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Protein K: a New Major Outer Membrane Protein Found in Encapsulated *Escherichia coli*

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The protein composition of purified outer membranes of 47 *Escherichia coli* strains was examined by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis. Of 33 encapsulated strains, all contained an outer membrane protein distinguishable from previously reported proteins. The 14 non-encapsulated strains with one exception lacked this protein. Because of its apparent association with encapsulation (K antigen) we have named it K protein. The protein was purified nearly to homogeneity by chromatography in the presence of detergents, and its composition was determined. Its amino acid composition does not differ significantly from that reported for protein I, another *E. coli* major outer membrane protein. Furthermore, the N-terminal amino acid sequence of protein K indicates that it is related to protein I.

In recent years, numerous studies have been published concerning the major outer membrane proteins of *Escherichia coli*. The first to give detailed information about these proteins were Braun and Rehn (3), who described a lipoprotein bound covalently to peptidoglycan. Schnaitman later described that the purified outer membrane (OM) contained a major protein band with an apparent molecular weight of 44,000 when subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (30). Henning et al. (13, 14) were the first to identify all four major OM proteins as they are understood today, and have characterized proteins I, II*, and III (9-11, 29). Table 1 summarizes the findings of most authors working in the field and indicates the most probable correlation of the different electrophoretic classifications (2, 4, 6, 15-17, 19, 22, 27, 28, 31, 32, 35). Ic is a newly described third variant of protein I (16). Protein 2 was isolated by Schnaitman (6, 32) from a phage PA-2 lysogen of *E. coli* K-12 and is shown to be dependent on the presence of this phage.

These studies concentrated on established laboratory strains, and little information is available on the OM protein composition of strains freshly isolated from patients. In this paper, we describe a new major OM protein found in encapsulated *E. coli* strains obtained mostly from human neonates with meningitis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains for this study are described in Table 2. Bacteria were subcultured for 17 h on plates of Mueller-Hinton (Difco) medium or on nutrient agar base at 37°C. For growth in liquid culture, ca. 5×10^8 bacteria were suspended in 1,000 ml of Fraser-Jerrel medium (8). Cells were grown at 37°C on a rotary platform shaker (115 rpm) to mid-exponential phase (about 3 h). Bacteria were harvested by centrifugation at 6,000 × *g* for 5 min.

Extraction procedure. About 5×10^{11} intact cells were washed by centrifugation with 80 ml of cold water and suspended in 35 ml of cold distilled water, and the OM was extracted as previously described for preparation I by Mizushima and Yamada (25). In some instances (strains 41 and 42, Table 2) insufficient amounts of OM were obtained in preparation I of Mizushima; Mizushima's preparation II was then used. The isolation and purification of the OM were carried out by an isopycnic sucrose density gradient centrifugation by the method of Miura and Mizushima (24). The sample in 500 μl of 36% (wt/wt) sucrose was overlaid on 4.5 ml of a 38 to 55% (wt/wt) sucrose gradient and centrifuged at 125,000 × *g* for 4 h at 4°C.

Gradients were collected from the bottom of the tubes; fractions of ca. 200 μl were collected. Protein concentration was determined by the method of Lowry et al. (21), and fractions corresponding to the OM (at densities from 1.196 to 1.253 g per cm^3, depending on the strain) were pooled, diluted with 2 volumes of distilled water, and recovered by centrifugation at 77,000 × *g* for 30 min. The OM was suspended in distilled water to a protein concentration of 10 mg/ml and stored at −20°C.

SDS-PAGE. Proteins in the OM preparations were analyzed by SDS-PAGE according to the method of Maizel (23). A continuous gradient gel from 7% on the top to 30% (wt/vol) acrylamide at the bottom was prepared. Samples to be electrophoresed were boiled at 100°C for 5 min in a solution of 50 μl of 1% SDS

† Reprint requests.

