JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1137–1143
0021-9193/95/$04.00+0

Vol. 177, No. 5

Characterization and Localization of the KpsE Protein of *Escherichia coli* K5, Which Is Involved in Polysaccharide Export

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Received 29 August 1994/Accepted 19 December 1994

In *Escherichia coli* with group II capsules, the synthesis and cellular expression of capsular polysaccharide are encoded by the *kps* gene cluster. This gene cluster is composed of three regions. The central region 2 encodes proteins involved in polysaccharide synthesis, and the flanking regions 1 and 3 direct the translocation of the finished polysaccharide across the cytoplasmic membrane and its surface expression. The *kps* genes of the K5 polysaccharide, which is a group II capsular polysaccharide, have been cloned and sequenced. Region 1 contains the *kpsE*, *-D*, *-U*, *-C*, and *-S* genes. In this communication we describe the KpsE protein, the product of the *kpsE* gene. A truncated *kpsE* gene was fused with a truncated β-galactosidase gene to generate a fusion protein containing the first 375 amino acids of β-galactosidase and amino acids 67 to 382 of KpsE (KpsE′). This fusion protein was isolated and cleaved with factor Xa, and the purified KpsE′ was used to immunize rabbits. Intact KpsE was extracted from the membranes of a KpsE-overexpressing recombinant strain with octyl-β-glucoside. It was purified by affinity chromatography with immobilized anti-KpsE antibodies. Cytofluorometric analysis using the anti-KpsE antibodies with whole cells and spheroplasts, as well as sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) of proteins from spheroplasts and membranes before and after treatment with proteinase K, indicated that the KpsE protein is associated with the cytoplasmic membrane and has an exposed periplasmic domain. By Tn*phoA* mutagenesis and by constructing β-lactamase fusions to the KpsE protein, it was possible to determine the topology of the KpsE protein within the cytoplasmic membrane.

The pathogenicity of an *Escherichia coli* strain is determined to a large extent by its capsular polysaccharides (K antigens). Particular K antigens are associated with certain infections, and they generally mediate resistance of the bacteria to complement-mediated bacteriolysis and phagocytosis (8, 9, 22–24). The capsular polysaccharides of *E. coli* have been divided into groups I and II on the basis of microbiological, biochemical, and genetic findings (22, 24). Group II capsular polysaccharides are usually produced by extraintestinal *E. coli*. They are expressed at 37°C but not at 18°C (36), and they are linked to phosphatidic acid at their reducing end (22).

The *E. coli* K5 antigen, a group II capsular polysaccharide with the structure -4)-β-D-GlcA-(1,4)-α-D-GlcNAc-(1- (46), is identical to *N*-acetyl heparosan, the first polymeric precursor of heparin (35).

Group II capsular polysaccharide expression in *E. coli* is determined by the chromosomal *kps* gene cluster, which consists of three regions. The central region 2 encodes proteins involved in the synthesis of the capsular polysaccharide and is type specific. Proteins encoded by regions 1 and 3, which are conserved between the different *kps* clusters, are involved in the translocation of the polysaccharide across the cytoplasmic membrane and in its transport to the cell surface (5, 6, 28, 29, 40, 41, 44, 47).

Region 3 of the *kps* cluster contains two genes, *kpsM* and *kpsT* (43). Analysis of the predicted amino acid sequences of the KpsM and KpsT proteins indicated that they belong to a subclass (ABC-2 [39]) of the ABC transporter family and are likely to constitute a polysaccharide export system of the cytoplasmic membrane, energized by ATP hydrolysis (20). The ABC-2 subfamily also includes the transporter proteins BexA and BexB of *Haemophilus influenzae* type b (26, 27) and CtrD and CtrC of *Neisseria meningitidis* (18, 19).

The nucleotide sequence of region 1 of the *kps* gene cluster (GenBank accession number X74567) revealed five genes, termed *kpsE*, *-D*, *-U*, *-C*, and *-S*, which are probably organized in a single transcriptional unit (37, 38). The *kpsU* gene encodes a CMP–3-deoxy-D-*manno*-octulosonic acid synthetase, which accounts for the elevated activity of this enzyme in *E. coli* expressing group II capsules (16, 17, 41a). The *kpsC* and *kpsS* genes encode proteins which seem to play a role in stabilizing polysaccharide biosynthesis on the cytoplasmic membrane, while the KpsE and KpsD proteins appear to be engaged in the export of polysaccharide from the cytoplasmic membrane to the cell surface (3, 4). The *kpsE* genes of *E. coli* K1 and K5 have been sequenced, and the predicted amino acid sequence of the KpsE protein is homologous to those of both the CtrB and BexC proteins (7, 37, 38), which are encoded by the capsule gene clusters of *H. influenzae* and *N. meningitidis*, respectively (18, 19, 26, 27). These proteins, which have been tentatively assigned to the cytoplasmic membrane, are believed to play a role in the export of polysaccharide in these two microorganisms (16, 18, 19, 26). On the basis of their possible function and location within the cytoplasmic membrane, it has been suggested that the CtrB and BexC proteins may be third components of the ABC-2 transporter for the export of polysaccharide across the cytoplasmic membrane (39). In this communication we report the purification of the KpsE protein together with its location and topology within the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used are shown in Table 1. For the expression of the recombinant plasmids pH18 and pCR6, *E. coli* K-12 strain

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TABLE 1. Plasmids used in this study

Plasmid vector	Relevant characteristic(s)	Reference
pROS	Fusion vector, IPTG inducible	11
pCE30	Temperature-inducible expression vector	12
pCR4	Subclone of a 0.95-kb *Dra*I fragment from K5 region 1 that encodes KpsE′ in pROS	This study
pCR6	Subclone of 2.2-kb *Sma*I-*Hin*cII fragment from K5 region 1 that encodes KpsE in expression vector pCE30	This study
pH18	Subclone of 10.4-kb *Hin*dIII fragment from K5 region 1 and part of region 2 in vector pUC18	2

JA221 (F$^-$ *leuB6 trpE5 hsdR hsdM recA1 lacY*) (provided by B. Bachmann) was used. For plasmid pCR4, *E. coli* K-12 strain BMH71/18 [*supE thi* Δ(*lac-proAB*) F′ (*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] (25) was used as a host and *E. coli* DS410 was a source of minicells (48). The bacteria were grown in L broth. Recombinant *E. coli* JA221(pCR6) was grown with 50 μg of ampicillin per ml, and *E. coli* BMH71/18(pCR4) and JA221(pH18) were grown with 100 μg of ampicillin per ml.

