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Nucleotide Sequence and Mutational Analysis of the Gene Encoding KpsD, a Periplasmic Protein Involved in Transport of Polysialic Acid in *Escherichia coli* K1

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The 17-kb *kps* gene cluster encodes proteins necessary for the synthesis, assembly, and translocation of the polysialic acid capsule of *Escherichia coli* K1. We previously reported that one of these genes, *kpsD*, encodes a 60-kDa periplasmic protein that is involved in the translocation of the polymer to the cell surface. The nucleotide sequence of the 2.4-kb *Bam*HI-*Pst*I fragment accommodating the *kpsD* gene was determined. Sequence analysis showed an open reading frame for a 558-amino-acid protein with a typical N-terminal prokaryotic signal sequence corresponding to the first 20 amino acids. KpsD was overexpressed, partially purified, and used to prepare polyclonal antiserum. A chromosomal insertion mutation was generated in the *kpsD* gene and results in loss of surface expression of the polysialic acid capsule. Immunodiffusion analysis and electron microscopy indicated that polysaccharide accumulates in the periplasmic space of mutant cells. A wild-type copy of *kpsD* supplied in *trans* complemented the chromosomal mutation, restoring extracellular expression of the K1 capsule. However, a *kpsD* deletion derivative (*kpsDΔC11*), which results in production of a truncated KpsD protein lacking its 11 C-terminal amino acids, was nonfunctional. Western blot (immunoblot) data from cell fractions expressing KpsDΔC11 suggest that the truncated protein was inefficiently exported into the periplasm and localized primarily to the cytoplasmic membrane.

Escherichia coli that synthesizes the K1 capsular polysaccharide continues to be an important pathogen in the neonate (46). Strains that produce the K1 capsule account for 80% of *E. coli* neonatal meningitis and compose the majority of isolates from neonatal septicemia and pediatric pyelonephritis (22, 39). The K1 capsular polysaccharide is an α2,8-linked homopolymer of sialic acid (*N*-acetylneuraminic acid [NeuNAc]). The polysialic acid (PSA) capsule is an essential virulence determinant allowing *E. coli* to evade normal host defense mechanisms (46). The capsule provides the bacterium with an antiphagocytic barrier characterized by its ability to inhibit complement activation by the alternative pathway (11). Purified PSA is also a poor immunogen in humans and other mammals and does not stimulate synthesis of antibodies required for phagocytic removal of the organism (59). The poor immunogenicity has been explained by the structural identity between the K1 polysaccharide and polysialosyl chains found on host tissue (12, 13).

Biosynthesis of the PSA capsule of *E. coli* K1 involves synthesis, activation, and polymerization of sialic acid subunits into a large polymer that must be transported across the inner and outer membranes and anchored to the cell surface (3, 46, 54). The genes encoding proteins necessary for these events have been isolated and characterized (4, 45, 55). The 17-kb *kps* gene cluster is divided into three functionally distinct regions, a motif reiterated by other capsule-producing gram-negative bacteria (4, 15, 16, 25, 45, 55). The central region, region 2, contains information for synthesis, activation, and polymerization of NeuNAc (40, 49, 60). This region of the cluster is genetically distinct and unique for each *E. coli* K serotype (3, 4, 40). In contrast, the flanking regions, regions 1 and 3, involved in transport of the polymer from the cytoplasm to the bacterial cell surface (4, 46), are highly conserved and functionally equivalent between various encapsulated *E. coli* strains (4, 40, 44, 46). Region 3 includes two genes, *kpsM* and *kpsT*, that function in transport of PSA across the cytoplasmic membrane (33, 47). The *kpsM* gene product is a 29-kDa hydrophobic, integral membrane protein, while *kpsT* encodes a 25-kDa hydrophilic protein that contains a consensus ATP-binding site (33, 47). They belong to the large superfamily of ABC (ATP-binding cassette) transporters (also referred to as traffic ATPases) that includes the periplasmic permeases of enteric bacteria and the medically important mammalian P glycoprotein and cystic fibrosis gene product (CFTR) (1, 10, 21).

Region 1 of the *kps* gene cluster includes genes that are involved in the transport of polymer across the outer membrane to the bacterial cell surface (Fig. 1) (4, 46). This 8.4-kb region accommodates five genes, producing proteins of 75 kDa (KpsC), 60 kDa (KpsD), 45 kDa (KpsS), 43 kDa (KpsE), and 27 kDa (KpsU) (Fig. 1) (4, 34, 45). We previously reported that *kpsD* encodes a 60-kDa periplasmic protein that is necessary for extracellular expression of PSA (44). Cells harboring a Tn*1000* insertion in the *kpsD* gene of the K1 cosmid, pSR23, did not synthesize the 60-kDa protein and did not express polysaccharide on the cell surface (44). Immunodiffusion and rocket immunoelectrophoresis of cell extracts, however, demonstrated that PSA was synthesized by these cells (44). The *kpsD* gene was localized to a 2.4-kb *Bam*HI-*Pst*I restriction fragment (Fig. 1). In this communication we present the

