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Escherichia coli Outer Membrane Protein K Is a Porin

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Protein K is an outer membrane protein found in pathogenic encapsulated strains of *Escherichia coli*. We present evidence here that protein K is structurally and functionally related to the *E. coli* K-12 porin proteins (OmpF, OmpC, and PhoE). Protein K was found to cross-react with antibody to OmpF protein and to share 8 out of 17 peptides in common with the OmpF protein. Strains that are OmpC porin$^-$ and OmpF porin$^-$ and contain protein K as their major outer membrane protein have increased rates of uptake of nutrients and a faster growth rate relative to the parental porin$^-$ strain. The protein K-containing strains are at least 1,000-fold more sensitive to colicins E2 and E3 than is the porin$^-$-deficient strain. These data suggest that protein K is a functional porin in *E. coli*. The porin function of protein K was also demonstrated in vitro, using black lipid membranes. Protein K increased the conductance in these membranes in discrete, uniform steps characteristic of channels with a size of about 2 nS.

Porins are among the predominant or major proteins found in the outer membranes of gram-negative bacteria. Their function is to form pores that allow the diffusion of small hydrophylic molecules across the outer membrane barrier (3, 12, 16, 17, 20–22). The transmembrane nature of porins is demonstrated by their ability to serve as receptors for bacteriophages or colicins (3) and their tight, noncovalent association with peptidoglycan (3, 15–18, 29). The OmpF and OmpC proteins are the porins usually found in *E. coli* K-12 strains. *Escherichia coli* B has only the OmpF-like porin, protein I (29).

Recently, Paakkanen et al. (25) described protein K as an outer membrane protein found in each of 33 encapsulated strains of *E. coli* but absent in 13 of 14 unencapsulated *E. coli* strains. They compared the first 10 *N*-terminal amino acids of protein K to porin from *E. coli* B (protein I) and found the sequences to be identical, with the exception of residue 8.

In this paper, we provide further evidence that protein K is structurally related to porins. In addition, we find that protein K may function in vivo and in vitro as a porin in the *E. coli* outer membrane.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains are described in Table 1. Strain N63 is an *E. coli* K1 strain originally isolated from cerebrospinal fluid and given to us by Emil Gotschlich from the Rockefeller University. Strains N63-1, N63-2, N63-3, and N63-4 are independently isolated unencapsulated K1 derivatives of strain N63 selected as resistant to bacteriophage E. This bacteriophage is specific for encapsulated K1$^+$ strains (11). The presence of the K1 antigen was detected by precipitin formation on L agar plates containing 10% meningococcal group B antiserum (23).

Media and culture conditions. Strains were grown in either Franz medium (9, 10) or L broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl; pH 7.0) to mid-exponential phase in a shaking water bath at 37°C. Bacteria were harvested by centrifugation at 6,000 × *g* for 10 min. Plating medium contained 15 g of agar per liter.

Preparation of colicins. Colicins E2 and E3 were prepared from mitomycin C-induced cultures of appropriate strains as described previously (7).

Envelope fractionation. Inner and outer membrane proteins were separated by extraction of total cell envelopes with either Sarkosyl (6) or Triton X-100 (29). Peptidoglycan-associated proteins are those proteins not solubilized by 2% sodium dodecyl sulfate (SDS) at 60°C for 30 min as described by Rosenbusch (28).

Purification of protein K. Strain JS17 is an *ompF ompC ompA* strain lysogenic for a derivative of phage lambda (λ 540) into which we have cloned the structural gene for protein K from *E. coli* K1 strain N63 (Sutcliffe and Foulds, J. Cell. Biochem., in press). This strain produces protein K as its only porin.