Tn*phoA* mutagenesis. Tn*phoA* mutagenesis was performed as described previously (8). The precise site of Tn*phoA* insertion within the *kpsE* gene was determined by nucleotide sequence analysis with oligonucleotide primers to *phoA*.

Generation of β-lactamase fusions. Fusions of *blaM* to the *kpsE* gene were generated by a modification of the method of Zhang and Broome-Smith (49). The transmembrane organization of the KpsE protein was analyzed by determining the level of ampicillin resistance (Amp^r) of individual cells.

Plasmid isolation. Small-scale preparation, large-scale preparation (alkaline lysis of cells), and CsCl gradient purification of plasmid DNA were performed as described by Maniatis et al. (34).

Transformation. Cells were transformed by electroporation (11).

Construction of a plasmid encoding a truncated β-galactosidase–KpsE fusion protein. Restriction enzymes were obtained from GIBCO BRL, DNA ligation system RPN 1507 was obtained from Amersham, and the Prep-A-Gene DNA Purification Kit was obtained from Bio-Rad.

Vector pROS (12) (5 μg) was cleaved with *Stu*I and dephosphorylated with alkaline phosphatase (CIP; Boehringer Mannheim) as recommended by the manufacturer. Plasmid pH18 was cleaved with *Dra*I, and the 950-bp fragment containing the truncated *kpsE* gene was isolated after electrophoresis on a 0.7% agarose gel. Vector DNA (150 ng) and the isolated fragment (50 ng) were ligated. The equivalent of 70 ng of DNA from the ligation reaction was used to transform *E. coli* BMH71/18. The recombinant *E. coli* BMH71/18(pCR4) was identified by minipreparations and *Sca*I digestion.

Construction of the KpsE-overexpressing plasmid pCR6. Plasmid pH18 was digested with *Sma*I and *Hin*cII, and the 2.1-kb fragment, containing *kpsE*, was isolated and ligated with *Sma*I-digested and dephosphorylated pCE30. Transformation was performed in JA221, and the recombinant *E. coli* JA221(pCR6) was identified by *Bam*HI digestion of plasmid DNA.

Analysis of plasmid-coded proteins by minicell analysis. Minicells were purified from plasmid-containing derivatives of *E. coli* DS410 as described elsewhere (48). Proteins were labeled with [^{35}S]methionine as described previously (38).

Purification and cleavage of the fusion protein. A 400-ml culture (optical density at 600 nm [OD_{600}], 0.5) of *E. coli* BMH71/18(pCR4) containing ampicillin (100 μg ml^{-1}) was induced at 37°C with isopropyl thiogalactosylpyranoside (IPTG) (10 mM). At an OD_{600} of 1.2, the bacteria were collected by centrifugation (10,000 × *g*, 10 min, 4°C) and disintegrated with a French press (Aminco) (four times at 75 kg cm^{-2} [11,000 lb in^{-2}]). The homogenate was centrifuged (12,000 × *g*, 10 min, 4°C). The sediment was suspended in 50 mM Tris (pH 8.3) containing 100 mM NaCl, 10 mM EDTA, and 0.5% Triton X-100, and the mixture was incubated for 15 min at room temperature. After centrifugation (10 min, 4°C, 12,000 × *g*), the pellet (fusion protein) was suspended in 20 mM Tris-HCl (pH 8.0) containing 8 M urea.

The fusion protein was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (31) with a vertical model 491 Prep cell (Bio-Rad). The running buffer (50 mM Tris containing 384 mM glycine and 0.1% SDS) was pumped through the elution chamber at a rate of 0.5 ml/min, and fractions (5 ml) were collected. The fractions containing the 80-kDa fusion protein, as determined by SDS-PAGE, were pooled, dialyzed against double-distilled water, and lyophilized. The yield of purified fusion protein was 3 mg from 2 ml of the Tris-urea solution containing 20 mg of protein.

The purified fusion protein was suspended in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 1 mM $CaCl_2$. Factor Xa (1/50 of the substrate by weight) was added, and the mixture was incubated for 1 h at 4°C. The truncated KpsE protein (KpsE′) was separated on an SDS–13% PAGE gel and negatively stained with imidazole-zinc (14). The 35-kDa band was cut from the gel, destained with 2% citric acid, and electroeluted.

Production of anti-KpsE antibodies. An antiserum was produced in New Zealand rabbits with the purified KpsE′ protein. It was absorbed with acetone-dried *E. coli* JA221 to eliminate natural anti-*E. coli* K-12 antibodies. The KpsE-specific antibodies were obtained from the absorbed antiserum on a column of protein A-agarose (Sigma). After removal of salts by chromatography on a PD10 column (Pharmacia) with double-distilled water, an antibody solution containing 3.8 mg of protein per ml was obtained.

Isolation of the KpsE protein and protein sequencing. For the isolation of the KpsE protein, *E. coli* JA221(pCR6) was induced by growth at 42°C for 2 h. Membranes obtained from a 400-ml bacterial culture were incubated for 15 min at room temperature in 4 ml of a mixture containing 48 mM octyl-β-glucoside and 50 mg of phenylmethylsulfonyl fluoride per ml in 10 mM Tris-HCl, pH 7.4. This mixture was centrifuged (180,000 × *g*, 60 min, 4°C), and the supernatant was incubated with anti-KpsE antibodies immobilized on mini-leak low-divinyl sulfone-activated agarose beads (Kem-En-Tec). After 15 min, the beads were removed by centrifugation. Four hundred microliters of sample buffer was added to the washed beads, and the mixture was heated to 95°C for 10 min. After centrifugation, the extract was subjected to SDS-PAGE (10% acrylamide). Proteins were electrophoretically transferred to a mini-Problot membrane (Applied Biosystems), and the bands reactive with anti-KpsE antibodies were cut out and used for automated amino-terminal amino acid sequencing on an Applied Biosystems model 476A gas phase analyzer. Samples were analyzed by high-pressure liquid chromatography through a narrow-bore RP column (C18 micron; 220 by 1.2 mm) in a gradient of acetonitrile (33% for 10 min, 38% for 17 min, 90% for 9.0 min). The flow rate was 0.325 ml min^{-1} at 55°C, and detection was at 269 nm.

