al.*, 1974; Lugtenberg *et al.*, 1975). As the relative amounts of several of these proteins are dependent on the growth conditions (Van Alphen & Lugtenberg, 1977; Braun *et al.*, 1976; Braun & Krieger-Brauer, 1977; Lugtenberg *et al.*, 1976; Overbeeke & Lugtenberg, 1980), this term can be misleading. The outer membrane protein pattern of *Escherichia coli* K12 is characteristic in that usually three major bands are observed in the molecular weight range between 30000 and 42000. The nomenclature of these proteins, designated b, c and d in our laboratory (Lugtenberg *et al.*, 1975), was different in other laboratories (Bassford *et al.*, 1977; Henning & Haller, 1975; Uemura & Mizushima, 1975). Recently, a new nomenclature has been agreed in which the proteins are called after their structural genes (Reeves, 1979), i.e. *ompF*, *ompC* and *ompA*, respectively, for the proteins b, c and d.

The *ompA* protein, which plays a role in acceptor cells in F-pilus-mediated conjugation (Van Alphen *et al.*, 1977; Skurray *et al.*, 1974), is partially degraded when cell envelope preparations are incubated with trypsin, resulting in a major degradation product with a molecular weight of about 18000 (Henning *et al.*, 1973). The *ompF* and *ompC* proteins form tight complexes with peptidoglycan which resist incubations in 2% SDS at 60°C (Lugtenberg *et al.*, 1976; Rosenbusch, 1974). The latter proteins are involved in the formation of hydrophilic pores through which nutrients and other solutes with a molecular weight of up to about 700 can pass the outer membrane (Van Alphen *et al.*, 1978*a, b*;

Bavoil *et al.*, 1977; Lutkenhaus, 1977; Nakae, 1976); they are therefore called porins (Nakae, 1976). There are considerable differences between these two pore proteins with respect to the patterns of their cyanogen bromide and proteolytic fragments (Ichihara & Mizushima, 1978; Verhoef *et al.*, 1979), but their total amino acid composition and their amino termini are very similar (Ichihara & Mizushima, 1978; Verhoef *et al.*, 1979; Henning *et al.*, 1977). Immunological studies with antisera raised against the purified proteins showed an antigenic relationship between the *ompF* protein and the *ompC* protein, while antiserum raised against the *ompA* protein only reacted with the homologous protein (Overbeeke *et al.*, 1980).

As most studies of the outer membrane proteins of *E. coli* have been carried out with strain K12, very little is known about the outer membrane proteins of other *E. coli* strains. Because we were interested to know whether the situation in other strains of human origin resembles that known for strain K12, we compared several biochemical and immunological properties of the outer membrane proteins of strain K12 with those of many recently isolated hospital strains. In this study, we show that the major outer membrane proteins of the latter strains, in spite of considerable heterogeneity of their patterns on gels, resemble those of *E. coli* K12 in several biochemical and immunological properties.

METHODS

Strains and growth conditions. The multi-auxotrophic *E. coli* K12 strain PC0479 (Verhoef *et al.*, 1979) was used as a reference. The 45 *E. coli* strains used were isolated from human faeces, blood and urine. They were obtained from W. C. van Dijk and J. Verhoef, Department of Microbiology, State University, Utrecht, The Netherlands. Their relevant properties are listed in Table 1. Cells were grown in yeast broth (Lugtenberg *et al.*, 1976) at 37 °C under vigorous aeration to the late exponential phase, harvested at 4 °C and washed with 0.9 % (w/v) NaCl.

Isolation and characterization of membrane fractions. Cell envelopes were prepared by differential centrifugation after disruption of the cells by ultrasonic treatment (Lugtenberg *et al.*, 1975). A fraction containing the outer membrane proteins was isolated from cell envelopes by a slight modification (Lugtenberg *et al.*, 1976) of the Triton X-100 extraction procedure described by Schnaitman (1974). A fraction containing proteins tightly associated with the peptidoglycan was prepared by incubation of cell envelopes at 60 °C in the presence of 2 % SDS followed by ultracentrifugation (Lugtenberg *et al.*, 1977). Trypsin-treated cell envelopes were prepared by incubation of cell envelopes (300 to 400 µg protein) in 25 mM-Tris/HCl buffer pH 7.2 containing 50 µg trypsin ml⁻¹, in a final volume of 100 µl. After incubation for 1 h at 37 °C, the mixture was chilled in ice-water, then 100 µl buffer (50 mM-Tris/HCl pH 8.5 containing 2 mM-EDTA and 0.2 M-KCl) was added, and cell envelopes were harvested by centrifugation for 15 min at 4 °C in an Eppendorf table centrifuge and washed once with 50 mM-Tris/HCl pH 7.2. Procedures used for the purification of heat unmodified, biologically active *ompA* protein (Van Alphen *et al.*, 1977), SDS and heat modified *ompC* and *ompF* protein (Verhoef *et al.*, 1979) and for lipopolysaccharide (LPS) (Galanos *et al.*, 1969) have been described previously. These purified proteins were used as the antigens. Unless otherwise indicated, the protein patterns of membrane fractions were analysed using the SDS-polyacrylamide gel electrophoresis system described by Lugtenberg *et al.* (1975). In some cases 4 M-urea was added to the running gel in order to obtain a different resolution (M. Achtman, personal communication).

