Major Outer Membrane Proteins: Common Antigens in Enterobacteriaceae Species

By H. HOFSTRA* AND J. DANKERT

Laboratory for Medical Microbiology, State University Groningen and Division of Hospital Infections, University Hospital A.Z.G., Oostersingel 59, 9713 EZ Groningen, The Netherlands

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The major outer membrane (OM) proteins of 23 enterobacterial strains (principally clinical isolates) and five non-Enterobacteriaceae species were investigated by the sodium dodecyl sulphate–polyacrylamide gel immunoperoxidase (SGIP) technique to evaluate antigenic cross-reactivity among these proteins. All enterobacterial strains contained one or more peptidoglycan-associated major OM proteins, cross-reactive with the peptidoglycan-bound protein I of *Escherichia coli*, and one non-peptidoglycan-bound heat-modifiable protein, cross-reactive with protein II* of *E. coli*. Results indicated that antigenic cross-reactivity of the major OM proteins is a general phenomenon in the family Enterobacteriaceae, independent of any molecular weight variation of the corresponding proteins in different bacterial strains. SGIP experiments carried out with OM preparations of other species showed no cross-reactivity of any of their OM proteins with enterobacterial major OM proteins. The significance of the immunological relatedness of OM proteins for the classification of some Enterobacteriaceae is discussed.

INTRODUCTION

The outer membrane (OM) of members of the family Enterobacteriaceae contains two to four major proteins in the 30000 to 42000 molecular weight range (Lugtenberg *et al.*, 1977). In *Escherichia coli* K12 these proteins have been well characterized. Some, designated Ia and Ib (Schmitges & Henning, 1976) or b and c (Lugtenberg *et al.*, 1975), are associated with the peptidoglycan layer. Another protein, the heat-modifiable protein II* (Garten *et al.*, 1975) or d (Lugtenberg *et al.*, 1975), is not peptidoglycan-associated and can be solubilized in buffer containing sodium dodecyl sulphate (SDS) at 37 °C (Reithmeier & Bragg, 1974). The presence of peptidoglycan-bound and non-peptidoglycan-bound proteins has been detected in the OM of various species of Enterobacteriaceae (Ames *et al.*, 1974; Lugtenberg *et al.*, 1977; Rottem *et al.*, 1979).

The antigenic cross-reactivity of the major OM proteins in Enterobacteriaceae species was previously investigated using the interfacial immunoprecipitation test (Hofstra & Dankert, 1979). In these experiments, carried out with antiserum prepared against whole formaldehyde-fixed bacteria, a weak though significant cross-reactivity was detected among the major OM proteins of several *E. coli* serotypes, while a weaker cross-reactivity was found with OM proteins of *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Proteus vulgaris*. The antiserum applied in these experiments contained a low titre of antibodies against OM proteins, as shown recently using the enzyme-linked immunosorbent assay (Hofstra & Dankert, 1980a). Furthermore, the compulsory use of SDS as a solubilizing agent in interfacial immunoprecipitation experiments performed with OM preparations may have reduced the sensitivity of this test.

The SDS-gel immunoperoxidase (SGIP) technique, developed by Van Raamsdonk *et al.*

(1977), was used for the immunochemical characterization of *Neisseria meningitidis* OM (Poolman *et al.*, 1978). Using this method it was possible to demonstrate the antigenic relationship between OM proteins of a wild-type strain of *E. coli* and four species of the genus *Proteus* (Hofstra & Dankert, 1980b), although CNBr cleavage experiments failed to demonstrate any similarities between the peptidoglycan-bound major OM proteins of *E. coli* K12 and *P. mirabilis* (Datta *et al.*, 1976).

In this study we have used the SGIP technique to investigate the antigenic relationship of the major OM proteins in seven serotypes of *E. coli*, sixteen related Enterobacteriaceae species and five Gram-negative rod-like bacteria not belonging to the Enterobacteriaceae.

