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Outer Membrane Protein K of *Escherichia coli*: Purification and Pore-Forming Properties in Lipid Bilayer Membranes

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Protein K, a recently described outer membrane protein correlated with encapsulation in *Escherichia coli* (Paakkanen et al., J. Bacteriol. **139**:835–841, 1979), has been purified to apparent homogeneity. Purification was based upon the noncovalent association of protein K with peptidoglycan, and the purified protein was shown to form sodium dodecyl sulfate-resistant oligomers on polyacrylamide gels. Incorporation of small amounts (10^{-10} to 10^{-11} M) of purified protein K into artificial lipid bilayers resulted in an increase, by many orders of magnitude, in membrane conductance. The increased conductance resulted from the formation of large, water-filled, ion-permeable channels exhibiting single-channel conductance in 1.0 M KCl of 1.83 nS. The membrane conductance showed a linear relationship between current and applied voltage and was not voltage induced or regulated. The channel was permeable to large organic ions (e.g., $Tris^+$ Cl^-) and, based upon a pore length of 7.5 nm, a minimum channel diameter of 1.2 nm was estimated; these properties resemble values for other enteric porins. The possible biological role of the pores produced by protein K is discussed.

The outer membranes of *Escherichia coli* and other members of the *Enterobacteriaceae* contain several characteristic major proteins (2, 18). The relative amounts of these proteins may vary considerably and are dependent, in part, upon culture conditions (19, 27, 40). The outer membrane of *E. coli* K-12 contains three major polypeptides in the molecular weight range of 33,000 to 42,000; these proteins have been named OmpA, OmpF, and OmpC after their respective structural genes (31).

The OmpA protein serves a function in F-pilus-mediated conjugation (36, 39) and is characteristically degraded during trypsin treatment of outer membranes (13). Both OmpC and OmpF proteins form tight complexes with peptidoglycan and are not dissociated by treatment with 2% sodium dodecyl sulfate (SDS) at 60°C (22, 32). These proteins form large water-filled pores through the hydrophobic portion of the outer membrane and have been collectively termed porins (reviewed in reference 23). Thus, the porin proteins confer on the outer membrane a function as a molecular sieve with defined exclusion limits for hydrophilic molecules (23).

Recent studies have shown that encapsulated *E. coli* isolates have outer membrane protein profiles similar to that of *E. coli* K-12 but have demonstrated heterogeneity in protein migration during SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (1, 14–16, 28, 29). Subdivision of encapsulated *E. coli* isolates into discrete groups based upon outer membrane protein profiles, however, correlates well with serotype and other properties (1). A new major outer membrane protein present in encapsulated *E. coli*, regardless of K (capsular) serotype, but rarely found in nonencapsulated bacteria, has been recently described (1, 29, 41). This protein, consequently named protein K (29), has an apparent molecular weight of 40,000 and, in contrast to other major outer membrane proteins, shows a remarkably constant migration on SDS-PAGE. Protein K is resistant to solubilization by SDS at 37°C but susceptible at 100°C and is not degraded during trypsin treatment of outer membranes (1). These properties considered together with shared amino acid homology with OmpC and OmpF proteins (29) suggest that protein K may be a porin. Furthermore, the correlation between encapsulation of *E. coli* and the presence of protein K in the outer membrane suggests that protein K may play an important role in the synthesis or assembly of capsular polymers or in the anchorage of such polymers to the cell surface.

The following study was initiated to gain a better understanding of the function of protein K in an attempt to resolve its role in capsule synthesis. We describe the purification of protein K, using existing porin techniques, and

report on the properties of protein K as a porin in a black lipid bilayer model system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* CS146, N63-2, and JS17 were kindly provided by Joyce Sutcliffe (Abbott Laboratories). *E. coli* CS146, originally from the culture collection of Carl Schnaitman, is a K-12 strain lacking the major outer membrane proteins OmpA, OmpC, and OmpF. Strain N63-2, a nonencapsulated mutant of strain N63, contains protein K in the outer membrane (29). *E. coli* JS17 is a CS146 derivative which carries the structural gene for protein K in the genome (J. Sutcliffe, personal communication); the source of the protein K gene was strain N63-2.

Bacteria were subcultured on plates of brain heart infusion agar (Difco Laboratories) at 37°C. Cells were grown with aeration at 37°C in L-broth containing (per liter of distilled water): 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 10 g of sodium chloride, and one pellet of sodium hydroxide.