Preparation of membranes and cytosol fractions. Membranes and cytosol fractions were prepared by disruption of *E. coli* JA221(pH18), separation of membranes and cytosol by ultracentrifugation, and separation of inner and outer membranes by sucrose gradient centrifugation as previously described (15). Immunoblotting of the fractions from the gradient was performed after electrotransfer, as described previously (45). Their protein concentrations were determined by OD_{280}, and their NADH oxidase activities were assayed as described previously (31).

Incubation with proteinase K. Spheroplasts (1) (right side out) and membrane vesicles obtained from bacterial French press homogenates (about 85% inside out [15]) were incubated with proteinase K (Merck) (250 and 500 mg ml^{-1}, respectively) for 10 min at 37°C. After the addition of phenylmethylsulfonyl fluoride (50 mg ml^{-1}), the mixtures were analyzed with SDS-PAGE and Western blotting (immunoblotting). Samples without proteinase K served as controls.

Preparation of spheroplasts for cytofluorometric (FACS) analysis. For analysis by fluorescence-activated cell sorter (FACS), a culture of *E. coli* JA221(pH18) (75 ml; OD_{600}, 0.3) was centrifuged at 10,000 × *g* for 10 min at 4°C. The sedimented bacteria were suspended in phosphate-buffered saline (PBS)–10 mM EDTA (2×10^{10} cells per ml) and converted into spheroplasts with lysozyme (0.5 mg ml^{-1}). The spheroplasts formed were collected by centrifugation at 6,000 × *g* for 10 min and resuspended in PBS to a density corresponding to about 2×10^{10} cells per ml.

Cytofluorometric (FACS) analysis. *E. coli* JA221(pH18) cells from a 75-ml culture (OD_{600}, 0.3) were collected by centrifugation (10,000 × *g*, 10 min, 4°C) and suspended in 10 ml of PBS. Five milliliters of this suspension and 5 ml of the suspension of spheroplasts described above were incubated for 45 min each, at room temperature and with gentle shaking, with 50 μl of antibody obtained from the absorbed anti-KpsE antiserum. After centrifugation (12,000 × *g*, 3 min, room temperature), the sediments were washed twice with PBS, resuspended in 5 ml of PBS, and incubated for 30 min at room temperature with 50 μl of a solution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (1 mg ml^{-1}). After centrifugation (12,000 × *g*, 3 min, room temperature) and washing with PBS, the sediments were suspended in 4 ml of PBS which had been filtered through a 45-μm-pore-size membrane and were analyzed in a FACScan flow cytometer (Becton Dickinson, Sunnyvale, Calif.). *E. coli* JA221 with no plasmid was subjected to the same procedure as a control.

RESULTS

Construction and purification of the β-Gal′–KpsE′ fusion protein. A gene fusion was constructed that coded for a hybrid protein containing amino acids 1 to 375 of β-galactosidase (β-Gal′) linked to amino acids 67 to 382 of KpsE (KpsE′) (Table 1; Fig. 1) by the recognition sequence for factor Xa protease. Plasmid pCR4, carrying the gene fusion, was introduced into *E. coli* BMH71/18. Induction with IPTG resulted in the synthesis of large amounts of the fusion protein (Fig. 2, lanes 1 and 2), which formed insoluble inclusion bodies. After isolation by centrifugation (Fig. 2, lane 3), the pellet was resuspended in 8 M urea–20 mM Tris, pH 8.0. The fusion protein was purified by preparative gel electrophoresis (Fig. 2, lane 4)

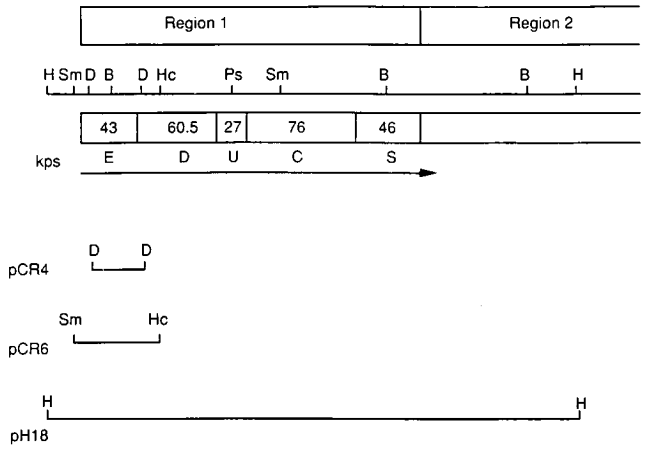

FIG. 1. Physical maps of subclones of the K5 region 1 genes. Solid lines indicate the DNA present in the plasmids described in the text. Boxes E, D, U, C, and S are the five genes encoded in region 1, and the arrow denotes the direction of transcription. Restriction site abbreviations: H, *Hin*dIII; Sm, *Sma*I; D, *Dra*I; B, *Bam*HI; Hc, *Hin*cII; Ps, *Pst*I.

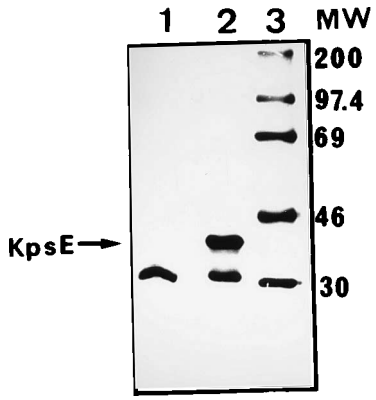

FIG. 3. Autoradiogram of a gel containing ^{35}S-labeled proteins encoded by plasmids pCR6 and pCE30 in minicells. Minicells were isolated from strain DS410 carrying pCE30 (lane 1) or pCR6 (lane 2), and plasmid-encoded proteins were labeled with [^{35}S]methionine. The labeled proteins were separated by SDS-PAGE, which was followed by autoradiography. The numbers on the right refer to the molecular weight (MW) protein markers (lane 3) (in thousands).

and cleaved with factor Xa (Fig. 2, lane 5). The KpsE′ protein thus obtained was electroeluted from SDS-PAGE gels (Fig. 2, lane 6) and was used for the immunization of rabbits.