Protein K was purified from the total cell envelope fraction as described by Nakae (21). Briefly, the cell envelope fraction was prepared by centrifugation (100,000 × *g* for 1 h) of a French press lysate of strain

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TABLE 1. Bacterial strains

Strain	Genotype or comment	Reference
N63	Wild-type $K1^+$	25
N63-1	Unencapsulated $K1^-$, derivative of N63	This manuscript
N63-2	Unencapsulated $K1^-$	This manuscript
N63-3	Unencapsulated $K1^-$	This manuscript
N63-4	Unencapsulated $K1^-$	This manuscript
D699	Wild-type O1:K1:H7	23
D703	Unencapsulated $K1^-$, derivative of D699	23
CS146	*ompA154 ompC165 ompF168*	C. Schnaitman
JF568	K-12 *aroA357 ilv-277 xyl-14 metB65 his-53 purE41 cyc-1 lacY29 rpsL97 tsx63 proC24*	8
JF773	JF568 *ompC ompA260*	8
JS17	CS146 lysogenic for λ_{540} protein K^+	This manuscript

JS17. The pellet was extracted with 2% (wt/vol) SDS in 0.05 M Tris-hydrochloride, pH 7.7–5 mM EDTA at 37°C for 30 min.

The peptidoglycan and associated proteins (primarily protein K and lipoproteins) remained insoluble and were separated by centrifugation at 100,000 × *g* for 1 h. The pellet was washed and suspended in 0.05 M Tris-hydrochloride, pH 7.7–0.4 M NaCl–5 mM EDTA–0.05% 2-mercaptoethanol and incubated at 37°C for 2 h. The insoluble peptidoglycan and covalently linked lipoprotein were separated from the solubilized material (primarily protein K and free lipoprotein) by centrifugation at 100,000 × *g* for 1 h. The supernatant fraction was then chromatographed on a Sepharose-6B column equilibrated and eluted with 0.05 M Tris-hydrochloride containing 0.4 M NaCl–5 mM EDTA–0.05% 2-mercaptoethanol. The fractions containing pure protein K were identified by electrophoresis in polyacrylamide gels containing SDS and 4 M urea, pooled, and exhaustively dialyzed against 0.01 M Tris-hydrochloride, pH 7.5.

Polyacrylamide gel electrophoresis. All protein samples were heated at 100°C for 5 min in 2% SDS–2.5% beta-mercaptoethanol–0.001% bromophenol blue before application to the gel. To distinguish protein K from strain K-12 porins, a continuous acrylamide (45/1 ratio of acrylamide to bis-acrylamide) gradient gel of 7% at the top to 20% (wt/vol) at the bottom was prepared in 0.375 M Tris-hydrochloride, pH 8.8, containing 0.1% SDS and 4 M urea. A stacking gel of 3% acrylamide (45/1.2 ratio of acrylamide to bis-acrylamide) in 0.125 M Tris-hydrochloride, pH 6.8, was added to the top of the polymerized gradient gel. The reservoir buffer for this gel system was 0.38 M glycine–0.05 M Tris-hydrochloride, pH 8.8–0.1% SDS.

To distinguish protein K from other proteins of similar molecular weight in K1 strains, the Weintraub modification (33) of the Laemmli gel system (14) was used.

Immunological comparison of OmpF protein and protein K. The OmpF protein used to elicit antibody was purified as a single band from SDS-polyacrylamide gels of outer membrane proteins prepared from strain JF773. Approximately 40 μg of OmpF protein in 0.4 ml was mixed with an equal volume of Freud complete adjuvant and injected intradermally into a 6- to 8-lb (ca. 2.8- to 3.7-kg) New Zealand white female rabbit. The rabbit was bled every 1 to 2 weeks to monitor the titer of anti-OmpF antibody. Additional injections of antigen, equivalent to the initial injection, were given every 4 weeks and again after 6 weeks. Antiserum was collected every 2 weeks from 4 to 10 weeks and pooled. These anti-OmpF antisera did not react with purified lipopolysaccharide, SDS, phospholipids, or lipoprotein (kindly provided to us by Henry Wu). A 10- to 15-μl sample of purified OmpF protein (containing about 1 mg/ml) or protein K (purified from *E. coli* K1 strain N63 by the same method as OmpF protein) was added to each well of an Ouchterlony double-diffusion plate, and 15 μl of undiluted antiserum was placed in the center well. The Ouchterlony plate was prepared as a 1% agarose gel containing 20 mM Tris-hydrochloride, pH 7.9–0.5% Triton X-100. Antigen-antibody complexes were visible after overnight incubation in a moist chamber at room temperature.