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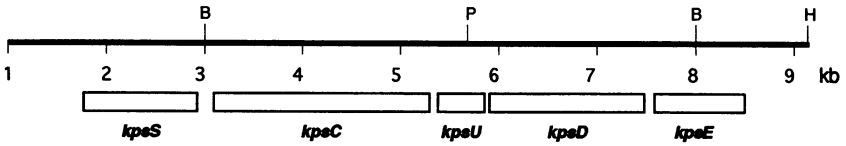


FIG. 1. Restriction map and genetic organization of region 1 of the K1 *kps* gene cluster (4, 34, 55). The locations of the *kps* genes are indicated by the open boxes. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I.

nucleotide sequence of *kpsD*. The KpsD protein was gel purified and used as an antigen to raise polyclonal antiserum. We describe a chromosomal insertion mutation in *kpsD* which results in the loss of surface expression of the K1 capsule and the accumulation of the polysaccharide in the periplasmic space. A *kpsD* deletion derivative (*kpsDΔC11*) which results in production of a KpsD protein lacking its 11 C-terminal amino acids was nonfunctional and localized primarily to the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and media. The bacterial strains and plasmids used in the study are described in Table 1. Bacteriophage E is specific for *E. coli* strains that express extracellular K1 polysaccharide (19). Bacterial cultures were grown at 37°C in Luria-Bertani broth or on Luria-Bertani agar supplemented with antibiotics as required.

DNA manipulations and sequencing. DNA manipulations were carried out according to protocols of Sambrook et al. (41). Plasmid DNA isolation was performed by the rapid alkaline extraction procedure as described elsewhere (41). Genomic DNA was isolated by hexadecyltrimethyl ammonium bromide precipitation (2). Southern blot analysis was performed by capillary transfer to Zeta-Probe nylon membranes (Bio-Rad, Richmond, Calif.) and hybridizations using the formamide method as described by the manufacturer's protocol. Autoradiography was performed with X-Omat film (Eastman Kodak, Rochester, N.Y.) at −70°C.

Both DNA strands of the 2,406-bp *Bam*HI-*Pst*I restriction endonuclease fragment containing *kpsD* were sequenced from overlapping fragments cloned into the M13mp18 and M13mp19 phage vectors (29). The dideoxy-chain termination method was used with the M13 universal sequencing primer (Pharmacia P-L Biochemicals, Inc., Piscataway, N.J.) to generate the nucleotide sequence (42). Alternatively, double-stranded DNA was isolated by polyethylene glycol precipitation (24) or by the Magic Miniprep protocol (Promega, Madison, Wis.) and sequenced with synthesized internal and flanking oligonucleotide primers. Oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. DNA and protein sequence analyses were performed on the VAX computer system using programs provided by University of Wisconsin Genetics Computer Group software package (8).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype, or description	Source or reference
E. coli K-12		
HB101	F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mlt-1 supE44 leu*	41
DH5α	F⁻ *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ*lacU169* (φ80 *lacZ*ΔM15)	41
SM10	F⁻ *araD thi-1 thr-1 leuB6 tonA21 lacY1 supE44 recA*::RP4-2-Tc^r::Mu Km^r	30
SY327	Δ(*lac-pro*) *argE*(Am) Rif^r *nalA recA56*	30
JC10240	Hfr PO45 *srlC300*::Tn*10* (Tc^r) *recA56 thr-300 ilv-318 rpsE300*	7
JL3664	*argA22 galP23 rha-200*	56
XL1-Blue	F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10* (Tc^r)] *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*	41
E. coli B [strain BL21(DE3)]	F⁻ *ompT hsdS*Δ*5 gal* λ-*lacUV5*-T7 gene 1	50
E. coli K-12–K1		
EV1	PA360 (*kps*$^+$ *serA*$^+$ *malA*$^+$) Sm^r	55
RS2275	EV1 *kpsD5* Ap^r	This study
RS2276	EV1 *kpsD5* Ap^r *srlC300*::Tn*10* (Tc^r) *recA56*	This study
EV36	*galP36 rpsL9* (*argA*$^+$ *rha*$^+$ *kps*$^+$) Sm^r	55
RS2484	EV36 Δ*kpsD*	This study
RS2439	EV36 *kpsM*::IS*1*	This study
Plasmids		
Bluescript KS+	2.95-kb Ap^r phagemid cloning vector	Stratagene
pACYC184	4.2-kb Tc^r Cm^r low-copy-number cloning vector	6
pGP704	3.7-kb Ap^r suicide vector containing *oriR6K* and *mobRP4*	30
pCVD442	6.2-kb Ap^r pGP704 derivative containing the *Bacillus subtilis sacB* gene	9
pT7-5	2.4-kb Ap^r T7 expression vector	53
pSR123	Cm^r; 2.4-kb *Bam*HI-*Pst*I fragment cloned into pACYC184	51
pSR309	Ap^r; 2.4-kb *Bam*HI-*Hin*dIII fragment of pSR123 cloned into pT7-5	This study
pSR345	Ap^r; 867-bp *Hin*cII *kpsD* fragment cloned into pGP704	
pSR359	Cm^r; 2.15-kb *Bam*HI-*Pvu*II fragment of pSR123 into the 3.9-kb *Bam*HI-*Cla*I fragment of pACYC184	This study
pSR439	Ap^r; Δ*kpsD* (Δ1,065-bp *Hin*cII fragment) cloned into pCVD442	This study