Construction of the KpsE-overexpressing plasmid pCR6. For the isolation of KpsE, overexpressing plasmid pCR6 (Table 1; Fig. 1) was constructed, using promoter vector pCE30 (13). This vector contains the *c*I857ts gene and both strong λ promotors p_R and p_L arranged in tandem to promote transcription in the same direction. Minicell analysis of plasmid pCR6 revealed, after a temperature shift, the presence of a single nonvector radiolabeled protein of 43 kDa (Fig. 3). Plasmid pCR6 was introduced into *E. coli* JA221, and the resulting *E. coli* JA221(pCR6) was grown at 30°C. A temperature shift of the culture to 42°C resulted in overexpression of KpsE in the recombinant bacteria. A Western blot of homogenates from *E. coli* JA221(pCR6) before and after temperature induction demonstrated overexpression of the KpsE protein (data not shown).

Isolation of the KpsE protein and N-terminal sequencing. Membranes from *E. coli* JA221(pCR6), in which the production of the KpsE protein had been induced, were obtained by disintegration of the bacteria with a French press and differential centrifugation. The KpsE protein was extracted from these membranes with octyl-β-glucoside and immunoabsorption on immobilized anti-KpsE′ antibodies. The KpsE protein exhibited two bands, both of which were reactive with the anti-KpsE antibodies (Fig. 4). The immunprecipitated proteins were transferred (45) to a polyvinylidene difluoride membrane, and both bands that were reactive with the anti-KpsE antiserum were used for sequential Edman degradations in an automated amino acid analyzer. The sequence of the 16 amino-terminal amino acids obtained with the protein fractions of both bands is MLIKVKSAVSWMRARL, which corresponds exactly to the predicted amino acid sequence previously reported (37, 38).

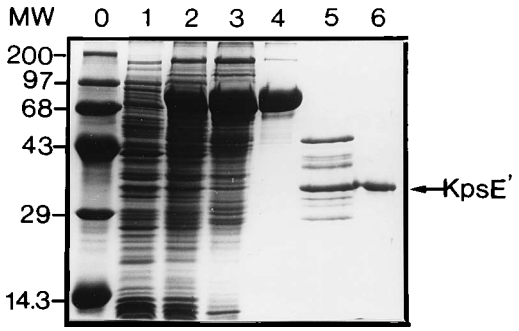

FIG. 2. Coomassie blue-stained gel monitoring the purification of the truncated KpsE protein (KpsE′). *E. coli* BMH71/18(pCR4) was grown at 37°C (lane 1). At an OD_{600} of 0.5, IPTG was added to induce the expression of the fusion protein (lane 2). After disintegration of the bacteria in a French pressure cell, the fusion protein was isolated by centrifugation (lane 3) and preparative SDS-PAGE (lane 4). The purified fusion protein was cleaved with factor Xa (lane 5), and the truncated KpsE protein (KpsE′) was isolated by electroelution (lane 6). The numbers on the left refer to molecular weight (MW) markers (lane 0) (in thousands).

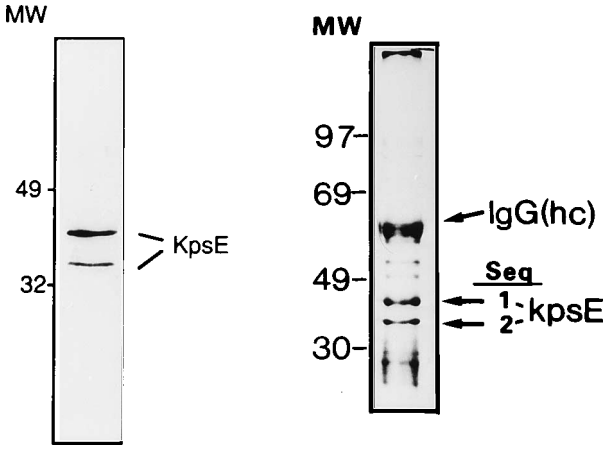

FIG. 4. SDS-PAGE (right) and Western blot (left) analyses of immunoprecipitated KpsE. Membranes of *E. coli* JA221(pCR6) were solubilized with octyl-β-glucoside, and the extract was incubated with immobilized anti-KpsE′ antibodies. After centrifugation and washing, the beads were heated in sample buffer and loaded on an SDS gel. One part of the gel (right panel) was stained with Coomassie blue. IgG(hc), heavy chain of immunoglobulin G. Seq 1 and 2, sequenced bands of the KpsE protein. The proteins of the other part of the gel (left panel) were transferred to a polyvinylidene difluoride membrane and analyzed with anti-KpsE antibodies. The numbers on the left of each gel refer to molecular weight (MW) markers (in thousands).