Chymotryptic peptide maps. The proteins were radioiodinated in polyacrylamide gel slices as described by Elder et al. (5). The iodinated proteins were digested with chymotrypsin, and the resulting peptides were separated by two-dimensional thin-layer chromatography and electrophoresis (30, 32).

Cell surface labeling. Strain N63-1 was grown in mid-exponential phase in L broth, the bacterial cells were harvested by centrifugation, and the surfaces of whole cells were iodinated by lactoperoxidase procedures (13, 26). The cells were broken by passage through a French pressure cell, and inner and outer membrane fractions were prepared with Sarkosyl (6). Approximately 40 μg of protein from inner or outer membrane preparations was applied to a 12% polyacrylamide gel prepared by the method of Weintraub et al. (33). Proteins that had been labeled by $Na^{125}I$ were visualized by autoradiography of the dried gel, using X-ray film (Kodak X-Omat AR).

Uptake of nutrients. Bacterial strains were grown in 10 ml of L broth to a density of 5×10^8 cells per ml. Cells were harvested by centrifugation and suspended in 5 ml of Davis minimal medium (Difco Laboratories) plus 0.2% glucose and 100 μg of chloramphenicol per ml. The cell suspension was divided into 1-ml portions and allowed to equilibrate at 37°C for 15 min. The radioactive substrate (0.5 μM, final concentration) was added, and 100-μl samples were removed at 30-s intervals and filtered through Whatman GF/F glass fiber filters. The filters were immediately washed with 1 ml of minimal medium at 37°C, dried, and counted. The initial uptake rates were determined over the period of time where the uptake was linear, usually 3 to 5 min. The substrate was chosen after preliminary experiments, using concentrations from 0.2 to 10 μM proline.

Black lipid membrane experiments. Black lipid membranes were formed by spreading the membrane-forming solution under water across a circular hole (1-mm diameter) in a Teflon cup (19). Each chamber contained KCl at 1 M and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at

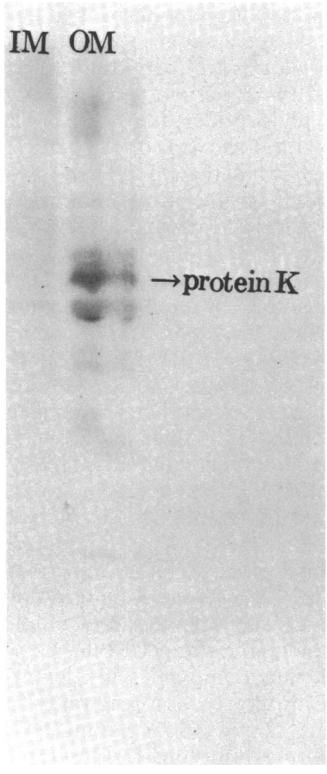

FIG. 1. SDS-polyacrylamide gel electrophoresis of membrane proteins prepared from intact cells labeled with $Na^{125}I$. Cells of strain N631 were labeled with $Na^{125}I$ by the lactoperoxidase procedure. Cells were broken by passage through a French pressure cell, and inner and outer membrane fractions were prepared with Sarkosyl. Protein samples (40 μg) were electrophoresed on a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was dried, and the labeled proteins were visualized by autoradiography. IM, inner membrane fraction; OM, outer membrane fraction.

pH 7.4. Measurements were made in a voltage clamp apparatus (31). The membrane-forming solution was oxidized cholesterol (1). A sample of the protein K stock solution was added to one chamber of the voltage clamp apparatus, and changes in current were measured with a very sensitive current amplifier.

RESULTS

Protein K is a transmembrane protein in the outer membrane. Since no bacteriophages or colicins are known to use protein K as a receptor, we demonstrated that a portion of the protein K molecule was exposed to the cell surface by labeling whole cells with $Na^{125}I$ by the lactoperoxidase procedure. Figure 1 shows an SDS-PAGE pattern of inner and outer membrane proteins that were labeled with ^{125}I and visualized by autoradiography. The inner membrane fraction was virtually unlabeled by this procedure and contained less than 0.5% of the ^{125}I incorporated. Protein K is one of the outer membrane proteins that has surface tyrosyl residues accessible to the ^{125}I label. The ^{125}I label did not penetrate the outer membrane as evidenced by the observation that no inner membrane proteins were labeled. Thus, we conclude that a portion of protein K is exposed on the outer surface of the outer membrane.

