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

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In *Escherichia coli* with group II capsules, the synthesis and cellular expression of capsular polysaccharides 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 TnphoA 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→3-β-D-GlcNAc-(1→6)-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...
Proteins were labeled with [35S]methionine as described previously (38). Identified from plasmid-containing derivatives of E. coli identified by minipreparations and ScaI digestion. System RPN1507 was obtained from Amersham, and the Prep-A-Gene DNA protein. lysis of cells), and CsCl gradient purification of plasmid DNA were performed as described previously (48). The bacteriawere 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(pH81) were grown with 100 μg of ampicillin per ml.

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

**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 (AmpR) 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. (54).

**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.

The purified fusion protein was suspended in 50 mM Tris-HCl (pH 8.0) containing 8 M urea and 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 homogenization of E. coli JA221(pCR6) containing plasmids 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 electro-transfer, as described previously (45). Their protein concentrations were determined by OD₅₀₀ 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 m⁻³, respectively) for 10 min at 37°C. After the addition of phenylmethylsulfonyl fluoride (50 μg m⁻³), the mixtures were analyzed with SDS-PAGE and Western blotting (immunoblotting). Samples without proteinase K served as controls.

**Preparation of spheroplasts for cyttofluorometric (FACS) analysis.** For analysis by fluorescence-activated cell sorter (FACS), a culture of E. coli JA221(pCR6) (75 μl; OD₅₀₀ 0.3) was centrifuged at 18,000 × g for 10 min at 4°C. The membrane fractions were separated by electrophoresis on a 13% polyacrylamide gel. The bands were stained with 0.1% Coomassie blue and destained with 2% citric acid, and electroeluted.

**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 re-suspended in 8 M urea—20 mM Tris, pH 8.0. The fusion protein was purified by preparative gel electrophoresis (Fig. 2, lane 4)
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 cI857ts gene and both strong λ promoters pR and pL 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-D-glucoside and immunabsorption on immobilized anti-KpsE antibodies. The KpsE protein exhibited two bands, both of which were reactive with the anti-KpsE antibodies (Fig. 4). The immunoprecipitated 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).
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 fluorescence 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. 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. (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%.

![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 (IM band), fractions 10 and 11 contain both inner and outer membranes (M band), and fractions 13 to 18 contain the outer membrane (OM 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.
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 hydrophathy blot of KpsE, derived from the DNA sequence of kpsE (37, 38), Tn<sup>phoA<sup>1</sup> insertion mutations at amino acid positions 54 and 56 and bla<sup>M</sup> fusions at amino acids 24, 211, 345, 361, and 381 were generated (Fig. 8). The following levels of Amp<sup>r</sup> were determined: fusion at position 24, <5 μg ml<sup>-1</sup>; fusions at positions 361 and 381, 10 and 15 μg ml<sup>-1</sup>, respectively; fusions at positions 211 and 345, 150 and 100 μg ml<sup>-1</sup>, respectively (Fig. 8).

**DISCUSSION**

In this communication, we report on the isolation and characterization of the KpsE protein of recombinant <i>E. coli</i> 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<sup>E</sup>) facilitated the preparation of anti-KpsE antibodies. These could be used in the purification of KpsE after extraction with octyl-β-D-glucoside from membranes of <i>E. coli</i> 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<sup>r</sup> conferred by bla<sup>M</sup> fusions at positions 361 and 381 suggests that these fusions are in the C-terminal membrane domain of the KpsE protein. The low level of Amp<sup>r</sup> conferred by bla<sup>M</sup> fusions at positions 361 and 381 suggests that these fusions are in the C-terminal membrane domain of the.
KpsE protein, as predicted from the hydrophathy plot. The fact that the fusion at amino acid position 381 conferred Amp" 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 translocation startpoint. 


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