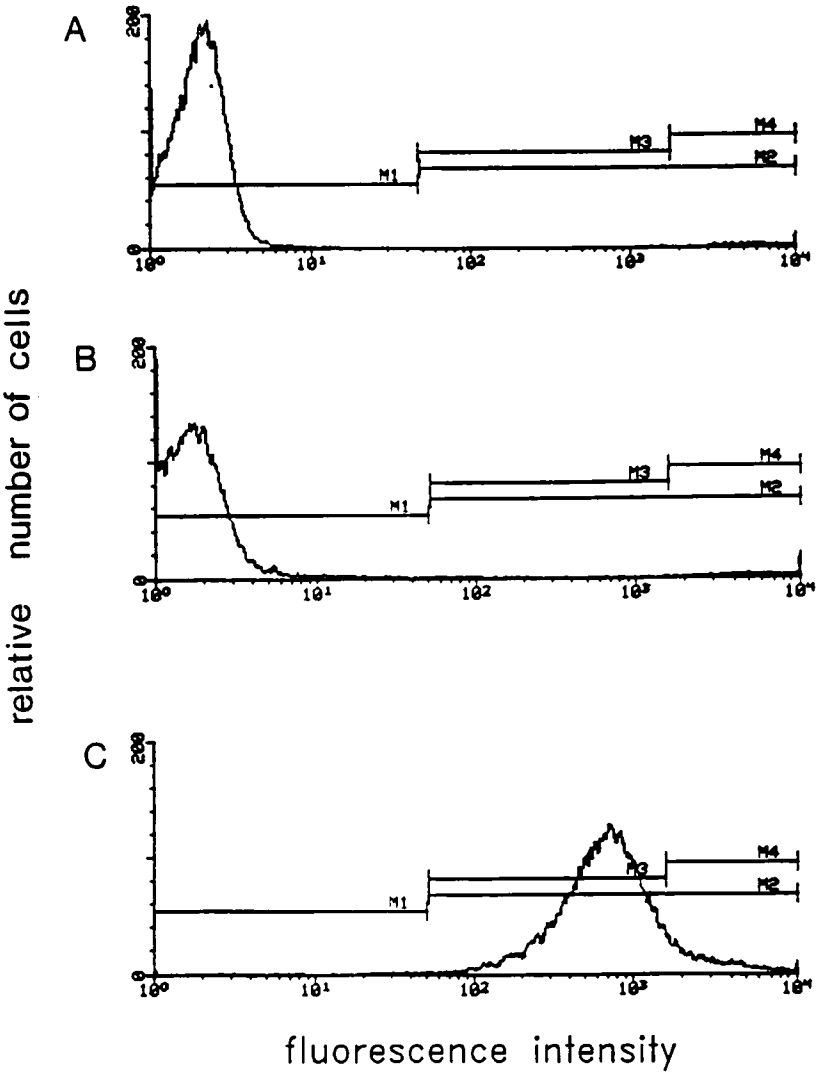

FIG. 5. Cytofluorometric (FACS) analysis of *E. coli* JA221(pH18) and of spheroplasts from *E. coli* JA221(pH18). In each histogram, the vertical axis represents the relative number of cells and the horizontal axis represents fluorescence intensity. Segments M1 to M4 show the percentages of counted cells with a given fluorescence intensity. (A) Labeling of *E. coli* JA221(pH18) spheroplasts with FITC-conjugated anti-rabbit antibodies. M1, 99.08%; M2, 0.92%; M3, 0.03%; M4, 0.9%. (B) Labeling of *E. coli* JA221(pH18) with anti-KpsE antibodies and FITC-conjugated anti-rabbit antibodies. M1, 97.76%; M2, 2.24%; M3, 0.49%; M4, 1.75%. (C) Labeling of *E. coli* JA221(pH18) spheroplasts with anti-KpsE antibodies and FITC-conjugated anti-rabbit antibodies. M1, 0.99%; M2, 98.99%; M3, 89.07%; M4, 10.08%.

Localization of the KpsE protein in *E. coli* JA221(pH18). The anti-KpsE antibodies were used to localize and characterize the KpsE protein in *E. coli* JA221(pH18) (Fig. 1). This strain, which contained the *kpsE* gene on a multicopy plasmid but did not overproduce it, was used instead of the overproducer *E. coli* JA221(pCR6) in order to avoid artifacts due to overproduction. Cytofluorometric (FACS) analysis with whole bacteria indicated that the anti-KpsE antibodies do not bind to the cell surface (there was no fluorescence with a fluorescein-conjugated second antibody). In contrast, spheroplasts from *E. coli* JA221(pH18) exhibited distinct immunofluorescence (Fig. 5). Spheroplasts from *E. coli* JA221 with no plasmid did not show fluorescense in this assay.

Cytoplasmic and membrane fractions of *E. coli* JA221 (pCR6) were analyzed by Western blotting. Only the membrane fraction was reactive with the anti-KpsE antibodies (not shown). The cytoplasmic and outer membranes were separated by sucrose density gradient centrifugation, and the fractions

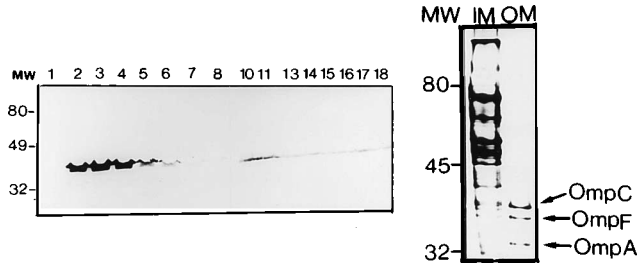

FIG. 6. Western blot analysis of fractions from the isopycnic sucrose gradient centrifugation of membranes from *E. coli* JA221(pH18). Samples of fractions were examined by Western blotting with anti-KpsE antibodies. Lanes 1 to 7 refer to the fractions containing the inner membrane (L band), fractions 10 and 11 contain both inner and outer membranes (M band), and fractions 13 to 18 contain the outer membrane (H band). The peak fractions (3 and 15), representing the inner (IM) and outer (OM) membranes, respectively, were analyzed with SDS-PAGE (right panel). MW, molecular weight markers in thousands.

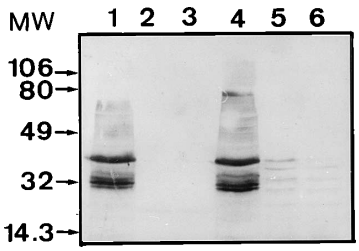

FIG. 7. Western blot of inverted membranes and spheroplasts from *E. coli* JA221(pH18) treated with proteinase K. *E. coli* JA221(pH18) was converted to spheroplasts (right-side-out vesicles) or disintegrated with a French press (inside-out vesicles). Spheroplasts were incubated without proteinase K (lane 1) and with proteinase K at 250 μg ml^{-1} (lane 2) and 500 μg ml^{-1} (lane 3). Inverted membranes were incubated without proteinase K (lane 4) and with proteinase K at 250 μg ml^{-1} proteinase K (lane 5) and 500 μg ml^{-1} (lane 6). MW, molecular weight markers in thousands.

were characterized by their densities, SDS-PAGE patterns, and NADH oxidase activities (not shown). Proteins from fractions with different densities were separated by SDS-PAGE, transferred (46) to a polyvinylidene difluoride membrane, and detected with anti-KpsE antibodies. The results showed that the KpsE protein is associated predominantly with the cytoplasmic membrane (Fig. 6).