METHODS

Bacterial strains and growth conditions. *Escherichia coli*, serotypes O1K⁻, O26K60, O55K59 and O75K⁻, *Edwardsiella tarda*, *Salmonella typhimurium* VI 260, *Citrobacter freundii*, *Klebsiella pneumoniae* (*K. aerogenes*), *Enterobacter aerogenes*, *Ent. cloacae*, *Serratia marcescens*, *Proteus vulgaris*, *P. morganii* (*Morganella morganii*), *P. rettgeri*, *P. mirabilis*, *Providencia stuartii* (*Proteus stuartii*), *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Acinetobacter calcoaceticus* subtype *anitratus* and *Alcaligenes faecalis* were isolated from faecal samples obtained from healthy subjects. *Escherichia coli* O4K2 was isolated from an infected wound in the oral cavity of a seal. *Shigella flexneri*, *Salmonella panama*, *S. braenderup* and *S. derby* were isolated from faecal samples obtained from patients with diarrhoea. *Pseudomonas fluorescens* was isolated from a contaminated sink. *Escherichia coli* O111K58 and its galactose epimerase-deficient mutant strain J5 were supplied by Dr B. Witholt, Laboratory of Biochemistry, State University, Groningen. Strains of *E. coli* and *Salmonella* were serotyped by the Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands. The other strains were identified in our laboratory according to Cowan (1975) and Lennette *et al.* (1978). Nomenclature according to the *Manual of Clinical Microbiology* (Lennette *et al.*, 1978) was used in this study. Alternative names for some strains, as preferred by Cowan (1975), are given in parentheses. Bacteria were grown as previously described (Hofstra & Dankert, 1979).

Isolation of outer membrane. Outer membranes (OM) of *E. coli* strains were isolated by Triton X-100 extraction of a crude envelope fraction according to Schnaitman (1971) as described in detail for *E. coli* O26K60 (Dankert & Hofstra, 1978). From the other bacterial species, OM were isolated by a simple and quick method developed by P. Sloots and B. Witholt, Laboratory of Biochemistry, State University, Groningen (unpublished procedure). Briefly, late-exponential phase cells from a 3 l culture were washed with 0.9 % (w/v) NaCl and suspended in 50 ml 0.1 M-Tris/HCl buffer (pH 8.0) containing 2.5 mM-EDTA. Subsequently, cells were subjected to a series of short sonic oscillations (20 to 30 s; MSE, London), each period being followed by cooling on ice. Sonic treatment was continued until the absorbance of the suspension, measured at 450 nm (Beckman, Fullerton, Calif., U.S.A.), had decreased to approximately 10 % of the original value. Unbroken cells were removed by centrifugation (6000 g, 10 min, 4 °C). The supernatant was then centrifuged at 30000 g for 20 min at 4 °C. The sediment was washed twice with deionized water and lyophilized (Edwards, Crawley, Sussex). The resulting preparation was almost pure OM as analysed by polyacrylamide gel electrophoresis. Subsequent Triton X-100 extraction of these fractions according to Schnaitman (1971), to remove contaminating cytoplasmic membrane material, did not significantly change the electrophoretic profile of the major OM protein range (25000 to 42000 molecular weight). This extraction step was therefore usually omitted.

Extraction of non-peptidoglycan-bound OM proteins. OM were dispersed to a concentration of 1 mg ml⁻¹ in 62.5 mM-Tris/HCl buffer (pH 6.7) containing 2 % (w/v) SDS, followed by incubation at 37 °C for 30 min. The insoluble material was sedimented by centrifugation at 110000 g for 30 min at 15 °C. To investigate possible heat-modifiability of the extracted proteins the supernatants were divided in two equal parts, one of which was heated at 100 °C for 10 min, and the other kept at room temperature. Subsequently both samples were supplemented with glycerol (10 %, v/v) and Bromophenol Blue (0.001 %, w/v) and analysed by polyacrylamide gel electrophoresis.