Membrane preparation. Cells were harvested in mid- to late-log phase and washed once in 10 mM Tris-hydrochloride, pH 8.0. Routinely, washed cells from 1 liter of L-broth were suspended in 5 ml of 10 mM Tris-hydrochloride, pH 8.0, and disrupted by two passages through a French pressure cell (Aminco) at 10,000 lb/in^2. Intact cells were removed from the lysate by centrifugation at 3,000 × *g* for 20 min. Membranes were sedimented from the cell-free supernatant by centrifugation at 100,000 × *g* for 30 min, washed once, and suspended to a final protein concentration of approximately 5 mg/ml in 10 mM Tris-hydrochloride, pH 8.0.

Extraction of outer membrane. Partially purified outer membrane was isolated on the basis of its insolubility in the detergent Sarkosyl (sodium lauroyl sarcosinate; Sigma Chemical Co.). To 1 ml of membrane suspension prepared as above, 0.1 ml of 20% (wt/vol) Sarkosyl in 100 mM Tris-hydrochloride, pH 7.6, was added. After vigorous mixing, extraction was performed for 30 min at room temperature. Sarkosyl-insoluble outer membranes were isolated by centrifugation for 5 min in an Eppendorf bench-top centrifuge and suspended in 0.2 ml of distilled water.

Extraction of protein K. Protein K was extracted from strain JS17 by using the technique established by Nakae et al. (21, 22) for the purification of *E. coli* porins. Briefly, membranes prepared from 2 liters of late-log cells were suspended in 80 ml of 10 mM Tris-hydrochloride, pH 8.0, containing 2% (wt/vol) SDS and incubated at 32°C for 30 min. The insoluble fraction containing peptidoglycan and associated proteins (including porins) was isolated as a pellet after centrifugation at 100,000 × *g* for 30 min. The pellet was washed once with distilled water and suspended at a protein concentration of 4 mg/ml in 50 mM Tris-hydrochloride, pH 8.0, containing 0.4 M NaCl, 5 mM EDTA, 3 mM NaN_3, and 0.05% 2-mercaptoethanol. Peptidoglycan-associated proteins were released after incubation at 37°C for 2 h and freed from insoluble material by centrifugation at 100,000 × *g* for 30 min.

The supernatant was applied to a Sepharose-6B column equilibrated and run in 50 mM Tris-hydrochloride, pH 8.0, containing 0.4 M NaCl, 5 mM EDTA, 3 mM NaN_3, and 0.05% 2-mercaptoethanol. Fractions of 2.5 ml were collected at a flow rate of 10 ml/h and monitored for protein. Those fractions containing pure protein K, as determined by SDS-PAGE, were pooled and dialyzed for 1 week against 3 mM NaN_3 at room temperature.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (17), using discontinuous gels with 12% (wt/vol) acrylamide. Samples were dissolved in Laemmli sample buffer (17) before electrophoresis.

Protein determination. Protein was measured in the presence of detergent, using the method of Sandermann and Strominger (33).

Black lipid bilayer experiments. Detailed descriptions of the methods used for black lipid bilayer experiments have been described elsewhere (5–7). The chamber used for membrane experiments was made of Teflon, and the two compartments of the chamber connected by a small circular hole (0.1 and 2.0 mm^2 for single-channel and macroscopic conductance measurements, respectively). A lipid bilayer was formed across the hole by painting on a solution of 1 to 2% (wt/vol) oxidized cholesterol or diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in *n*-decane, and its formation was indicated by the membrane turning black to incident light. Conductance through pores was measured by using a pair of Ag/AgCl electrodes immersed in aqueous salt solutions at both sides of the membrane. Current was amplified, monitored on a storage oscilloscope, and recorded on a strip chart recorder. Experiments were performed at 25°C.

RESULTS

Purification and properties of protein K. Sarkosyl-insoluble outer membranes from *E. coli* JS17 show a major polypeptide (protein K) with an apparent molecular weight of 40,000 on SDS-PAGE after solubilization in SDS sample buffer at 100°C for 5 min (Fig. 1, lane B). Protein K is a major component of the outer membrane of strain N63-2 but is absent in CS146 (lanes C and A, respectively). Migration of protein K was slightly retarded in strain N63-2, which contains the other major outer membrane proteins OmpC and OmpF.