The transmembrane organization of KpsE was investigated by treatment with proteinase K of spheroplasts (right side out) and of French press vesicles followed by SDS-PAGE and Western blotting. In spheroplasts the KpsE protein was extensively degraded by proteinase K, and in French press vesicles it was converted to slightly smaller proteins (Fig. 7). These results, together with those described above, indicated that the KpsE protein is integrated in the cytoplasmic membrane, with the greater part of the molecule being exposed to the periplasm.

Analysis of the topology of KpsE within the inner membrane. On the basis of the hydropathy blot of KpsE, derived from the DNA sequence of *kpsE* (37, 38), Tn*phoA*$^+$ insertion mutations at amino acid positions 54 and 56 and *blaM* fusions at amino acids 24, 211, 345, 361, and 381 were generated (Fig. 8). The following levels of Amp^r were determined: fusion at position 24, <5 μg ml^{-1}; fusions at positions 361 and 381, 10 and 15 μg ml^{-1}, respectively; fusions at positions 211 and 345, 150 and 100 μg ml^{-1}, respectively (Fig. 8).

DISCUSSION

In this communication, we report on the isolation and characterization of the KpsE protein of recombinant *E. coli* K5. This protein is known to be involved in the cell surface expression of the K5 capsular polysaccharide (3–6). The generation of a fusion protein containing a truncated form of KpsE (KpsE′) facilitated the preparation of anti-KpsE antibodies. These could be used in the purification of KpsE after extraction with octyl-β-glucoside from membranes of *E. coli* JA221(pCR6) and also in the localization of KpsE in the cell.

The results of the Western blot analysis of separated inner and outer membranes, together with the sensitivity of KpsE in spheroplasts to protease treatment compared with that of inverted vesicles as well as FACS analysis with anti-KpsE antibodies, indicated that KpsE is localized in the cytoplasmic membrane and to a large extent is exposed to the periplasm. This is in agreement with previous data from DNA sequencing of the *kpsE* gene (7, 37, 38) and the prediction that the KpsE protein may be anchored in the cytoplasmic membrane via its amino and/or carboxy termini. The low level of Amp^r conferred by a BlaM fusion at position 24 suggests that this portion of KpsE is likely to be cytoplasmic. The presence of six positively charged amino acids in the first amino acids also suggests that the N terminus of the KpsE protein remains cytoplasmic (10). The isolation of PhoA$^+$ insertions which were mapped at amino acids 54 and 56 suggests that the N-terminal membrane-spanning domain of KpsE is located between amino acids 30 and 50. This is in agreement with the hydropathy plot and structural prediction (30), which indicate a hydrophobic region with an α-helical configuration (Fig. 8). The high level of Amp^r conferred by BlaM fusions at amino acids 211 and 345 confirms the periplasmic location of this region of the KpsE protein. The low level of Amp^r conferred by the BlaM fusions at positions 361 and 381 suggests that these fusions are in the C-terminal membrane domain of the

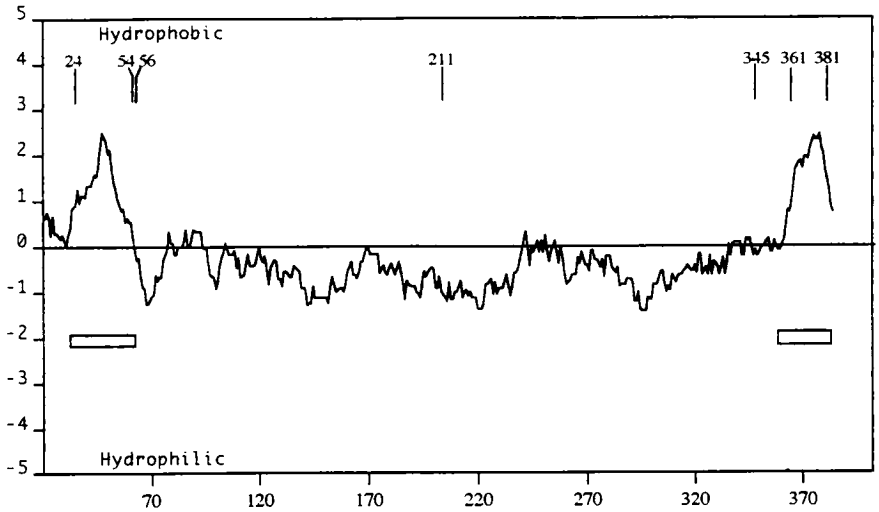

FIG. 8. Hydropathic analysis of the predicted amino acid sequence of the KpsE protein. The method of Kyte and Doolittle (30) with a window size of 20 was used. Residue numbers are on the horizontal axis, and hydrophobicity is on the vertical axis, with increasing hydrophobicity depicted by increasing positive values. The two boxes denote the hydrophobic areas of α-helicity. The numbered vertical lines define the amino acid positions of BlaM fusions, except for numbers 54 and 56, which refer to PhoA$^+$ fusions.

KpsE protein, as predicted from the hydropathy plot. The fact that the fusion at amino acid position 381 conferred Amp^r to 15 μg ml^{-1}, as opposed to the resistance to 5 μg ml^{-1} conferred by the BlaM fusion at position 24, might suggest that the C terminus of the KpsE protein is membrane-associated rather than a transmembrane domain and that the C terminus is not exposed in the cytoplasm. The lack of a net positive charge at the C terminus would be in keeping with this model. These experiments on the topology of the KpsE protein suggest that there is a large periplasmic domain of approximately 300 amino acids.

The N-terminal amino acid sequence of the purified KpsE protein corresponds exactly to that predicted from the DNA sequence reported for the *E. coli* K5 *kpsE* gene (37, 38). This is in contrast to the deduced amino acid sequence predicted for KpsE in *E. coli* K1 (7), which was predicted to begin at a methionine 36 amino acids downstream from the actual translational start point.

However, the observation that a *kpsE* deletion in *E. coli* K1 that affected capsule expression could be repaired with the corresponding gene from *E. coli* K7 (41) suggests a common function for KpsE in *E. coli* strains expressing chemically different group II capsular polysaccharides. The implication of KpsE in polysaccharide export was recently demonstrated by the periplasmic accumulation of the K5 polysaccharide in a *kpsE kpsD* deletion mutant (3, 4). The function of the KpsE protein in the export mechanism, however, is still unclear.