Isolation of OM proteins of *E. coli* O26K60. Major OM proteins I and II* and murein-lipoprotein were isolated and purified as described previously (Hofstra & Dankert, 1980a).

Isolation of lipopolysaccharide (LPS). LPS of *E. coli* O26K60 was isolated according to Westphal *et al.* (1952) as described previously (Hofstra & Dankert, 1979).

Preparation of antisera. Antisera against the isolated OM proteins I and II* of *E. coli* O26K60 were elicited by intramuscular administration to rabbits. Two pairs of rabbits received 5 mg protein I or II*, respectively, suspended in 0.5 ml 0.9 % (w/v) NaCl and the same volume of Freund's complete adjuvant. The antigens

were administered in the upper hindleg region at days 0 and 28. Blood was collected 3 weeks after the second injection. Sera were obtained after centrifugation of the clotted blood and stored at -70°C . Both types of antiserum contained antibodies against LPS O26. In addition, a considerable amount of anti-murein-lipoprotein was present in the antiserum against protein I. Antisera monospecifically directed against protein I or II* were prepared by absorption of the sera with LPS O26 and murein-lipoprotein (Hofstra & Dankert, 1980a). The monospecificity of the absorbed antisera was controlled using the enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay. This was carried out as described by Hofstra & Dankert (1980a).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This was carried out according to Laemmli (1970) on slab gels as described by Ames (1974). Staining, destaining and photography of the gels, as well as the application of standard proteins for molecular weight estimation, were described by Dankert & Hofstra (1978).

SDS-gel immunoperoxidase technique (SGIP). This was performed essentially according to Van Raamsdonk *et al.* (1977) and Poolman *et al.* (1978). Briefly, OM preparations of all strains studied were subjected to SDS-PAGE. The unstained gel was cut into 3×4 cm pieces, each containing three electrophoretic profiles approximately covering the 20000 to 50000 molecular weight range. These gel segments were sliced into $40\text{ }\mu\text{m}$ thick sections (American Optical Cryocut, Buffalo, U.S.A.) at -30°C , and the sections were incubated for 2 h at 37°C in 1 ml specific antiserum diluted 1:20 with phosphate-buffered saline (PBS). Sections were washed with fresh PBS, spread on microscope slides, covered with goat anti-rabbit-IgG conjugated with horseradish peroxidase (Nordic, Tilburg, The Netherlands) and incubated for 1 h at room temperature. After washing with PBS, sections were covered with peroxidase substrate, composed of 10 ml deionized water, 41 mg sodium acetate, 4 mg 3-amino-9-ethylcarbazole (Sigma), 0.5 ml *N,N*-diethylformamide and $10\text{ }\mu\text{l}$ 30% (v/v) H_2O_2 (pH 5.0). Those areas of the gel sections where rabbit antibodies had reacted with gel-fixed antigens during the first incubation of the SGIP procedure turned bright red after 5 to 10 min at room temperature. Finally, sections were rinsed with deionized water and dried on microscope slides. SGIP experiments were carried out using antisera against proteins I and II* of *E. coli* O26K60 which had been absorbed with LPS and murein-lipoprotein. Preimmunization sera were applied as controls. Sections were photographed as described for SDS-PAGE slab gels.

RESULTS

Outer membrane protein profiles of several Gram-negative bacteria

Polyacrylamide gel electrophoresis (PAGE) profiles of the major outer membrane (OM) proteins in the 25000 to 45000 molecular weight range of the bacteria studied are shown in Fig. 1. All members of the Enterobacteriaceae showed at least two major OM protein bands (tracks 1 to 23). The regularity of the patterns in the 33000 to 41000 molecular weight range found within the Enterobacteriaceae (except *Serratia marcescens*, track 18) was not observed in the OM profiles of the other Gram-negative bacteria studied (tracks 24 to 28). No major protein bands were detected in the OM of our strain of *Aeromonas hydrophila* (track 26). Profiles of *E. coli* strains, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* were discussed previously (Hofstra & Dankert, 1979). Our estimates of molecular weight are generally in agreement with those reported by others, though small differences exist for some bacteria. According to our calculations, the molecular weights of the major OM proteins in *S. typhimurium* are higher than those reported by Ames *et al.* (1974) but lower than the values reported by Lugtenberg *et al.* (1977). In *P. mirabilis* our estimates are slightly higher than those given by Rottem *et al.* (1979).