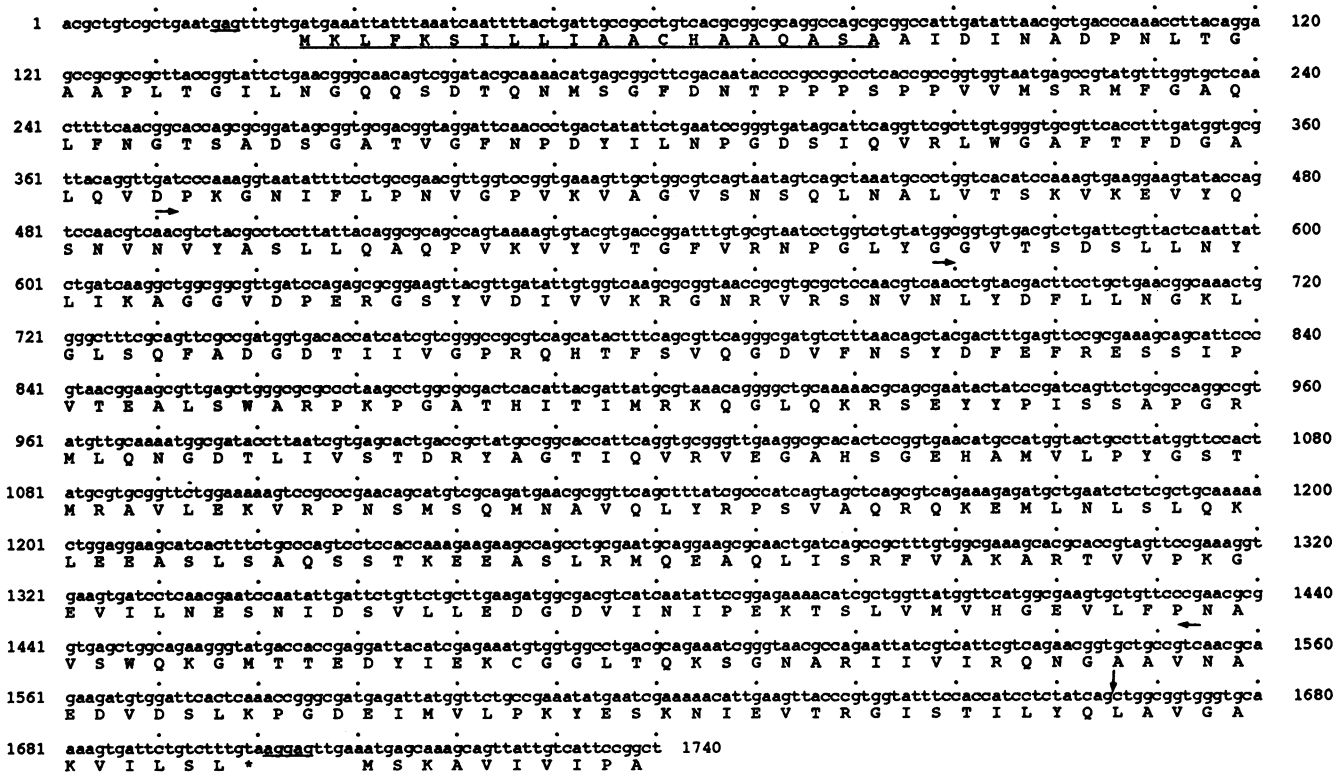


FIG. 2. Nucleotide sequence of the *kpsD* gene with the deduced amino acid sequence. The putative Shine-Dalgarno sequence (43) and the deduced signal peptide of KpsD are underlined. The *Hin*cII fragments used to construct the chromosomal insertion (bp 688 to 1554) and deletion (bp 490 to 1554) mutations in *kpsD* are bracketed by the horizontal arrows. The *Pvu*II cleavage site (bp 1665) used to generate *kpsDΔC11* is identified by the vertical arrow. The partial DNA and amino acid sequences of *kpsU* are indicated with the putative ribosome binding site underlined.

SDS-PAGE. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was done according to the method of Laemmli (26) as previously described (33).