The location of KpsE at the outer face of the cytoplasmic membrane points to its probable role in polysaccharide export after its translocation across the cytoplasmic membrane. Translocation also requires the region 3 proteins KpsM and KpsT, which are thought to form a specialized ABC transporter (20) subclass, the ABC-2 translocator (39, 42). It is possible that the KpsE protein functions in concert with this translocator, accepting the polysaccharide on the outer face of the cytoplasmic membrane and passing it on to the cell surface. The K5 polysaccharide has been reported (28) to exit the cell at (specific) sites where cytoplasmic and outer membranes come into an apparently close apposition and maybe even transiently fuse (2, 20). It will be interesting to study whether the KpsE protein participates in the (probably transient) membrane association.

ACKNOWLEDGMENTS

This work was supported by grant Ja 115/28 from the Deutsche Forschungsgemeinschaft (to K.J.) and by grants from the Lister Institute of Preventive Medicine, Medical Research Council and Science and Engineering Council of the United Kingdom, and the Commonwealth Scholarship Commission of the United Kingdom (to I.S.R.).

We thank Rita Carsetti for help with the cytofluorometric analysis.

REFERENCES

1. **Akiyama, Y., and K. Ito.** 1985. The SecY membrane component of the bacterial protein export machinery: analysis by new electrophoretic methods for integral membrane proteins. EMBO J. **4:**3351–3356.
2. **Bayer, M. H., G. P. Costello, and M. E. Bayer.** 1982. Isolation and partial characterization of membrane vesicles carrying markers of the membrane adhesion sites. J. Bacteriol. **149:**758–767.
3. **Bronner, D., V. Sieberth, C. Pazzani, I. S. Roberts, G. J. Boulnois, B. Jann, and K. Jann.** 1993. Expression of the capsular K5 polysaccharide of *Escherichia coli*: biochemical and electron microscopic analyses of mutants with defects in region 1 of the gene cluster. J. Bacteriol. **175:**5984–5992.
4. **Bronner, D., V. Sieberth, C. Pazzani, A. Smith, G. Boulnois, I. S. Roberts, B. Jann, and K. Jann.** 1993. Synthesis of the K5 (group II) capsular polysaccharide in transport-deficient recombinant *Escherichia coli*. FEMS Microbiol. Lett. **113:**279–284.
5. **Boulnois, G. J., and K. Jann.** 1989. Bacterial polysaccharide capsule synthesis, export and evolution of structural diversity. Mol. Microbiol. **3:**1819–1823.
6. **Boulnois, G. J., and I. S. Roberts.** 1990. Genetics of capsular polysaccharide production in bacteria. Curr. Top. Microbiol. Immunol. **150:**1–18.
7. **Cieslewicz, M. J., S. M. Steenbergen, and E. R. Vimr.** 1993. Cloning, sequencing, expression, and complementation analysis of the *Escherichia coli* K1 *kps* region 1 gene, *kpsE*, and identification of an upstream open reading frame encoding a protein with homology to GutQ. J. Bacteriol. **175:**8018–8023.
8. **Coleman, M. J., J. S. Milner, R. M. Cooper, and I. S. Roberts.** 1991. The use of Tn*phoA* in *Erwinia amylovora* to generate fusions of alkaline phosphatase to extracytoplasmic proteins. FEMS Microbiol. Lett. **80:**167–171.
9. **Cross, A. S.** 1990. The biological significance of bacterial encapsulation. Curr. Top. Microbiol. Immunol. **150:**87–96.
10. **Dalbey, R. E.** 1990. Positively charged residues are important determinants of membrane protein topology. Trends Biochem. Sci. **15:**253–257.
11. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* using electroporation. Nucleic Acids Res. **16:**6127.
12. **Ellinger, S., R. Glockshuber, G. Jahn, and A. Plückthun.** 1989. Cleavage of procaryotically expressed human immunodeficiency virus fusion proteins by factor X_a and application in Western blot (immunoblot) assays. J. Clin. Microbiol. **27:**971–976.
13. **Elvin, C. M., P. R. Thompson, M. E. Argall, P. Hendry, N. P. J. Stamford, P. E. Lilley, and N. E. Dixon.** 1990. Modified bacteriophage lambda promotor vectors for overproduction of proteins in *Escherichia coli*. Gene **87:**123–126.
14. **Fernandez-Patron, C., L. Castellanos-Serra, and P. Rodriguez.** 1992. Reverse staining of sodium dodecyl sulfate polyacrylamide gels by imidazole zinc salts: sensitive detection of unmodified proteins. BioTechniques **12:**564–573.
15. **Finke, A., D. Bronner, E. V. Nikolaev, B. Jann, and K. Jann.** 1991. Biosynthesis of the *Escherichia coli* K5 polysacharide, a representative of group II capsular polysaccharides: polymerization in vitro and characterization of the product. J. Bacteriol. **173:**4088–4094.
16. **Finke, A., B. Jann, and K. Jann.** 1990. CMP-KDO synthetase activity in *Escherichia coli* expressing capsular polysaccharides. FEMS Microbiol. Lett. **69:**129–134.
17. **Finke, A., I. Roberts, G. Boulnois, C. Pazzani, and K. Jann.** 1989. Activity of CMP-2-keto-3-deoxyoctulosonic acid synthetase in *Escherichia coli* strains expressing the capsular K5 polysaccharide: implication for K5 polysaccharide biosynthesis. J. Bacteriol. **171:**3074–3079.
18. **Frosch, M., U. Edwards, K. Bousset, B. Krause, and C. Weisgerber.** 1991. Evidence for a common molecular origin of the capsular gene loci in gram-negative bacteria expressing group II capsular polysaccharides. Mol. Microbiol. **5:**1251–1263.
19. **Frosch, M., D. Müller, K. Bousset, and A. Müller.** 1992. Conserved outer membrane protein of *Neisseria meningitidis* involved in capsule expression. Infect. Immun. **60:**798–803.
20. **Higgins, C. F., S. C. Hyde, M. M. Mimmack, U. Gileadi, D. R. Gil, and M. P. G. Gallagher.** 1990. Binding protein-dependent transport systems. J. Bioenerg. Biomembr. **22:**571–592.
21. **Ishidate, K., E. S. Creeger, J. Zrike, S. Deb, B. Glauner, and T. J. McAlister.** 1986. Isolation of differentiated membrane domains from *Escherichia coli* and *Salmonella typhimurium*, including a fraction containing attachment sites between the cytoplasmic and outer membranes and the murein skeleton of the cell envelope. J. Biol. Chem. **261:**428–443.
22. **Jann, B., and K. Jann.** 1990. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. Curr. Top. Microbiol. Immunol. **150:**19–42.
23. **Jann, K., and B. Jann.** 1985. Cell surface components and virulence: *Escherichia coli* O and K antigens in relation to virulence and pathogenicity, p. 157–176. *In* M. Sussman (ed.), The virulence of *Escherichia coli*. Academic Press, Inc., New York.
24. **Jann, K., and B. Jann.** 1992. Capsules in *Escherichia coli*, expression and biological significance. Can. J. Microbiol. **38:**705–710.
25. **Koenen, M., U. Rüther, and B. Müller-Hill.** 1982. Immunoenzymatic detection of expressed gene fragments cloned in lac Z gene of *E. coli*. EMBO J. **1:**509–512.
26. **Kroll, J. S., I. Hopkins, and E. R. Moxon.** 1988. Capsule loss in *H. influenzae* type b occurs by recombination-mediated disruption of a gene essential for polysaccharide export. Cell **53:**347–356.
27. **Kroll, J. S., B. Loynds, L. N. Brophy, and E. R. Moxon.** 1990. The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsule polysaccharide export. Mol. Microbiol. **4:**1853–1862.
28. **Kröncke, K. D., G. Boulnois, I. Roberts, D. Bitter-Suermann, J. R. Golecki, B. Jann, and K. Jann.** 1990. Expression of the *Escherichia coli* K5 capsular antigen: immunoelectron microscopic and biochemical studies with recombinant *E. coli*. J. Bacteriol. **172:**1085–1091.
29. **Kröncke, K.-D., J. R. Golecki, and K. Jann.** 1990. Further electron microscopic studies on the expression of *Escherichia coli* group II capsules. J. Bacteriol. **172:**3469–3472.
30. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**1005–1032.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
32. **Levengood, S. K., and R. E. Webster.** 1989. Nucleotide sequences of *tolA* and