Preparation of antisera. Antisera against the purified *ompA*, *ompC* and *ompF* proteins were raised in rabbits by H. Hofstra, Laboratory for Medical Microbiology, University Hospital, Groningen, The Netherlands, as described by Hofstra & Dankert (1980). Briefly, after purification of the proteins as described, 5 mg proteins was suspended in 0.5 ml 0.9 % NaCl and mixed with the same volume of Freund's complete adjuvant. The antigens were administered in the upper hindleg region. After 4 weeks this injection was repeated, and blood was collected 7 weeks after the first injection. As the antisera contained activity against LPS in a haemagglutination assay, using sheep red blood cells coated with purified LPS, the antisera were absorbed by two successive incubations of 0.9 ml serum with 0.1 ml of a sonically treated suspension of purified *E. coli* K12 lipopolysaccharide (10 mg ml⁻¹ in 10 mM-sodium phosphate buffer pH 7.2 containing 0.9 % NaCl) and the precipitates were removed by centrifugation. After this treatment anti-LPS activity was undetectable.

Iodination of Protein A. Protein A of *Staphylococcus aureus* (Pharmacia, Uppsala, Sweden) was labelled with ¹²⁵I (The Radiochemical Centre, Amersham) using chloramine T (Hunter & Greenwood, 1962) by a modified method described by Erlich *et al.* (1978).

Table 1. *Escherichia coli* strains of human origin

Strain*	Serotype†	Strain*	Serotype†
F1	O78 K—	S12	O2 K1
F2	O15 K?	S13	O18 K1
F3	O41	S14	O14 K—
F4	O88	S15	O4 K?
F5	O1 K1	S16	O78 K80
F6	O77 K96	S17	O106
F7	NT	S18	O15 K?
F8	O156	S20	O23 K18
F9	O127 K63	U3	O6 K53
F10	NT	U4	O134
F11	NT	U6	NT
F12	O2 K?	U7	O1 K1
S1	O75 K?	U8	O40
S2	O7 K?	U11	NT
S3	O18 K?	U13	AA
S4	O77 K96	U14	O18 K?
S5	O51	U15	AA
S6	O5 K?	U16	O18 K1
S7	O78 K80	U17	AA
S8	O4 K12	U18	O71
S9	O6 K—	U19	O9 K?
S10	O75 K—	U20	O15 K?
S11	O22 K24		

* F denotes strains isolated from faeces of healthy volunteers; S denotes strains isolated from blood cultures of patients with *E. coli* bacteraemia; U denotes strains isolated from patients with urinary tract infection. All strains were isolated in the Netherlands.

† Serotyping was performed by Dr P. A. M. Guinée, National Institute for Public Health, Bilthoven, The Netherlands, using tube agglutination. Abbreviations: K—, no K antigen detected using all K antisera; K?, strains did not react in any of the K antisera nor in their homologous O antiserum in the living state; NT, not typeable; AA, autoagglutinable.

Gel immuno-radioassay (GIRA). The procedure according to J. T. Poolman and others (personal communication) was slightly modified. The proteins of Triton X-100/MgCl₂-extracted cell envelopes were separated by SDS-polyacrylamide gel electrophoresis as described previously (Lugtenberg *et al.*, 1975), except that the gels were 3 mm thick and electrophoresis was carried out at 50 mA for 2.5 h. Subsequently 4.5 × 5 cm pieces, containing the region with the major membrane proteins, were cut out and immediately frozen at -70 °C. These pieces were sliced into 50 µm thin longitudinal sections at -30 °C using a cryostat microtome (Bright 5030). The gel slices were further handled as described in detail by Raamsdonk *et al.* (1977). After removal of SDS, the gel slices, spread on 8 × 8 cm glass slides, were incubated with 350 µl of an appropriate dilution (usually 1:250) of antiserum for 30 min at 37 °C, followed by incubation for 16 h at 4 °C. After floating the gel slices from the glass slides, they were transferred to Petri dishes and washed three to five times for 1 h with 10 mM-potassium phosphate buffer pH 7.2 containing 0.9 % NaCl, in order to remove excess and non-specifically bound IgG. The gel slices were again spread on the glass slides and ¹²⁵I-labelled Protein A was allowed to bind to IgG by incubating each slice for 2 h at room temperature with 350 µl of a solution containing ¹²⁵I-labelled Protein A at 3 µCi ml⁻¹ (110 kBq ml⁻¹). After intensive washing, the gel slices were spread on the glass slides again and dried. Membrane proteins with attached IgG were detected by exposing the slice for 40 to 60 h to Fuji X-ray film. To check for non-specific adsorption of IgG, control slices were incubated with normal rabbit serum.