Plasmid constructions. The KpsD overexpression vector was generated by fusing pSR123 and the T7 expression vector, pT7-5, at their *Bam*HI sites. The pSR123/pT7-5 chimeric plasmid was then digested with *Hin*dIII to remove the pACYC184 vector sequence in addition to a short sequence from the pT7-5 polylinker. The resultant plasmid, pSR309, contains the *kpsD* gene 532 bp downstream of the T7 RNA polymerase transcriptional start site. To construct truncated KpsD, a 2,145-bp *Bam*HI-*Pvu*II restriction fragment, containing *kpsD* lacking 36 bp from the 3′ end of the gene, was isolated from pSR123. This fragment was ligated to a pACYC184 vector that was cut with *Cla*I, filled in with Klenow enzyme, and then cut with *Bam*HI. This plasmid, pSR359, generated a *kpsD* derivative possessing the wild-type reading frame up to codon 547 (Fig. 2) followed by a new CGA codon (Ser) and a TGA stop codon.

Overexpression and isolation of KpsD. Overexpression of the KpsD protein was accomplished by modifying the procedures described by Tabor and Richardson (53). BL21(DE3) was used as the host cell for T7-mediated expression (50). Cultures, grown to an A_{600} of 1.5, were treated with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce T7 polymerase expression and incubated at 37°C for an additional 4 h. Cells were harvested and spheroplasted to release the periplasmic contents as described by Lindberg et al. (27). The periplasmic fraction was brought to 40% saturation with ammonium sulfate and centrifuged at 23,500 × *g* for 15 min at 4°C. The resulting pellet was resuspended in 10 mM sodium phosphate buffer (pH 7.0) and dialyzed extensively in 10 mM sodium phosphate buffer (pH 7.0). Samples were subjected to SDS-PAGE, and the KpsD protein was gel purified as the antigen source for antiserum production.

Polyclonal antiserum production and antibody preparation. Gel-purified KpsD was used to immunize two New Zealand White rabbits at East Acres Biologicals (Southbridge, Mass.). Anti-KpsD sera were collected, pooled, and stored at −20°C. Crude immunoglobulin fractions were isolated by ammonium sulfate precipitation as described by Harlow and Lane (20) and dialyzed extensively against phosphate-buffered saline. Nonspecific antibodies were removed by adsorption with an acetone powder prepared from strains JL3664, RS2276, and BL21(DE3) (20).

Cell fractionation for Western blot (immunoblot) analysis. Whole-cell lysates and membrane fractions were isolated by the procedure described by Swit et al. (52). Periplasmic fractions were obtained by the small-scale lysozyme spheroplasting procedure described by Koshland and Botstein (23). Protein content was determined by the Bio-Rad protein assay.

Immunological techniques. Precipitin halo formation was assayed on antiserum agar plates, and immunodiffusion was performed on 1.0% SeaKem GTG agarose (FMC Bioproducts, Rockland, Maine) in phosphate-buffered saline as described previously (45). For immunodiffusion experiments, whole-cell lysates were obtained by the freeze-thaw lysozyme lysis proce-

dure (55), while cellular periplasmic contents were obtained by the modified mini-osmotic-shock method based on the procedure of Neu and Heppel (31).

For Western blot analysis, proteins were immobilized to nitrocellulose sheets (Bio-Rad) by using a semidry electrophoresis transfer system (Millipore Corp., Bedford, Mass.). Immunodetection of KpsD was carried out by previously described procedures (2).

N-terminal amino acid sequencing of KpsD. Dialyzed extracts of ammonium sulfate-precipitated KpsD were separated on 8% polyacrylamide gels and transferred to Immobilon-P membrane (Bio-Rad) (28). The membrane was stained in 0.05% (wt/vol) Coomassie blue–20% methanol to visualize protein bands and destained in 20% methanol. The band corresponding to KpsD was excised from the membrane and stored in 20% methanol in preparation for N-terminal amino acid analysis. Automatic Edman degradation was performed with a PI 2020 microsequencer (Porton Instruments, Inc., Tarzana, Calif.). Phenylthiohydrantoin-amino acid degradation derivatives were separated and identified by the Beckman System Gold (Beckman Instruments, San Ramon, Calif.).

Insertional inactivation of chromosomal *kpsD*. The suicide vector system developed by Miller and Mekalanos (30) was used to insertionally inactivate a chromosomal copy of the *kpsD* gene. An 867-bp *Hin*cII fragment of *kpsD* (Fig. 2) was inserted into the suicide plasmid pGP704 to generate pSR345. SM10 harboring pSR345 was mated with strain EV1 (*kps*$^+$ Sm^r). Ampicillin and streptomycin resistance was used to select for transconjugant EV1 cells which integrated pSR345 into their genome. Southern blot hybridization of genomic DNA from the EV1 transconjugant, RS2275, confirmed insertion of the suicide plasmid into the chromosomal copy of *kpsD* (data not shown). Generation of a *recA56* derivative of the RS2275 was accomplished by broth mating with the Hfr strain JC10240, which carries the *srlC300*::Tn*10* and *recA56* mutations (7). Mating mixtures were selected for resistance to ampicillin and tetracycline. Sensitivity to UV irradiation was used to screen for *recA56* recombinants.