TABLE 1. *OM protein bands described by several authors and the most probable correlation between the classifications*

Author	Reference	Protein band designations at mol wt ($\times 10^5$):									
		39	37			33			17	10
Bragg	2, 27		A1	A2		B				
Henning	8, 13		Ia	Ib	Ic	II*			III	IV
Lugtenberg	22	a	b	c		d				
Mizushima	35	0–7	0–9	0–8		0–10	0–11			
Rosenbusch	28		Matrix protein							
Schnaitman	31, 6	3b	1a	1b	2	3a				
	32, 1									
Koplow	19		B			C, D				
Chai	4					G				
Inouye	17		Peak 4			Peak 6	Peak 7			

(wt/vol) and 1% 2-mercaptoethanol (wt/vol). After cooling, 1 μl of 1% bromophenol blue and some crystals of sucrose (J. T. Baker) were added. Ten microliters of sample containing 10 μg of protein was usually loaded into the gel slot. The Laemmli (20) (12.5% acrylamide) and the Bragg-Hou (2) (10% acrylamide) PAGE systems were also used for comparative purposes. Stains-All staining was performed as described by King and Morrison (18).

Purification of protein K. Strain N63 (K1) and BOS 12 (K92) were grown in 6 liters of modified Frantz medium (7, 12) at 37°C for 4 h and collected by centrifugation. The cell paste was suspended in 500 ml of 0.05 M Tris-hydrochloride buffer (pH 8.0) containing 0.01% (wt/vol) disodium EDTA and 2% (wt/vol) hexadecyltrimethyl-ammonium bromide (Cetavlon) and placed in a boiling-water bath for 10 min. After centrifugation at 20,000 × *g* for 20 min, 258 g of $(NH_4)_2SO_4$ was added, and the mixture was allowed to sit at room temperature overnight. The solution was centrifuged at 5,000 × *g* for 20 min, and the floating oily layer was removed with a spatula, dissolved, and dialyzed against 0.02 M potassium phosphate buffer (pH 8.0) containing 2% Cetavlon. After removal of insoluble debris, the solution was applied to a hydroxylapatite column (1.6 by 20 cm) (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.) equilibrated with the same buffer. The column was washed with initial buffer and eluted with a 300-ml linear gradient with a final concentration of 1 M potassium phosphate (pH 8.0) and 1% Cetavlon. The eluate was monitored by 280 nm absorbance and SDS-PAGE. Protein which had a mobility identical to protein K in purified OM eluted in two peaks—at the midpoint of the gradient (0.5 M) and near the end of the gradient (0.85 M). Both peaks were separately pooled, dialyzed, and subjected again to the same chromatographic procedure. On rechromatography the two proteins eluted at about the same ionic conditions as they had done the first time.

Amino acid and amino terminal sequence analysis. Amino acids were analyzed by the method of Spackman et al. (34) using a model D500 Durrum amino acid analyzer (Durrum Instruments Co., Palo Alto, Calif.). Protein (0.5 mg) was hydrolyzed with 300 μl of 4 N methane sulfonic acid at 100°C for 24 h with and without prior performic acid oxidation (33). Automated sequence analysis was carried out on ca. 50 nmol of protein on a Beckman 890B sequencer (Beckman Instruments Co., Palo Alto, Calif.) using a modified Quadrol program no. 011576 of Beckman Instruments. The phenylthiohydantoin amino acids were identified and quantitated by high-pressure liquid chromatography (36) using a Hewlett Packard model 1084A instrument (Hewlett Packard Co., Cupertino, Calif.).

Test for lysogenicity. Overnight cultures of each strain were diluted tenfold in meat extract broth and incubated at 37°C for a further 90 min. Mitomycin C was added to a final concentration of 5 μg/ml, and incubation was continued for 3 h with shaking. Chloroform (1 drop/5 ml) was added, and shaking was continued for an additional 10 min. After centrifugation the supernatant was tested as such and at several dilutions for production of plaques on indicator strains (Table 2, strains 1, 5, 12, and 20 to 40).

RESULTS

Electrophoretic profiles of OM of *E. coli* strains. When the isolated OM of the *E. coli* strains were examined by SDS-PAGE, a simple protein profile was found in agreement with previous reports. However, there was a new protein of apparent electrophoretic homogeneity in all 33 encapsulated strains examined. We have named this band protein K because of its apparent association with the presence of K antigen. Staining of SDS-PAGE with Stains-All indicated that protein K is an ordinary protein without major glyco- or lipo-moieties. This protein was absent from six strains typed as nonencapsulated and also from all derivatives of the old laboratory strain K-12. The only exception to the association of this protein K with capsules was strain 38 (Table 2), classified as O18ac:K-:H7 (26), in which we found a band at the position of protein K. The molecular weight of the protein K was estimated to be about 40,000 on the basis of its relative position in the SDS-PAGE in comparison to the known major membrane proteins. A representative SDS-PAGE pattern