Non-peptidoglycan-bound outer membrane proteins

The presence of non-peptidoglycan-bound proteins in the OM preparations was investigated by extracting the OM with 2% (w/v) SDS at 37°C . Possible heat-modifiability of the extracted proteins was studied by heating half of the extract at 100°C for 10 min, followed by PAGE analysis of both the unheated and the heated samples (Table 1, first column of results). All enterobacterial strains tested contained one non-peptidoglycan-bound major OM protein which showed an apparent molecular weight shift after heating at 100°C . In all *E. coli* serotypes tested, except O1 K⁻, this protein, designated protein II* (Garten *et al.*, 1975), B (Reithmeier & Bragg, 1974) or d (Lugtenberg *et al.*, 1975), had an apparent molecular weight of 27000 after solubilization at 37°C , which shifted to 33000 after heating at

Fig. 1. PAGE patterns of outer membrane preparations of *E. coli* serotypes O1 K⁻ (1), O4 K2 (2), O26 K60 (3), O55 K59 (4), O75 K⁻ (5), O111 K58 (6), strain J5 (7), *Shigella flexneri* (8), *Edwardsiella tarda* (9), *Salmonella typhimurium* VI 260 (10), *S. panama* (11), *S. braenderup* (12), *S. derby* (13), *Citrobacter freundii* (14), *Klebsiella pneumoniae* (15), *Enterobacter cloacae* (16), *Ent. aerogenes* (17), *Serratia marcescens* (18), *Proteus vulgaris* (19), *P. morganii* (20), *P. rettgeri* (21), *P. mirabilis* (22), *Providencia stuartii* (23), *Pseudomonas aeruginosa* (24), *Ps. fluorescens* (25), *Aeromonas hydrophila* (26), *Alcaligenes faecalis* (27) and *Acinetobacter calcoaceticus* subtype anitratus (28).

Table 1. Outer membrane proteins of Gram-negative bacteria, their association with the peptidoglycan layer and their antigenic cross-reactivity with the major outer membrane proteins of *Escherichia coli* O26 K60

Outer membrane (OM) fractions, prepared as described in Methods, were extracted with buffer containing SDS (30 min, 37 °C) to solubilize the non-peptidoglycan-bound proteins. Peptidoglycan and peptidoglycan-bound proteins were removed by centrifugation (110000 g, 30 min, 15 °C). Heat-modifiability of the extracted proteins was investigated by heating one half of the supernatant (10 min, 100 °C), while the other half was kept at room temperature. Both samples were analysed by PAGE (first column of results). Polyacrylamide sections (40 µm thick) containing PAGE profiles of OM preparations were subjected to the SGIP technique. Proteins cross-reacting with protein I of *E. coli* O26 K60 are given in the second column of results and proteins cross-reacting with protein II* of this serotype in the third column. Minor proteins are given in parentheses.