Construction of a *kpsD* chromosomal deletion. The *kpsD* deletion was generated by the suicide vector system described by Miller and Mekalanos (30) as modified by Donnenberg and Kaper (9). This system uses a pGP704 derivative, pCVD442, that has a copy of the *Bacillus cereus sacB* gene. The *sacB* gene encodes levan sucrase, which is toxic to gram-negative organisms in the presence of sucrose. A 1,065-bp *Hin*cII fragment (Fig. 2) was removed from the *kpsD* gene, and a 1,341-bp *Xba*I-*Eco*RV fragment carrying the deletion was inserted into pCVD442, generating pSR439. A halo-negative Amp^s clone that was resistant to K1 bacteriophage infection and produced K1 capsule when complemented with plasmid-borne *kpsD* was isolated. This strain was designated RS2484. The deletion mutation was confirmed by PCR analysis of chromosomal DNA from RS2484.

STA assays. All sialyltransferase (STA) assays were performed using freshly isolated crude membrane fractions as described by Vimr and Troy (56). Assays were prepared in 50- or 100-μl volumes, with 250 μg of membrane protein and 0.02 μCi of CMP-[^{14}C]NeuNAc (302.80 Ci/mmol; NEN Products, Boston, Mass.) added per reaction, and mixtures were incubated at 33°C for 15 min. Colominic acid (Sigma Chemical Co., St. Louis, Mo.) was used as exogenous acceptor at a concentration of 7.5 mg/ml where required. Samples were subjected to paper chromatography and liquid scintillation counting to determine the extent of [^{14}C]NeuNAc incorporation into the polymers.

Preparation of bacteria for electron microscopy. Mid-logarithmic-phase cells from Luria-Bertani broth were pelleted in a microcentrifuge at 15,500 × *g* for 2 min at 4°C, the supernatants were removed, and glutaraldehyde-paraformaldehyde fixative was added (2.5 and 4%, respectively, in 0.2 M Na phosphate buffer, pH 7.0). These samples were fixed for 1 h at room temperature, the fixative was removed, and samples were washed twice with the same buffer, 30 min each. This was followed with a postfixation in 1% OsO_4 in the same buffer for 1 h at room temperature. The samples were rinsed twice with tap water with resuspension and pelleting at each step for 15 min each rinse. Samples were then subjected to en bloc staining with 1% aqueous uranyl acetate, pH 3.9, for 1 h at room temperature. Following en bloc staining, the samples underwent dehydration through a graded ethanol series and infiltration in Spurr's embedding resin (48). Samples were polymerized at 70°C and sectioned with a diamond knife (60 to 80 nm thick). The sections were mounted on naked grids, stained with uranyl acetate and lead citrate (37), examined with a Hitachi Hu-11-E-1 electron microscope at 75 kV, and photographed on Kodak SO-163 electron image film.

Nucleotide sequence accession numbers. The nucleotide sequence of the 2.4-kb *Bam*HI-*Pst*I restriction fragment was submitted to the GenBank/EMBL/DDBJ data bases under the assigned accession number of M64977.

RESULTS

Nucleotide sequence of *kpsD*. The 2,406-bp *Bam*HI-*Pst*I restriction fragment that contains the *kpsD* gene was sequenced. An open reading frame encoding a protein of 558 amino acids with a predicted molecular mass of 60,370 kDa was detected (Fig. 2). This is consistent with the size of the KpsD protein determined from minicell analysis and in vitro transcription-translation data (44). No obvious *E. coli* promoter consensus sequence was identified upstream of the *kpsD* gene, suggesting that *kpsD* is part of a polycistronic mRNA. A Shine-Dalgarno consensus sequence was observed 6 bp upstream of the initiation codon (Fig. 2) (43). KpsD is a periplasmic protein (44), and the N-terminal region of the protein contains a characteristic prokaryotic signal peptide required for secretion across the cytoplasmic membrane (Fig. 2). The cleavage site was determined by N-terminal amino acid sequencing of the mature protein by automatic Edman degradation. The N-terminal amino acid sequence was in agreement with the mature protein beginning at the alanine at codon position 21. Thus, cleavage of the signal peptide occurs between the two alanine residues at positions 20 and 21 of the protein. The processed form of KpsD has a predicted molecular weight of 58,301 and isoelectric point of 6.7.

The sequences of region 1 genes from the *E. coli* K5 capsule gene cluster have recently been reported (34). The *kpsD* nucleotide sequence from K1 is 97% identical to the sequence of the gene from K5. At the amino acid level, KpsD proteins from K1 and K5 serotypes are 99% homologous. We also detected the 3′ end of the *kpsE* gene upstream of *kpsD* and the 5′ end of *kpsU* downstream of *kpsD* (Fig. 1). Both proteins are also essentially identical to that reported for the *E. coli* K5 serotype (34).