To demonstrate that part of protein K is exposed to the periplasmic side of the outer membrane, we extracted total cell envelopes with 2% SDS at 60°C for 30 min. This procedure solubilizes all the inner membrane proteins and most of the outer membrane proteins (28). The insoluble residue represents peptidoglycan and those species of protein that either have a covalent attachment to peptidoglycan, such as lipoprotein, or a tight noncovalent association with peptidoglycan, such as porins. Figure 2 shows an SDS-PAGE pattern of the peptidoglycan-associated proteins from encapsulated and unencapsulated strains. Protein K was found to be associated with peptidoglycan in every strain

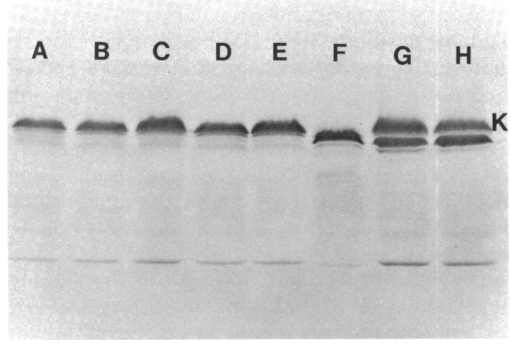

FIG. 2. SDS-polyacrylamide gel electrophoresis of peptidoglycan-associated proteins. Cell envelopes from strain N63 (lane A), strains N63-1, N63-2, N63-3, and N63-4 (lanes B to E, respectively), strain CHS75 (lane F), strain D699 (lane G), or strain D703 (lane H) were extracted with 2% SDS–10 mM Tris-hydrochloride, pH 7.3–10% glycerol–5% beta-mercaptoethanol at 60°C for 30 min. The peptidoglycan and associated proteins were first separated by differential centrifugation (100,000 × *g* for 1 h), and the pellet was suspended in 2% SDS–2.5% beta-mercaptoethanol–0.0001% bromophenol blue–10% glycerol–0.125 M Tris-hydrochloride, pH 6.8 and heated to 100°C for 5 min. The proteins solubilized by this heat treatment were visualized after electrophoresis on an SDS-polyacrylamide gel and staining with Coomassie brilliant blue R-250. K represents the location in the gel where protein K electrophoreses. Lane F is the peptidoglycan-associated proteins of strain CSH75, a K-12 strain. Note that this gel system does not separate OmpF and OmpC proteins which electrophorese slightly faster than protein K.

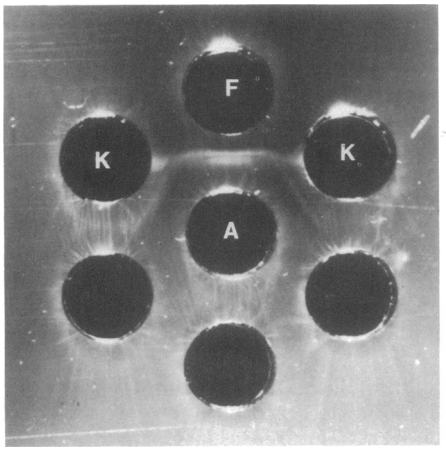

FIG. 3. Ouchterlony double-diffusion analysis of OmpF protein and protein K. Well A contains 15 μl of undiluted antiserum prepared by using purified OmpF protein; wells K contain 1 μg of purified protein K; well F contains 1 μg of purified OmpF protein.

and its association was not related to the strain's ability to produce K1 capsule. Protein K, like the OmpF and OmpC porin proteins, can be dissociated from peptidoglycan by extracting the insoluble residue with 0.4 M NaCl at 37°C for 2 h (data not shown). Thus, protein K, like *E. coli* K-12 porins, has a noncovalent tight association with peptidoglycan, at least in vitro.