Strain	10 ⁻³ × Molecular weight			
	Non-peptidoglycan-associated OM proteins		Peptidoglycan-associated OM proteins cross-reactive with protein I of <i>E. coli</i> O26 K60	Non-peptidoglycan-associated OM proteins cross-reactive with protein II* of <i>E. coli</i> O26 K60
	37 °C	100 °C		
<i>Escherichia coli</i> O1 K ⁻	28	35	37	35
<i>E. coli</i> O4 K2	27	33	36.5	38
<i>E. coli</i> O26 K60	27	33	36	(37)
<i>E. coli</i> O55 K59	27	33	36.5	33
<i>E. coli</i> O75 K ⁻	27	33.5	(36.5)	38
<i>E. coli</i> O111 K58	27	33	36.5	33
<i>E. coli</i> J5	27	33	36	37.5
<i>Shigella flexneri</i>	26.5	34	37	34
<i>Edwardsiella tarda</i>	27	36	37	38
<i>Salmonella typhimurium</i>	28	35	36.5	38
<i>S. panama</i>	28	35	36	38
<i>S. braenderup</i>	28	35	36	38
<i>S. derby</i>	28	34.5	35	38
<i>Citrobacter freundii</i>	28	34	35	36
<i>Klebsiella pneumoniae</i>	28	34	36.5	34
<i>Enterobacter cloacae</i>	28	34	35	34
<i>Ent. aerogenes</i>	28	34	38	34
<i>Serratia marcescens</i>	28	28 (34.5) (36)	40.5	28 (34.5) (36)
<i>Proteus vulgaris</i>	29	40	37	40
<i>P. morganii</i>	29	37	37.5	37
<i>P. rettgeri</i>	29.5	38.5	36	38.5
<i>P. mirabilis</i>	30.5	40	38	40
<i>Providencia stuartii</i>	31	39	35.5	39
<i>Pseudomonas aeruginosa</i>	33	33	None	None
<i>Ps. fluorescens</i>	33	33	None	None
<i>Aeromonas hydrophila</i>	None	None	None	None
<i>Alcaligenes faecalis</i>	None	None	None	None
<i>Acinetobacter calcoaceticus</i> subtype <i>anitratus</i>	(29)	(29)	None	None

100 °C. In *E. coli* O1 K⁻ both the unmodified and the heat-modified form of this protein showed a higher molecular weight than in the other *E. coli* serotypes. The apparent molecular weights of the unmodified forms of the corresponding proteins in the other Enterobacteriaceae ranged from 26500 in *Shigella flexneri* to 31000 in *Providencia stuartii*. Somewhat greater differences were found with the heat-modified forms, the estimations ranging from 34000 in several species to 40000 in *P. vulgaris* and *P. mirabilis*. From the OM preparations of *P. vulgaris* and *Ent. aerogenes* considerable amounts of their non-heat-modifiable proteins were also extracted at 37 °C. Lugtenberg *et al.* (1977) described this phenomenon for *P. vulgaris*. These proteins are probably more loosely bound to the peptidoglycan layer in these strains than the corresponding proteins of the other bacteria tested. The non-peptido-

Fig. 2. SGIP profiles of outer membrane preparations of several Enterobacteriaceae species. Polyacrylamide sections (40 μ m thick) contained electrophoretic OM profiles of *E. coli* O1 K⁻ (1), *E. coli* O4 K2 (2), *E. coli* O26 K60 (3), *Shigella flexneri* (4), *Edwardsiella tarda* (5), *Salmonella typhimurium* (6), *Citrobacter freundii* (7), *Klebsiella pneumoniae* (8), *Serratia marcescens* (9), *Proteus mirabilis* (10) and *Providencia stuartii* (11). Sections were treated with antiserum against OM protein I of *E. coli* O26 K60 (a), antiserum against OM protein II* of the same serotype (b) or a combination of both these antisera (c).

glycan-bound protein of *S. marcescens* showed an unexpected property concerning its heat-modifiability. Most of this protein was found in the unmodified form (molecular weight 28000) even after boiling in 2% (w/v) SDS for 30 min. Furthermore, the heat-modified form gave two weak bands with apparent molecular weights of 34500 and 36000, respectively. Consequently, three bands were found after boiling the protein extracted from *S. marcescens* OM at 37 °C. From the OM preparations of the species not belonging to the Enterobacteriaceae, few proteins were extracted with SDS at 37 °C. None of the extracted proteins showed a change in apparent molecular weight after heating to 100 °C.