Overexpression and partial purification of KpsD. We used the T7 promoter expression vector pT7-5 described by Tabor and Richardson (53) to overexpress the KpsD protein. The 2.4-kb *Bam*HI-*Pst*I fragment containing *kpsD* was inserted into pT7-5 to generate pSR309. The host cell for these experiments, BL21(DE3), is protease deficient and has an IPTG-inducible copy of the T7 polymerse in its chromosome (50). When induced, BL21(DE3) cells harboring pSR309 produced KpsD

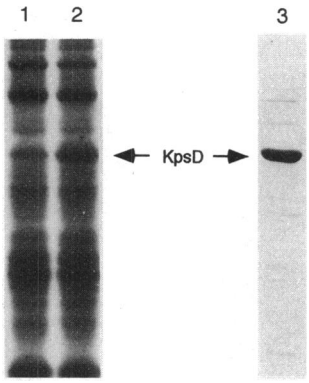


FIG. 3. Coomassie-stained SDS-polyacrylamide gels of whole-cell lysates of BL21(DE3) cells harboring the pT7-5 expression vectors (lane 1) and pSR309 (lane 2). Samples were mixed with 2× loading buffer (2) and boiled for 5 min prior to electrophoresis. Equivalent amounts (10 μl) of samples were loaded into each lane. Lane 3, partially purified KpsD extract. A total of 10 μg of protein was applied to the well. The protein in the vector control, migrating at a position similar to that of KpsD, was not purified under the conditions used to partially purify KpsD (data not shown).

that was detectable on SDS-PAGE (Fig. 3, lane 2). We partially purified the KpsD protein from the periplasmic extract of induced cells. Since the periplasmic proteins generally account for only 3 to 4% of total cell protein (31), the osmotic shock procedure provides an initial 30-fold purification. The KpsD protein was further enriched by ammonium sulfate precipitation. This material was dialyzed and run on preparative SDS-PAGE (Fig. 3, lane 3). The band corresponding to KpsD was excised from the polyacrylamide gel and used as the source of antigen for the production of polyclonal antiserum in rabbits. The antiserum has good specificity for KpsD and can detect the protein, by Western blot, from overproducing cells as well as wild-type strains (Fig. 4, lanes 1 and 2).

Characterization of a *kpsD* insertion mutant. Region 1 genes are postulated to be involved in transport of PSA to the cell surface. To study the role of KpsD in the transport process, we generated a chromosomal insertion mutation in *kpsD* as described in Materials and Methods. The strain harboring the *kpsD* insertion mutation, RS2275, did not produce precipitin halos on antiserum agar and was resistant to lysis by the

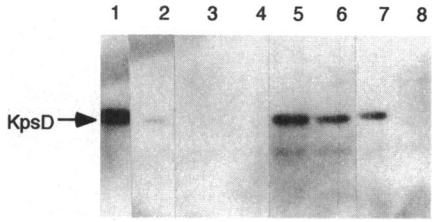


FIG. 4. Western blot analysis of KpsD. Approximately 25 μg of protein was loaded per lane. Anti-KpsD serum was used at a 1:200 dilution, and ^{125}I-labeled *Staphylococcus aureus* protein A (2 to 10 μCi/μg; ICN Radiochemicals, Irvine, Calif.) was used to detect immunocomplexes. Lane 1, partially purified KpsD (1 μg); lane 2, EV1 cell lysate; lane 3, RS2276 membranes; lane 4, RS2276 periplasmic extract; lane 5, RS2276/pSR123 membranes; lane 6, RS2276/pSR123 periplasmic fraction; lane 7, RS2276/pSR359 membranes; lane 8, RS2276/pSR359 periplasmic fraction.

TABLE 2. Capsule expression and STA activity of EV1 and mutant derivatives

Strain	Capsular phenotype		STA activity[a]	
	Halo[b]	K1 phage sensitivity[c]	Endogenous	Exogenous
EV1	+	+	100	100
RS2276	−	−	8.2	95
RS2276/pSR123	+	+	33.0	99
RS2276/pSR359	−	−	7.2	95

[a] STA assays were performed as detailed in Methods and Materials. Values are expressed as a percentage of the wild-type cell (EV1) value. Each value is an average calculated over three independent experiments.

[b] Cells were grown on H.46 antiserum agar and scored for the presence of precipitin halos surrounding colonies.

[c] A 10-μl drop of K1-specific bacteriophage E (10^8 PFU/ml) was placed on soft-agar overlays of each strain and incubated overnight at 37°C. Cells were scored for the presence or absence of a clear lytic zone where bacteriophage E was applied.

K1-specific bacteriophage. We noted, however, that the mutant reverted to the parental halo-positive phenotype at about 1% frequency when grown in the absence of ampicillin selection. Reversion was probably due to homologous recombination and precise excision of the suicide plasmid from the chromosome. To stabilize the *kpsD* insertion mutation, we introduced the *recA56* allele into RS2275. The RS2275 *recA56* derivative, RS2276, did not revert to the wild type at a detectable frequency. The K1$^+$ phenotype was restored by complementation with pSR123, a plasmid carrying the wild-type copy of the *kpsD* gene (Table 2). Transcription of *kpsD* in pSR123 is probably under control of a vector promoter.