TABLE 2. *E. coli strains examined*[a]

Strain no.	Strain designation (where isolated)	O	K	H	Source
1	K235		1[b]		MEN
2	N72 (Biloxi, Miss.)		1		MEN
3	NBM53		1		MEN/CSF
4	N71 (St. Paul, Minn.)		1		MEN/blood
5	4		1		MEN
6	SL19 (Salt Lake City, Utah)		1		MEN/blood
7	N76 (Denver, Colo.)		1		MEN/CSF
8	N65 (Salt Lake City, Utah)		1		MEN/CSF
9	93Eck		1		MEN
10	N68 (New York, N.Y.)		1		MEN/CSF
11	65		1		MEN/CSF
12	N63		1		MEN/CSF
13	C61-SH (Cincinnati, Ohio)		1		MEN
14	Easter		100		MEN
15	LH	75	1		MEN
16	C94	7	1	3	MEN
17	O4:K12	4	12	—[c]	MEN
18	O6:K13	6	13		MEN
19	44		92		MEN
20	EM36	119	—	4	MEN/CSF
21	EM37	2	2	?	Blood
22	EM38	2	+	4	Blood
23	EM39	16	1	6	MEN/CSF
24	EM40	18ac	1	7	MEN/CSF
25	EM42	?	34	—	MEN/CSF
26	EM48	77	96	—	Blood
27	F2388	9	29	—	
28	H509a	100	—	2	
29	H511	102	—	8	
30	C24/55	26	—	32	
31	1064	55	—	6	
32	SU65/42	4	12	4	
33	U4-41	4	3	5	
34	Bi7458-41	6	2ac	1	
35	Bi7509-41	7	1	—	
36	F10018-41	18ab	—	14	
37	E47a	25	19	12	
38	DM3219-54	18ac	—	7	
39	JC48-75	?	100	?	
40	JC44-75	?	100	?	
41	K-12, W1485F	—	?	48	
42	K-12, CS137	—	?	48	
43	BAM		—	—	
44	K-12, P400	—	?	48	
45	K-12, P530	—	?	48	
46	K-12, P530, 1gII	—	?	48	
47	K-12, P530, 3bII	—	?	48	

[a] MEN, Isolated from a patient with meningitis; CSF, cerebrospinal fluid. The strains were obtained from the following sources: Strain 1, W. F. Goebel, The Rockefeller University, New York, N.Y.; strains 2–19, John B. Robbins, Bureau of Biologics, Federal Drug Administration, Bethesda, Md.; strains 20–26, Matti Valtonen, Central Public Health Laboratory, Helsinki; strain 27, Klaus Jann, Max Planck Institut, Freiburg; Strains 28–38, Frits Ørskov and Ida Ørskov, State Serum Institute, Copenhagen; strains 39–40, John Robbins; strains 41–42, Carl A. Schnaitman and Anthony P. Pugsley, The University of Virginia, Charlottesville; strain 43, Charles Brinton, University of Pittsburg, Pittsburgh, Pa.; and strains 44–47, Ulf Henning, Max Planck Institut, Tübingen.

[b] Antigen designation.

[c] —, Definite absence of antigen.

is shown in Fig. 1. It can be seen that two different strains of K-12 exhibited the previously described OM proteins and that encapsulated strains (K1, K13, and K100) have a prominent protein band not seen in the laboratory strains. The electrophoretic homogeneity of the K protein from different strains was rather surprising in view of the fair amount of heterogeneity of

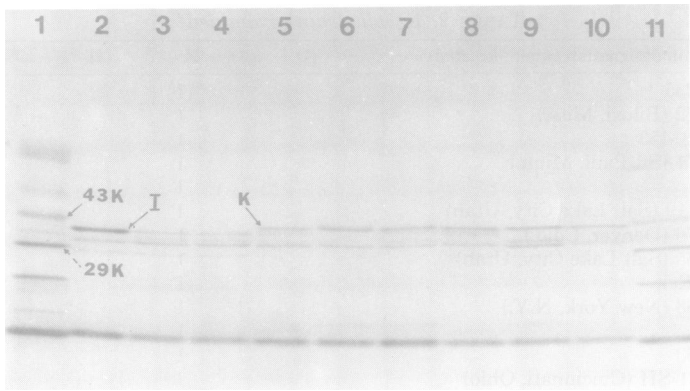

FIG. 1. *SDS-PAGE of OM preparations of E. coli strains. (1 and 11) Molecular weight markers (bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin, cytochrome c, and insulin); (2) K-12 BAM; (3 and 4) K-12 P400; (5) JC48-75 (K100); (6) 65 (K1); (7) SL19 (K1); (8) N63 (K1); (9) N68 (K1); (10) 06:K13 (K13).*