Antigenic cross-reactivity of outer membrane proteins

The results of SGIP experiments performed with polyacrylamide sections containing PAGE profiles of OM preparations from a representative selection of bacteria are shown in Fig. 2. The application of the antiserum specifically directed against protein I of *E. coli* O26 K60 resulted in SGIP profiles showing one or two major OM protein bands (Fig. 2a). In the profiles of *E. coli* serotypes, *Sh. flexneri*, *Edwardsiella tarda*, *S. typhimurium*, *Citrobacter freundii*, *K. pneumoniae* and *Ser. marcescens* the higher molecular weight bands were stained (tracks 1 to 9) while in *P. mirabilis* and *Prov. stuartii* the lower molecular weight bands had reacted with anti-protein I (tracks 10 and 11). When antiserum against protein II* of *E. coli* O26 K60 was used, SGIP profiles showed a positive reaction of the higher

molecular weight bands in *P. mirabilis* and *Prov. stuartii* (Fig. 2*b*, tracks 10 and 11), while in all other profiles the lower molecular weight bands were stained. In the *Ser. marcescens* OM profile (track 9) three bands reacted with anti-protein II*: the unmodified non-peptidoglycan-bound protein with an apparent molecular weight of 28000 and the two bands (molecular weights 34500 and 36000) possibly representing heat-modified forms of this protein. When a combination of antisera against OM proteins I and II* of *E. coli* O26 K60 was used, SGIP profiles obtained showed a complete set of major OM proteins (Fig. 2*c*), similar to the Coomassie Brilliant Blue-stained OM protein patterns shown in Fig. 1. Results obtained in SGIP experiments carried out with OM preparations of all bacteria studied are given in Table 1 (second and third columns). In all Enterobacteriaceae studied, the peptidoglycan-bound major OM proteins reacted with antiserum against protein I of *E. coli* O26 K60, while the non-peptidoglycan-bound, heat-modifiable proteins were cross-reactive with protein II* of *E. coli* O26 K60. In *Proteus* species (except *P. morganii*) and in *Prov. stuartii* the higher molecular weight protein was non-peptidoglycan-bound and cross-reactive with protein II*, while the lower band reacted with anti-protein I.

SGIP experiments carried out with OM preparations of bacteria not belonging to the Enterobacteriaceae showed no cross-reactivity of any of their OM proteins with proteins I or II* of *E. coli* O26 K60.

Preimmunization sera applied to SGIP sections as controls were always negative.

DISCUSSION

The major OM proteins of the 23 enterobacterial strains studied showed a high degree of regularity in their PAGE profiles and in their antigenic cross-reactivity with the major OM proteins of *E. coli* O26 K60. All strains tested contained one or more peptidoglycan-bound OM proteins and one non-peptidoglycan-bound, heat-modifiable major OM protein.

The presence of heat-modifiable, non-peptidoglycan-bound OM proteins in Enterobacteriaceae other than *E. coli* has been reported by several authors. Ames *et al.* (1974) and Nurminen (1978) described the heat-modifiability of the lower molecular weight major OM protein in *S. typhimurium*. Lugtenberg *et al.* (1977) reported that the lower molecular weight OM protein of *C. freundii*, *Ent. cloacae*, *K. aerogenes*, *S. typhimurium* and *Ser. marcescens* was not bound to the peptidoglycan layer, while in *P. vulgaris* and *P. mirabilis* the higher molecular weight major OM protein was not peptidoglycan-bound. This is in agreement with our findings. Rottem *et al.* (1979) described the heat-modifiability of the non-peptidoglycan-bound OM protein in *P. mirabilis*.