Protein K was purified to apparent homogeneity (Fig. 1, lanes D and E) from membranes of strain JS17 as described above. The procedure is based upon the preparation of peptidoglycan and its associated proteins, followed by release of the noncovalently bound proteins by treatment with salt and detergent. Preparations of highest purity (as demonstrated by SDS-PAGE) were obtained by using membranes which were twice extracted with detergent in the absence of salt. In typical preparations, 3 to 5 mg of highly purified protein K was obtained from 2 liters of original culture (approximately 250 mg of membrane protein).

Purified protein K exhibits an apparent molecular weight on SDS-PAGE of 40,000 (Fig. 1, lanes D and E) after solubilization in SDS at 100°C and corresponds exactly to the apparent

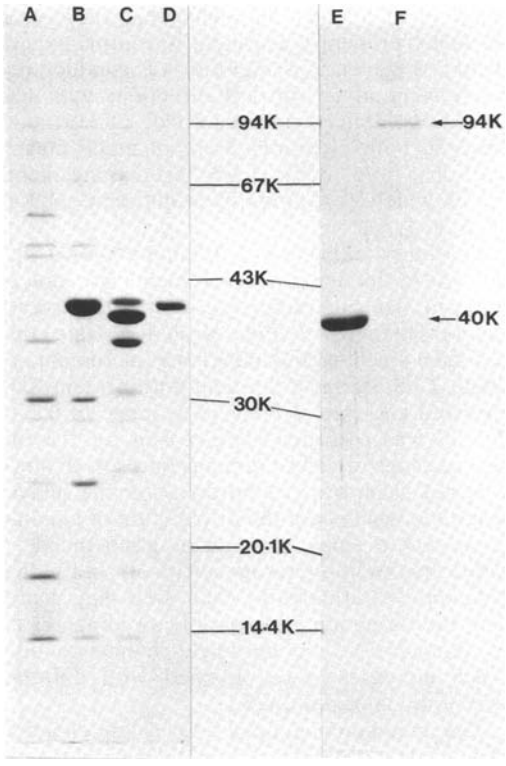

FIG. 1. SDS-PAGE of purified protein K and of outer membranes. Lanes A to C show Sarkosyl-insoluble outer membrane preparations from *E. coli* CS146, JS17, and N63-2, respectively; protein K purified from strain JS17 is shown in lane D. Lanes E and F demonstrate the effect of solubilization at 100°C (lane E) or 37°C (lane F) on the migration of purified protein K. Unless otherwise stated, proteins were solubilized at 100°C before electrophoresis. The positions of molecular weight standards are shown.

molecular weight of protein K in the outer membrane of strain JS17. Solubilization at temperatures below 60°C resulted in an apparently higher-molecular-weight form of purified protein K (approximately 94,000) (Fig. 1, lane F) and an absence of the 40,000-molecular-weight polypeptide from the outer membrane profile (data not shown). The higher-molecular-weight form showed less staining than would be expected for the amount of protein applied, an observation also made with *E. coli* PhoE and *Pseudomonas aeruginosa* porins (R. E. W. Hancock, unpublished data). The higher-molecular-weight form is assumed, by analogy with other enteric porins (22, 23), to represent the oligomeric (probably trimeric) form. Although the apparent molecular weight is lower than the calculated value for such a trimer, this could be accounted for by the anomalous migration of such complexes on SDS-PAGE (22).

Macroscopic conductance measurements. Addition of purified protein K to the aqueous phase bathing a lipid bilayer resulted in an increase in membrane conductance over several orders of magnitude (Fig. 2). A similar increase resulted whether the protein was added before membrane formation or after the membrane had turned black. After the membrane turned completely black (t = 0; Fig. 2), conductance increased with time, presumably indicating self-assembly of protein K into the lipid bilayer as a function of time. In agreement with this proposal, the rate of increase of conductance was dependent upon the concentration of protein K in the aqueous phase. In these experiments, membrane breakage occurred before a stationary conductance level could be achieved.

The membrane bilayer used in these experiments was formed from 1 to 2% (wt/vol) oxidized cholesterol in *n*-decane. Similar experiments performed with 1 to 2% diphytanoyl phosphatidylcholine in *n*-decane demonstrated that the channel-forming properties of protein K were not restricted to a single type of lipid bilayer. The single-channel conductance increment (see below) for protein K was independent of the nature of the lipid bilayer; however, the rate of incorporation of porin into the membrane was higher in experiments with oxidized cholesterol. This phenomenon has been previously reported for several other porins (5–8).