Protein K is immunologically related to strain K-12 porins. Figure 3 shows an immunodiffusion analysis using anti-OmpF antiserum and purified OmpF and K proteins. This antiserum is able to detect two components in its reaction to OmpF protein. Based on their diffusion in the gel and their behavior in the presence of detergents, the two components are likely to be the monomer and multimer species of OmpF protein. (The inclusion of 0.5% Triton X-100 in the agarose gel allows diffusion of OmpF protein from the well, but is not sufficient to totally dissociate OmpF protein into monomers.) Furthermore, this antiserum recognizes the same antigenic determinants in the multimer forms of protein K and OmpF protein as seen by the line of identity. However, other anti-OmpF antisera demonstrated that protein K was not antigenetically identical with OmpF protein (data not shown). Antisera to OmpF protein also cross-reacts with OmpC and PhoE porins (2, 24).

Protein K is structurally related to strain K-12 porins. We compared chymotryptic digest patterns of protein K with OmpF protein from *E. coli K-12*. Figure 4 is a composite representation of these chymotryptic peptide maps. There are 8 common peptides out of 17, and 6 of 8 are found in the region of the chromatogram where the more hydrophobic peptides are found (32). By using this same technique, we found that protein K also had 7 or 8 of 17 peptides in common with OmpC and PhoE porins, respectively. Of these seven or eight peptides, four were common to all porins (data not shown).

Protein K is functionally related to strain K-12 porins. Based on the protein K structural similarity to strain K-12 porins, it seems reasonable that protein K might also be functionally analogous to porins. A gamma-vector carrying the structural gene for protein K was constructed in strain CS146, an *ompF ompC ompA* mutant (cloning technique from T. Gregg, G. A. McDonald, S. S. Cross, W. R. Marcotte, D. J. Conard, and C. Schnaitman, personal communication). Strain CS146 does not grow on minimal glucose medium or minimal glucose medium supplemented with 0.2% Casamino Acids. The transductant producing protein K, strain JS17, grows on these media with a doubling time of 85 min (minimal glucose) to 46 min (minimal glucose plus 0.2% Casamino Acids). A difference in growth rate is also seen in more complex media. In Franz medium, strain CS146 has a doubling time of 300 min, compared with 33 min for strain JS17. In L broth, the doubling times are 44 min (CS146) and 22 min (JS17). Strain JS17 is also at least 1,000-fold more sensitive than strain CS146 to colicins E2 and E3, suggesting the presence of a functional porin (27). In addition, the rate of

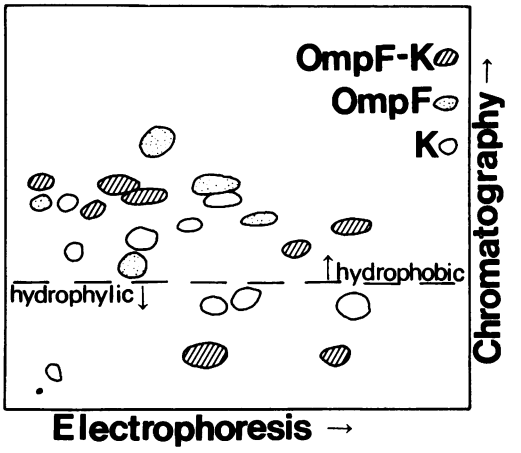

FIG. 4. Composite chymotryptic peptide map of OmpF protein and protein K. Protein K and the OmpF protein were separately iodinated in polyacrylamide gel slices. The ^{125}I-labeled proteins were digested with chymotrypsin. The resulting peptides were separating by two-dimensional thin-layer chromatography and electrophoresis. The direction of electrophoresis and ascending chromatography as well as the hydrophobic and hydrophylic regions of the chromatogram are as indicated.