The presence of PSA in culture supernatants and periplasmic fractions was examined by immunodiffusion against group B meningococcal antiserum (H.46). An immune precipitate was observed with the supernatants of the parental strain EV1 and RS2276/pSR123 but not RS2276 (Fig. 5, wells 1, 3, and 2, respectively). PSA was observed, however, in periplasmic extracts of both RS2276 and RS2276/pSR123 (Fig. 5, wells 2 and 3 respectively). These results are consistent with the view

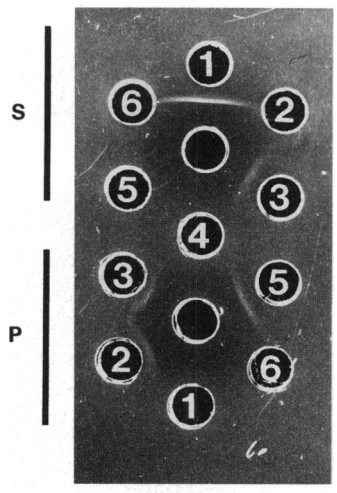
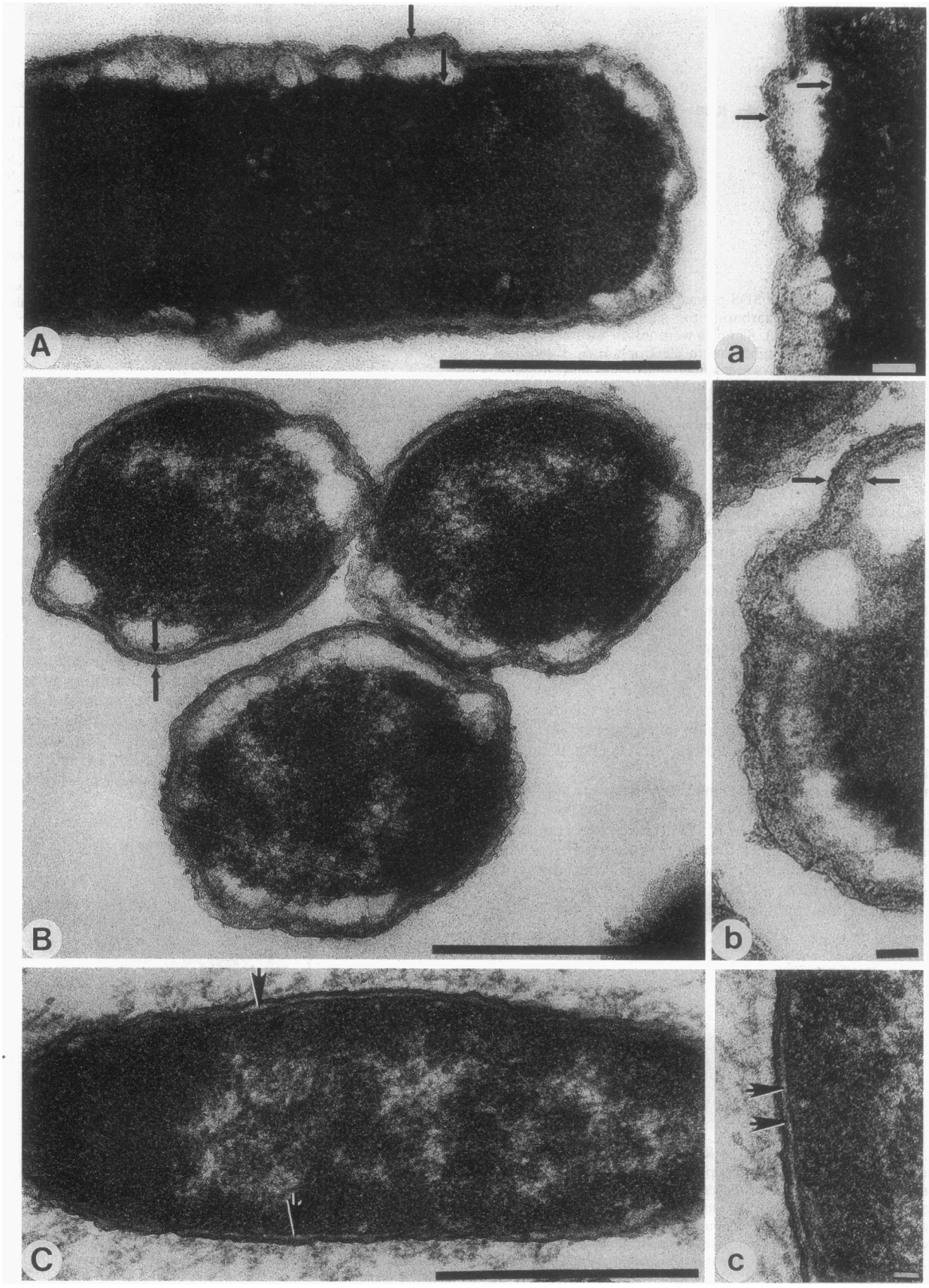


FIG. 5. Immunodiffusion with group B meningococcal antiserum (H.46, center wells) against culture supernatants (S) and periplasmic extracts (P) from strains RS2276 (*kpsD* insertion mutant) and RS2276 harboring either pSR123 or pSR359. Wells: 1, EV1; 2, RS2276; 3, RS2276/pSR123; 4, blank; 5, RS2276/pSR359; 6, DH5α.