With a membrane containing a significant number ($n > 100$) of channels, a plot of current measured versus voltage applied was linear (Fig. 3) and passed through zero current at zero voltage, indicating that the channels produced by protein K were neither induced nor regulated by voltage. Similar findings have been previous-

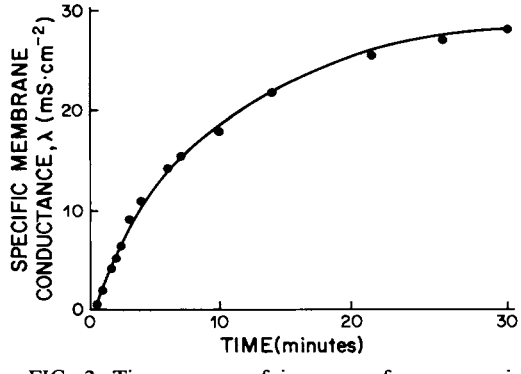

FIG. 2. Time course of increase of macroscopic conductance caused by the addition of 42 pg of purified protein K per ml to the aqueous solution bathing a lipid bilayer membrane. Experiments were performed at 25°C, using membranes made from 1% oxidized cholesterol in *n*-decane with 1.0 M KCl as the bathing solution and an applied voltage of 10 mV.

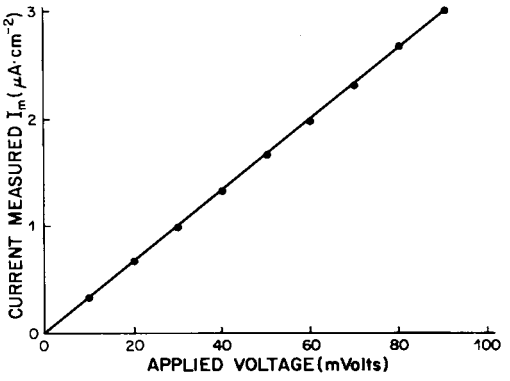

FIG. 3. Relationship between the mean current through single channels (I_m), averaged for a large number of conductivity increases ($n > 100$), and the voltage applied across the bilayer membrane. Otherwise, conditions were as described in the legend to Fig. 2.

ly reported (4–6). With slightly different techniques, however, the matrix protein of *E. coli* B has been proposed to be voltage regulated (34).

Single-channel experiments. Purified protein K, added in small concentrations (10^{-10} to 10^{-11} M) to the aqueous phase, resulted in an increase in membrane conductance in a stepwise manner. A typical recording is illustrated in Fig. 4. The stepwise increments in conductance resulted directly from channels produced by protein K and could not be induced by detergent, using levels of SDS 1,000-fold higher than those used in the experiments described. In routine experiments (Fig. 4), the majority of conductance steps were in an upward direction, with few downward steps being recorded; conductance steps in both directions were of equal magnitude. The ratio of initiating to terminating events varies widely for porins from different sources (5, 6, 8, 12).

Previous findings (5–8, 12) have shown that the single conductance increments are not of uniform size but rather are distributed over a range. Histograms (Fig. 5) demonstrate that although single-step conductance increments of about 2 nS are most frequent with protein K in oxidized cholesterol bilayers, a range of 0.5 to 3.5 nS was recorded. The reason for the observed range of values remains unresolved since it is not clear whether porins enter the bilayer singly, in which case the distribution of conductance values may arise from a chemical or conformational heterogeneity of individual channels. Alternatively, each step may represent the formation of a variably sized aggregate of channels (7). Identical single-channel conductance increases were obtained with different preparations of protein K.

The average conductance for single channels produced by protein K in lipid bilayers bathed in 1.0 M KCl was 1.83 nS. This value is similar to those obtained for *E. coli* outer membrane protein Ic (5; R. Benz and R. E. W. Hancock, unpublished data), also called e or PhoE (37), but slightly smaller than those obtained for other enteric porins (5). Despite variations in average conductance increase ($\bar{\Lambda}$) and specific conductance (σ) of the given aqueous salt solution, the ratios of $\bar{\Lambda}$ to σ for NaCl, KCl, and NH_4Cl remained relatively constant (Table 1). These