tolB genes and localization of their products, components of a multistep translocation system in *Escherichia coli*. J. Bacteriol. **171:**6600–6609.
33. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. **19:**119–123.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Navia, J. L., J. Riesenfeld, W. F. Vann, U. Lindahl, and L. Rodén.** 1983. Assay of N-acetylheparosan deacetylase with a capsular polysaccharide from *Escherichia coli* K5 as substrate. Anal. Biochem. **135:**134–140.
36. **Ørskov, F., V. Sharma, and I. Ørskov.** 1984. Influence of growth temperature on the development of *Escherichia coli* polysaccharide K antigens. J. Gen. Microbiol. **130:**2681–2684.
37. **Pazzani, C.** 1992. Molecular and genetic analysis of the *Escherichia coli* K5 capsule gene cluster. Ph.D. thesis. University of Leicester, Leicester, United Kingdom.
38. **Pazzani, C., C. Rosenow, G. J. Boulnois, D. Bronner, K. Jann, and I. S. Roberts.** 1993. Molecular analysis of region 1 of the *Escherichia coli* K5 antigen gene cluster: a region encoding proteins involved in cell surface expression of capsular polysaccharide. J. Bacteriol. **175:**5978–5983.
39. **Reizer, J., A. Reizer, and M. H. Saier, Jr.** 1992. A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. Protein Sci. **1:**1326–1332.
40. **Roberts, I., R. Mountford, N. High, D. Bitter-Suermann, K. Jann, K. Timmis, and G. Boulnois.** 1986. Molecular cloning and analysis of genes for production of K5, K7, K12, and K92 capsular polysaccharide in *Escherichia coli*. J. Bacteriol. **168:**1228–1233.
41. **Roberts, I. S., R. Mountford, R. Hodge, K. Jann, B. Jann, and G. J. Boulnois.** 1988. Common organization of gene clusters for production of different capsular polysaccharides (K antigens) in *Escherichia coli*. J. Bacteriol. **170:** 1305–1310.
41a. **Rosenow, C., I. S. Roberts, and K. Jann.** FEMS Microbiol. Lett., in press.
42. **Saier, M. H., Jr.** 1994. Computer-aided analysis of transport protein sequences gleaning evidence concerning function, structure, biogenesis, and evolution. Microbiol. Rev. **58:**71–93.
43. **Silver, R. P., W. Aaronson, and W. F. Vann.** 1987. Translocation of capsular polysaccharides in pathogenic stains of *Escherichia coli* requires a 60-kilodalton periplasmic protein. J. Bacteriol. **169:**5489–5495.
44. **Silver, R. P., W. F. Vann, and W. Aaronson.** 1983. Genetic and molecular analyses of *Escherichia coli* K1 antigen genes. J. Bacteriol. **157:**568–575.
45. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
46. **Vann, W. F., M. A. Schmidt, B. Jann, and K. Jann.** 1981. The structure of the capsular polysaccharide (K5 antigen) of urinary tract infective *Escherichia coli* O10:K5:H4. A polymer similar to desulfo-heparin. Eur. J. Biochem. **116:**359–364.
47. **Vimr, E. R., W. Aaronson, and R. P. Silver.** 1989. Genetic analysis of chromosomal mutations in the polysialic acid gene cluster of *Escherichia coli* K1. J. Bacteriol. **171:**1106–1117.
48. **Wilkins, B. M., G. J. Boulnois, and E. Lanka.** 1982. A plasmid DNA primase active in discontinuous bacterial DNA replication. Nature (London) **290:** 217–221.
49. **Zhang, Y., and J. K. Broome-Smith.** 1990. Correct insertion of a simple eukaryotic plasma-membrane protein into the cytoplasmic membrane of *Escherichia coli*. Gene **96:**51–57.