the other two main proteins I and II* as described by Schmitges and Henning (29) and also seen in our gels. Protein K is best seen in the gradient gel system, whereas in a Laemmli gel (see Materials and Methods) it overlaps protein Ib and in a Bragg-Hou gel it runs between I and II* (data not shown).

The mobility of protein K was compared to that of Schnaitman's protein 2 (Fig. 2). It is clear that the K band is not present in either strain W 1485 or CS 137 and that it differs in mobility from protein 2. Furthermore, protein 2 is dependent on a prophage PA-2. We looked for the presence of a comparable prophage in our K protein positive strains without finding any evidence of lysogenicity. Protein K thus seems to be a major OM protein not previously described.

Purification and preliminary characterization of protein K. Protein K was released from whole organisms by boiling in the cationic detergent Cetavlon and purified by chromatography on hydroxylapatite gel, in the presence of 1% (wt/vol) Cetavlon throughout. The protein, as defined by mobility on SDS-PAGE, eluted at two distinctly different phosphate concentrations, i.e., at about 0.5 and 0.85 M. Both forms of the protein were isolated from strain N63 (K1), and the protein eluting at 0.5 M was obtained from strain BOS12 (K92); their purity was assessed by SDS-PAGE (Fig. 2). We estimated that the proteins contained 10% or less contamination. No significant difference in mobility between protein K as found in the OM and purified material was evident.

The three preparations of protein K were subjected to amino acid analysis. No significant difference in the amino acid composition was noted between the two forms of K protein from strain N63 or the K protein derived from strain BOS12. Therefore, the amino acid composition listed in Table 3 is the average of the analyses performed. The amino acid composition is in close agreement with the published data for

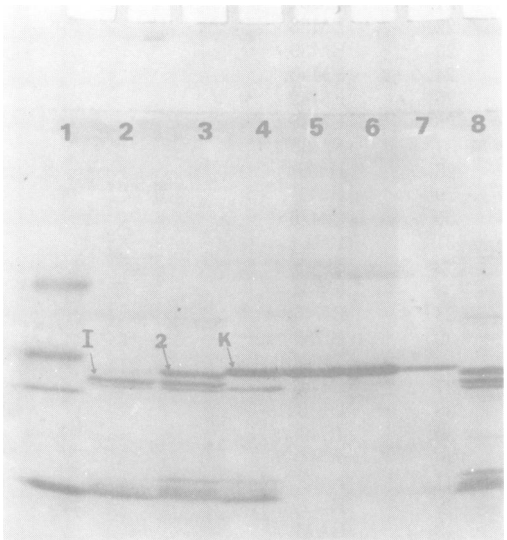

FIG. 2. *SDS-PAGE of OM preparations of E. coli and purified OM proteins. (1) Molecular weight markers (bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome c); (2) K-12 W1485; (3 and 8) K-12 CS137 (lysogen producing protein 2); (4) N63 OM; (5) purified K protein (from N63 eluting at 0.5 M phosphate); (6) purified K protein (from N63 eluting at 0.85 M phosphate); (7) purified K protein (from BOS12 eluting at 0.5 M phosphate).*

protein I (Table 3). No cysteic acid was found in performic acid oxidized samples. The three preparations of protein K were subjected to automatic amino acid sequencing (Fig. 3).

DISCUSSION

We describe here a new major OM protein and call it protein K because of its apparent association with the presence of a polysaccharide capsule. Protein K was present in all encapsulated strains examined which included capsular types K1, K2, K2ac, K3, K-12, K13, K19, K29, K34, K92, K96, and K100. Both the so-called K1+ and K1− variants of the K1 polysaccharide were examined. K1− polysaccharide is a poly-*N*-acetyl acid, and K1+ is a poly-*N*-acetyl-*O*-acetyl neuraminic acid (26). All the unencapsulated *E. coli* strains studied, except DM 3219-54 (strain 38, Table 2), were protein K negative.