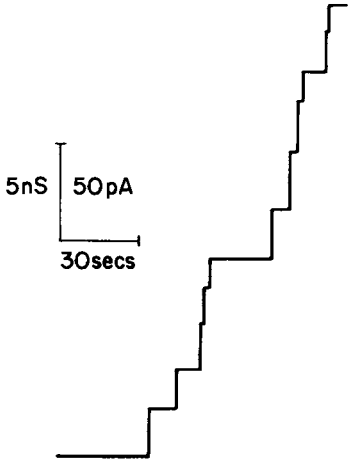

FIG. 4. Stepwise increase of the membrane current after addition of purified protein K to a final concentration of 5×10^{-11} M (6 pg · ml^{-1}) into the aqueous phase containing 1.0 M KCl; temperature, 25°C. The membrane was formed from a 1% (wt/vol) solution of oxidized cholesterol in *n*-decane. The applied voltage was 10 mV. The record begins at the left; usually only upward directed current increases were observed.

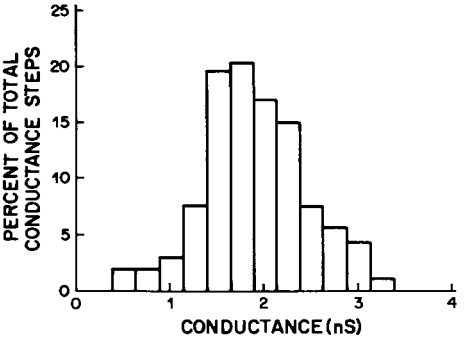

FIG. 5. Histogram of conductance steps in 1.0 M KCl. The membranes were made of 1% (wt/vol) oxidized cholesterol; applied voltage was 10 mV. Protein K (6 pg · ml^{-1}) was added to the aqueous phase. The total number of conductance increments examined was 158.

TABLE 1. Average conductance increment ($\bar{\Lambda}$) in different salt solutions[a]

Salt	Concn (M)	$\bar{\Lambda}$ (nS)	σ(nS · cm^{-1})	$\bar{\Lambda}/\sigma$	n
NaCl	1.0	1.23	84	1.46	102
KCl	1.0	1.83	112	1.63	121
NHCl	1.0	1.86	112	1.66	143
Tris$^+$ Cl$^-$	0.5	0.23	30	0.76	89

[a] The aqueous phase contained the indicated concentration of salt, and 10^{-10} to 10^{-11} M protein K was added to initiate single-channel conductance increments. Otherwise, conditions were as reported for Fig. 4; σ is the specific conductance of the given aqueous salt solution at 25°C. $\bar{\Lambda}$ was determined by recording a large number of conductance steps (n) and averaging.

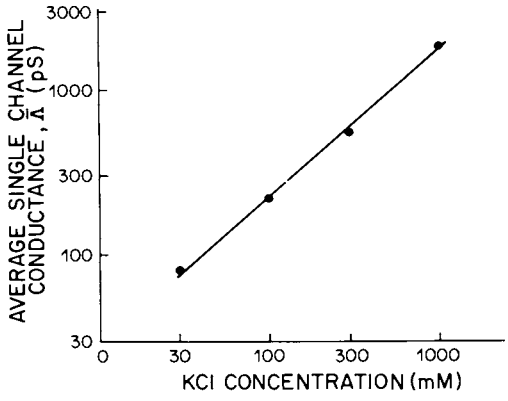

FIG. 6. Average single-channel conductance ($\bar{\Lambda}$) as a function of KCl concentration in the aqueous phase; temperature, 25°C. Membranes were formed from 1% (wt/vol) oxidized cholesterol in n-decane. Protein K (5×10^{-12} M) was added to the aqueous phase, and conductance increments were averaged for 74 to 158 individual events.

results indicate that the properties of the channels produced in lipid bilayers by protein K follow those expected for aqueous salt solutions. Thus, protein K produces large aqueous channels in the bilayer. The ratio of $\bar{\Lambda}$ to σ for Tris$^+$ Cl$^-$ was markedly reduced (Table 1), suggesting a small restriction in permeability to this salt. These results correlate well with those obtained for porins isolated from *E. coli* (8) and *Salmonella typhimurium* (6) and contrast with the larger aqueous channels produced by protein F isolated from *P. aeruginosa* (5).

Results obtained for the average conductance increments in different KCl concentrations are illustrated in Fig. 6. The average conductance increment was found to be a linear function of salt concentration. These results would be expected for large water-filled channels and support the conclusions derived from $\bar{\Lambda}/\sigma$ ratios.