TABLE 2. Uptake of nutrients by strains CS146 and JS17

Transport substrate (0.5 μM)	Relative initial uptake rate[a]	
	CS146	JS17
Proline	1.0 (3.2)	2.9
α-Methyl glucoside	1.0 (2.3)	1.4
Leucine	1.0 (0.23)	1.4
Lysine	1.0 (0.13)	1.9

[a] The initial uptake rate, in picomoles per 10^8 cells per minute, is given for strain CS146 within parentheses and is set at 1.0. The initial uptake rate for strain JS17 is relative to its parent strain CS146.

uptake of certain nutrients in strain JS17 is increased 1.5- to 3-fold over the rate of uptake in strain CS146 (Table 2).

Studies with black lipid membranes. To examine the pore-forming capacity of protein K in vitro, we turned to studies with black lipid membranes. This system has been used as an exquisitely sensitive assay system to study transport of ions mediated by transport entities such as carriers and channels. Figure 5 shows the effect of protein K on the conductance of an oxidized cholesterol black lipid membrane. Conductance changed in the form of discrete, uniform steps characteristic of channels (4). The unit channel size was about 2 nS. We occasionally observed steps of 4 and 6 nS, consistent with the cooperative opening or closing of two or three channels. The conductance of multichannel membrane appeared to be voltage independent (data not shown). We have observed similar discrete steps induced by protein K in planar bilayers made of phosphatidylethanolamine of tips of patch electrodes (data not shown).

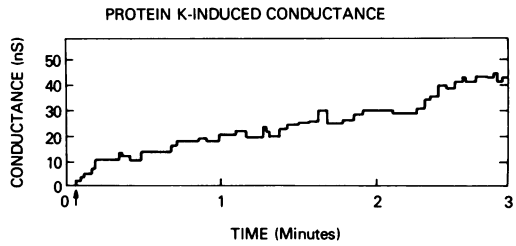

FIG. 5. Protein K-induced conductance changes in black lipid membranes. The black lipid membrane was formed from a 2% solution of oxidized cholesterol in *n*-decane. Both compartments contained 1 M KCl–10 mM HEPES at pH 7.4. Protein K was added at time zero to one compartment at a final concentration of 1 pg/ml. The voltage (20 mV) was applied from a DC battery switched at 30 s (arrow). Current was passed through the black lipid membrane by means of Ag/AgCl electrodes and fed into a current transducer that converted current into a voltage signal.

DISCUSSION

In the initial description of protein K, it was suggested that this protein was at least partially homologous to porin protein I from *E. coli* B. Our results support structural relatedness of protein K to porin proteins and extend the similarity to one of function. Data presented in this paper on the structural and functional nature of protein K are summarized as follows. (i) Protein K spans the outer membrane as demonstrated by its accessibility to surface-labeling reagent and its ability to interact with peptidoglycan in vitro. (ii) Protein K cross-reacts with anti-OmpF antisera. (iii) OmpF protein and protein K share 8 of 17 chymotryptic peptides. (iv) Protein K confers a faster growth rate and enhances uptake of nutrients when it is expressed in porin-deficient strains. (v) Protein K restores sensitivity to colicins E2 and E3 when it is expressed in porin-deficient strains. (vi) Protein K functions in vitro as a porin in black lipid membranes. The latter three findings suggest that protein K functions as a pore in vivo and in vitro.

Because of the apparent association of protein K with encapsulation in strains of *E. coli*, it was suggested (25) that protein K may be involved in capsular biosynthesis, expression, or attachment. The ability to express the K1 capsule in an *E. coli* K-12 strain that does not contain protein K suggests that protein K is not an absolute requirement for K1 capsule expression (J. Foulds and R. Silver, unpublished data). However, since protein K is structurally and functionally related to K-12 porins, a K-12 porin may substitute for putative role of protein K in capsular biosynthesis, excretion or attachment. We are currently investigating expression of K1 capsule in porin-deficient strains of *E. coli* K-12 in an attempt to answer this question.

During the preparation of this manuscript, we learned that C. Whitfield et al. (personal communication) had obtained similar data on the porin function of protein K in artificial lipid bilayers.

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We thank Carl Schnaitman in whose laboratory one of us (J.S.) carried out the initial experiments that led to the cloning of protein K by the procedure developed by Tom Gregg. We also thank Dr. Schnaitman for providing us with the manuscript describing this technique before publication.

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