The protein K of all encapsulated strains was found in the same position in SDS-PAGE, whereas there was much variation in the other main membrane proteins. The extent of the similarity or identity of the K proteins can be decided only by isolating the proteins from the other capsular types and comparing their analytical and serological properties with those of the protein K from type K1 and K92 described here. Protein K appears to have escaped the notice of previous workers because they did not concentrate on strains obtained from patients with invasive disease. Schnaitman has studied the OM proteins of some pathogenic *E. coli* strains (31), but none of his strains were of O-types bearing acidic polysaccharide capsules (26).

The results obtained by amino acid analysis and N-terminal sequence analysis indicate that K protein is homologous to protein I. The sequence of the first 10 residues of protein I have been reported by Chen et al. (5) and, with the exception of residue 8, are identical to those found in protein K. The heterogeneity of protein K evident in the chromatographic behavior was confirmed in the sequence analysis. It was found that the two proteins differed in regard to the amino acid at position 12 (Fig. 3).

The finding of protein K immediately raises two further questions. First, does this protein have a role in the synthesis or in the structure of *E. coli* capsule? We hope to investigate this

TABLE 3. *Amino acid composition of E. coli OM proteins*[a]

Amino acid	K	2	I	II*	III	IV
Lysine	6.5	6.0	5.2	5.4	4.8	8.8
Histidine	1.6	1.2	0.6	1.8	0.7	0.0
Arginine	2.7	3.9	3.4	4.0	4.8	7.0
Aspartic	16.4	11.8	15.3	12.0	11.2	24.5
Threonine	6.4	7.0	6.1	6.2	6.9	3.5
Serine	3.1	5.4	4.9	4.7	6.3	10.5
Glutamic	9.4	8.8	7.9	9.1	9.1	8.7
Proline	1.0	1.2	2.1	5.8	2.8	0.0
Glycine	11.7	12.1	13.1	10.9	12.6	0.0
Alanine	10.3	9.7	9.2	8.7	8.4	15.8
Valine	6.4	9.7	6.7	7.2	6.9	7.0
Methionine	1.4	1.2	1.2	1.8	2.8	3.5
Isoleucine	3.6	2.1	3.9	4.4	4.2	1.8
Leucine	6.3	6.7	7.1	7.2	4.9	7.0
Tyrosine	6.8	6.7	6.1	5.1	6.9	1.7
Phenylalanine	5.5	6.7	5.2	2.5	4.2	0.0
Tryptophan	0.8	ND	1.5	2.2	1.4	0.0
Cysteine	0.0	0.0	0.3	0.7	0.7	0.0

[a] In moles/100 moles. Data for protein 2 are from reference 6 and data for proteins I, II*, III, and IV are from reference 10. ND, Not determined.

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                          1          5          10
Protein I                 Ala-Glu-Ile-Tyr-Asn-Lys-Asp-Gly-Asn-Lys-
                          1          5          10                  15                  20
K Protein N63 (0.5 M)     Ala-Glu-Ile-Tyr-Asn-Lys-Asp-Ser-Asn- Lys-Leu-Tyr-Leu-Tyr-Gly-Lys-Val-Asn-Ala-X-His-Tyr-
                          1          5          10                  15                  20
K Protein N63 (0.85 M)    Ala-Glu-Ile-Tyr-Asn-Lys-Asp-Ser-Asn- Lys-Leu-Asp-Leu-Tyr-Gly-Lys-Val-Asn-Ala-X-His-Tyr-
                          1          5          10                  15                  20
K Protein BOS12 (0.5 M)   Ala-Glu-Ile-Tyr-Asn-Lys-Asp-Ser-Asn- Lys-Leu-Asp-Leu-Tyr-Gly-Lys-Val-Asn-Ala-X-His-Tyr-
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FIG. 3. *N-terminal amino acid of protein K. Sequence data for protein I is from reference 5.*

by isolating and analyzing a series of mutants blocked in the synthesis of the capsular polysaccharide. Secondly, what is its significance to the virulence of the bacteria? The protein K seems to be related to the capsule, which is a known virulence factor. However, further studies are needed to learn whether it is present in all strains bearing acidic capsular polysaccharides or is specific for *E. coli* strains causing purulent meningitis.

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