DISCUSSION

The data presented in this paper demonstrate that outer membrane protein K from *E. coli* is a porin. The pores produced by protein K are large water-filled channels as indicated by the linear relationship between current measured and voltage applied in membranes containing significant numbers of channels and by the relatively constant $\bar{\Lambda}/\sigma$ ratios for several salts. Thus, these channels resemble those produced in lipid bilayers by other porins isolated from *E. coli* and *S. typhimurium*.

Single-channel experiments showing the slight restriction in permeability to Tris$^+$ Cl$^-$ gave an indication of pore diameter. A calculated molecular diameter of 0.67 nm has been reported for Tris$^+$ (6), and the experiments reported here suggest that this must be approaching the upper exclusion limit of protein K pores, resulting in the restriction observed. Based upon data obtained in single-channel experiments with 1.0 M KCl and assuming a pore length of 7.5 nm (5), we calculated an effective pore diameter of 1.2 nm for protein K. This value is slightly smaller than those reported for other *E. coli* porins (5) but remains much larger than the 0.5-nm (calculated from ion sizes) protein P channels from *P. aeruginosa* (12). Purified protein K was extremely active in single channel formation; indeed, most experiments were performed with 10^{-10} to 10^{-11} M porin, based upon a monomeric molecular weight of 40,000. Pore conductance increments for specific salts were in general somewhat lower than the equivalent values for other *E. coli* porins (8).

We have not attempted in this study to resolve the question of ion selectivity for protein K channels. By analogy with other *E. coli* porins, a weak preference might be expected. Both OmpF and OmpC proteins have been shown to have a slight preference for cations over anions as purified proteins in lipid bilayers (5, 8) or in liposome swelling assays (24), and these results have been confirmed by experiments with whole cells (25). Such observations are thought to result from the presence of negative changes in, or near, the pore (5). A new *E. coli* porin, PhoE, which is induced in cells starved for phosphate (27, 37), has an apparent preference for anions (24). Protein PhoE has a monomeric molecular weight (on SDS-PAGE) of 40,000, identical to that of protein K. The relationship between K and PhoE remains unclear, but these proteins seem to be induced under different culture conditions, a phenomenon which has been described for the *E. coli* porins OmpC, OmpF, and protein K (1, 23).

Encapsulation of *E. coli* exhibits an apparent

correlation with the presence of protein K in the outer membrane (1, 29, 41), and it has been suggested that the protein may serve an important role in the synthesis, or anchorage, of capsular polysaccharides (29). Since the majority of current evidence points towards membrane phospholipids as the components which bind these polymers to the cell surface (11, 35), it is unlikely that protein K is involved in an anchorage function. Whether porins may mediate the export of a polysaccharide from its site of synthesis at the inner membrane (38) to its final location outside the outer membrane remains a matter for speculation. Calculated pore sizes suggest an exclusion limit for *E. coli* porins at the trisaccharide level. These values would be appropriate for a trisaccharide free in solution; however, it is debatable whether a nascent growing polysaccharide chain might pass in a sequential fashion through a pore in a mechanism analogous to that proposed for membrane protein synthesis and insertion (26). As an example, a growing chain of a linear homopolymer, such as the *E. coli* K1 capsule comprising polysialic acid (38), might present a diameter equivalent to that of the terminal monosaccharide, depending upon its folding characteristics in such a situation.

Regulation of extracellular polysaccharide synthesis by membrane proteins has been suggested for *E. coli* K-12. The presence of protein a (18, 19), also called 3b (3), in the outer membrane is correlated with repression of synthesis of the slime polysaccharide colanic acid (M antigen) (10). Any precise role for this protein is, however, obscured by the pleiotropic effects of the *capR* (*lon*) mutation (20), which derepresses polysaccharide synthesis and represses the expression of protein a (10). There is, however, no evidence implicating the *lon* locus in control of strain-specific capsular polysaccharide (K antigen). With respect to the apparent coregulation of protein K and capsular polymers in *E. coli*, it is interesting to note that both porin (30) and capsule (9) have been suggested to be essential for survival of the bacterium in nature. It is possible that both are synthesized in response to the same selective pressure.

During the preparation of this manuscript, it came to our attention that another group was pursuing similar studies to those presented here. By using a combination of purified protein and whole cell experiments, Sutcliffe et al. (36a) have confirmed our own conclusions regarding the porin function of protein K.

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