RESULTS

Major outer membrane protein patterns

The pattern of the major outer membrane proteins of *E. coli* K12 was compared with those of recently isolated strains from various human sources (see Table 1). Preparations containing the outer membrane proteins, but not those of the cytoplasmic membrane, were obtained as a Triton X-100/MgCl₂-insoluble cell envelope fraction. Such a preparation

Fig. 1. SDS-polyacrylamide gel electrophoresis of outer membrane proteins of F strains (A), U strains (B, C) and S strains (D, E) in the absence (A, B, D) or presence (C, E) of 4 M-urea in the running gel. Only the relevant part of the gel, containing the major outer membrane proteins, is shown.

contained all major proteins present in the cell envelope (not shown). Preliminary experiments showed that of several SDS-polyacrylamide gel electrophoresis systems tested, the best resolution, with respect to the number of major protein bands, was obtained with the two systems described previously. The patterns obtained for the major outer membrane proteins with apparent molecular weights between about 30000 and 42000 are shown in Fig. 1. The results show that the patterns from the strains from faeces (F) (Fig. 1A), urine (U) (Fig. 1B, C) and blood (S) (Fig. 1D, E) are all different from that of *E. coli* K12. In a number of cases a better resolution was obtained by including 4 M-urea in the running gel, as can be observed for strains U3, U11 and U14 by comparing Fig. 1(B) and 1(C) and for strains S1, S2, S4, S8, S9 and S11 by comparing Fig. 1(D) and 1(E). Based on the number of major protein bands, which ranged from two to five, and on their electrophoretic mobilities, 36 different patterns were observed among the 45 strains tested (Fig. 1). Attempts to correlate a certain type of protein pattern with a certain serotype for the O and/or K antigens, and vice versa, revealed that, except for the five strains with a K1 antigen, such a tight correlation did not appear to exist. The five K1 strains used in this study came from different sources (Table 1) and had indistinguishable protein patterns (strains F5, S12, S13, U7 and U16, see Fig. 1). However, examination of the outer membrane proteins of a larger number of K1 strains revealed that different patterns can also be observed among strains of this K serotype (L. Van Alphen, personal communication).

Biochemical characterization of major outer membrane proteins

The *ompA* protein of strain K12 is unique in that it is the only major outer membrane protein which is degraded during incubation of cell envelopes with trypsin, leaving a large degradation product with a molecular weight of about 18000 associated with the cell envelope (Henning *et al.*, 1973). The known porins of *E. coli* K12 are the only proteins

Fig. 2. SDS-polyacrylamide gel electrophoresis of membrane fractions of strain U13. With the cell envelope preparation (C) as the starting material, the following fractions were obtained: Triton X-100/MgCl₂-insoluble material, containing the outer membrane proteins (D), a soluble (A) and an insoluble (B) fraction obtained after extraction in 2 % SDS at 60 °C, and a cell envelope sample treated with trypsin (E).

Fig. 3. SDS-polyacrylamide gel electrophoresis of outer membrane proteins of strain U13 on a 3 mm thick gel. After slicing, one section was stained (A), whereas others were incubated with anti-*ompF* protein serum (B), anti-*ompC* protein serum (C), anti-*ompA* protein serum (D) and normal rabbit serum (E) according to the GIRA technique (see Methods).

that remain attached to the peptidoglycan during extraction of cell envelopes with 2 % SDS at 60 °C (Lugtenberg *et al.*, 1976). The outer membrane proteins of the recently isolated *E. coli* strains were tested for these properties in an attempt to correlate their protein patterns with that of strain K12. The results, which were similar for all strains (for an example, see Fig. 2), can be summarized as follows. For all strains the fastest moving band (in both gel systems) is the only major protein that is soluble in 2 % SDS at 60 °C (Fig. 2A) and degraded by trypsin (Fig. 2E). After the latter treatment a new major band appeared with an apparent molecular weight of about 18000. A similar situation was observed in *E. coli* K12 where the new protein is known to be a degradation product of the *ompA* protein (Lugtenberg *et al.*, 1976). Additional evidence for the notion that the new protein generated by trypsin treatment is a degradation product of the fastest moving major protein came from the observation that the differences in the electrophoretic mobility of this protein among the various strains (Fig. 1C, E) were mimicked by the pattern of the protein bands which were generated by trypsin (results not shown). Except for the fastest moving protein, all other major proteins of all F, S and U strains, present in one to four bands, shared the properties that they were resistant to trypsin treatment (Fig. 2E) and tightly associated with peptidoglycan (Fig. 2B). In these respects they behave like the known porins of strain K12 (Lugtenberg *et al.*, 1976).