Results of SGIP experiments indicate that antigenic cross-reactivity of the major OM proteins is a general phenomenon in the Enterobacteriaceae independent of variation in the molecular weight of the corresponding proteins in different bacterial strains. However, the cross-reactivity of the peptidoglycan-associated proteins should probably be treated with some reserve. Proteolytic cleavage experiments (Lee *et al.*, 1979) gave strong evidence that OM proteins 1a and 1b of *E. coli* K12 (b and c according to Lugtenberg *et al.*, 1975) are clearly different, which is in agreement with results obtained by Ichihara & Mizushima (1978). Their results indicate that antigenic identity of these porin proteins of *E. coli* K12 is unlikely, although the presence of shared antigenic determinants is not excluded by their experiments. In our SGIP experiments all peptidoglycan-bound major OM proteins of all Enterobacteriaceae species studied reacted with the antiserum elicited against the peptidoglycan-bound protein I of wild-type *E. coli* O26 K60. This was also the case in those strains possessing more than one peptidoglycan-bound major OM protein, e.g. *E. coli* O4 K2, *Edwardsiella tarda*, *Salmonella* serotypes and *C. freundii*, which indicates that in these strains all peptidoglycan-bound proteins are antigenically related to protein I of *E. coli* O26 K60. However, it should be noted that protein I of *E. coli* O26 K60 is not composed of a single polypeptide. About 90% of it is represented by the protein of 36000 molecular weight and

approximately 10% consists of a polypeptide of 37000 molecular weight (not visible in Fig. 1). Possibly, antibodies against the minor 37000 molecular weight component were also present in our antiserum against protein I. Therefore we cannot completely exclude the possibility that some of the peptidoglycan-bound OM proteins of heterologous enterobacterial strains cross-reacted with the minor 37000 molecular weight protein of *E. coli* O26 K60 rather than with its major 36000 molecular weight protein. The SGIP method would be very useful for investigating the antigenic relationship of the numerous major and minor OM proteins of *E. coli* K12 described so far.

A comparison of the results obtained by the SGIP technique with those of interfacial immunoprecipitation experiments (Hofstra & Dankert, 1979, 1980*a*) shows that the former method is both more specific and much more sensitive than the latter. However, the SGIP technique is not a quantitative assay. It discriminates strictly between antigenically related and unrelated proteins. Furthermore, this procedure implies a previous separation of the proteins on polyacrylamide gels after boiling the OM samples in SDS-containing buffer. Therefore results obtained with the SGIP method do not indicate the actual immunological relationship of the OM proteins in their native configuration, i.e. as organized within the OM structure and more or less covered by lipopolysaccharide or capsular materials. In immunofluorescence experiments, antibodies against protein II* of *E. coli* O26 K60 were shown to adhere to intact cells of the homologous strain (Hofstra *et al.*, 1979). Results obtained in these experiments with an antiserum against a denatured preparation of protein I, as used in the SGIP technique, were disappointing. Antibodies elicited against the denatured form of protein I hardly reacted with this protein in its native configuration, in contrast to antibodies prepared against the undenatured protein (H. Hofstra & J. Dankert, unpublished results).

The classification of the Enterobacteriaceae largely rests on biochemical characteristics (Clancy, 1974; Cowan, 1975; Ewing & Martin, 1978) and on the relationships of bacterial DNAs (Brenner & Falkow, 1971). It is tempting to draw parallels between this classification and the immunological relatedness of the major OM proteins within the subgroups (tribes) of the family Enterobacteriaceae. In general, the properties of the OM proteins (the similarity of PAGE profiles and the antigenic cross-reactivity) are in agreement with the accepted classification. However, in the case of the tribe Proteaceae, which contains four *Proteus* species and two species of the genus *Providencia* according to Ewing & Martin (1978), our observations would favour the classification according to Cowan (1975), who preferred the nomenclature *Proteus stuartii* instead of *Providencia stuartii* and who distinguished Morgan's bacillus number 1 (Morgan, 1906) as a separate genus (*Morganella*) rather than as a *Proteus* species (*P. morganii*).

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