Immunological characterization of major outer membrane proteins

Although the biochemical properties tested have been well established for the *E. coli* K12 proteins, they are not very well understood. Therefore, we also tested the possible immunological relationships between the major outer membrane proteins of strain K12 and the

new strains using the gel immuno-radioassay technique. This method is extremely sensitive and has the advantage that, as many slices can be cut from the same gel, the stained pattern of 10 to 12 strains can directly be compared with the patterns observed after reaction with the different antibody preparations (see Fig. 3). The results showed that, for all strains, antibodies against the purified *ompA* protein reacted specifically with the fastest moving band (Fig. 3D), whereas normal rabbit serum showed no reaction at all (Fig. 3E). These results indicate that the trypsin-sensitive proteins are immunologically related to the *ompA* protein of *E. coli* K12. The major peptidoglycan-associated proteins reacted with antibodies against the purified *ompC* and/or *ompF* proteins, while different degrees of affinity of the two antisera towards the various proteins were often observed. For example, Fig. 3 shows that the anti-*ompF* protein serum has more affinity for the peptidoglycan-associated proteins 1 and 2 (Fig. 3B), whereas anti-*ompC* protein serum has more affinity for the peptidoglycan-associated protein 3 (Fig. 3C). The results showed that all major peptidoglycan-associated proteins of all recently isolated strains have antigenic determinants in common with at least one of the *E. coli* K12 porins.

DISCUSSION

The patterns of major outer membrane proteins of 45 *E. coli* strains were investigated and compared with that of strain K12. The patterns of all recent isolates differed from that of strain K12. Surprisingly, the patterns of the former strains were extremely heterogeneous in that, based only on the electrophoretic mobilities of the major proteins, 36 different types were distinguished. The interpretation of these results could vary between the following extremes: (i) many *E. coli* strains basically differ from each other with respect to the structures of their major proteins; (ii) these proteins differ mainly from each other in apparent molecular weight due to evolutionary processes but proteins with very similar structures and functions are present in all strains. In order to distinguish between these two extremes, the outer membrane proteins were tested for several biochemical and immunological properties known for the major outer membrane proteins of strain K12. The results showed that in all recent isolates the major protein with the highest electrophoretic mobility behaved like the *ompA* protein of strain K12 (Fig. 2 and 3). All other major proteins were peptidoglycan-associated (Fig. 2 and text) and react with anti-*ompC* protein serum and/or anti-*ompF* protein serum (Fig. 3 and text).

It should be noted that recent results with *E. coli* K12 outer membrane proteins have shown that anti-*ompA* protein serum reacts only with the homologous protein whereas anti-*ompC* and anti-*ompF* protein sera react with both porins (Overbeeke *et al.*, 1980). Thus, we conclude that all major outer membrane proteins of the recent isolates are closely related either to the *ompA* protein or to one or both of the porins of strain K12. Similar results were observed when the relationship between the major outer membrane proteins of *E. coli* K12 was compared with those of other *Enterobacteriaceae* (Hofstra & Dankert, 1979; Overbeeke *et al.*, 1980). Thus, all peptidoglycan-associated major outer membrane proteins of *Enterobacteriaceae* in the molecular weight range between 30000 and 42000 are structurally related with at least one of the K12 porins and therefore probably function as general pores.

Many of the strains tested are encapsulated (see Table 1). Recently, Paakkanen *et al.* (1979) reported the presence of a specific major outer membrane protein in encapsulated strains of *E. coli* with an apparent molecular weight in gradient gels of 40000. With respect to its amino acid composition and amino terminus, this K protein is very similar to the porins of strain K12. Comparison of the outer membrane protein patterns of the strains used in the present study does not show the presence of a band common for all encapsulated strains (Fig. 1). The difference in electrophoretic mobilities of the *ompA*-like proteins and of the porin-like proteins observed in the various strains even suggest that a protein with

a certain function has been subject to different patterns of evolution in various strains, resulting in differences in electrophoretic mobilities. Although our results certainly do not exclude the existence of a K protein, they make it likely that such a protein would not have exactly the same electrophoretic mobility in all encapsulated strains.

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