A
a
B
b
C
c

that cells harboring mutations in the *kpsD* gene are able to export PSA across the cytoplasmic membrane but are unable to transport the polymer across the outer membrane to the cell surface.

The phenotype of mutations in regions 1 and 3 is the intracellular accumulation of polymer in vivo and a reduction of endogenous STA activity in vitro (55, 56). The endogenous STA assay measures the transfer of labeled sialic acid onto preexisting acceptors within the membrane, while the exogenous assay measures the transfer of labeled sugar onto exogenously added PSA (54). We determined STA activity of RS2276 and found that the level of endogenous activity was approximately 12-fold lower than that of the parental strain, EV1 (Table 2). Exogenous STA activity was at wild-type levels.

Electron microscopy. We used transmission electron microscopy to confirm the location of polymer in cells lacking the KpsD protein. For these experiments we compared the localization of polymer in RS2484 with that of RS2439. Strain RS2484 contains a 1-kb deletion of the *kpsD* gene that was constructed by allelic exchange as described in Materials and Methods. RS2439 is a spontaneously derived acapsular mutant that carries an IS*1* element in the *kpsM* gene (36). The phenotype of both strains is the intracellular accumulation of polymer. Thin sections of glutaraldehyde-paraformaldehyde- and osmium tetroxide-fixed cells embedded in resin revealed the characteristic accumulation of intracellular polysaccharide that appears as electron-lucent spaces (Fig. 6A and B) (5, 35). Wild-type EV36 showed no such accumulation of polymer (Fig. 6C and c). As can be seen in Fig. 6A, polymer in RS2484 accumulates along the long axis of the periplasmic space, disrupting the continuity between the inner and outer membranes. The inner membrane appears as an electron-dense margin below the areas of polymer accumulation (Fig. 6A and a). In contrast, polymer in RS2439 accumulates as a cavitation below the cytoplasmic membrane. Both membranes remain contiguous throughout the cell, and the integrity of the periplasmic space is maintained (Fig. 6B and b).

Generation and characterization of a truncated KpsD protein. The C-terminal region of KpsD, corresponding to amino acids 540 to 558, was identified as a putative transmembrane α-helix (38). To study this region of the molecule, we constructed a deletion of *kpsD* which resulted in the production of a protein lacking the 11 carboxy-terminal amino acids. The plasmid, pSR359, generated a *kpsD* derivative (*kpsDΔC11*) possessing the wild-type reading frame up to codon 548 followed by a new CGA codon (Ser) and ending with a TGA stop codon. pSR359, however, did not complement the chromosomal mutant RS2276 (Table 2). PSA was detected in the periplasmic fraction of these cells (Fig. 5, well 5), and the endogenous STA activity was approximately 7% of wild-type levels, similar to the levels obtained from RS2276 alone (Table 2).

The location of KpsD and KpsDΔC11 in RS2276 harboring either pSR123 or pSR359 was studied by Western blot analysis using the polyclonal KpsD antiserum. We detected KpsD in both the membrane and periplasmic fractions (Fig. 4, lanes 5 and 6). In contrast, we did not detect KpsDΔC11 in periplasmic extracts, although the protein was detected in the membrane fraction (Fig. 4, lanes 7 and 8). The requirement for an intact C terminus for efficient export of proteins has been documented for two other periplasmic proteins, β-lactamase and alkaline phosphatase (17, 23).

DISCUSSION

Region 1 of the *kps* gene cluster is involved in the transport of polymer to the bacterial cell surface. *kpsD*, a region 1 gene, encodes a 60-kDa periplasmic protein that is involved in the transport process (44). Hybridization studies using a *kpsD*-specific probe detected similar genes in K2, K5, K7, K12, K13, and K92 serotypes (44). A KpsD homolog has not been described, however, for other gram-negative encapsulated organisms. In this study we have presented the nucleotide sequence of the *kpsD* gene from *E. coli* K1 and have shown that cells harboring a mutation in *kpsD* accumulate PSA in the periplasmic space. Although its specific role in the translocation of polymer to the cell surface remains unknown, our data could be interpreted as evidence of transport of PSA via the periplasmic space. Other ABC transporters, such as the histidine and oligopeptide permeases of *Salmonella typhimurium* and the maltose permease of *E. coli* (1, 21), include a periplasmic binding protein in addition to the membrane transport proteins. KpsD may function as the periplasmic binding element of the PSA transport system, in which KpsD transiently interacts with the membrane component of the transporter, binds polysaccharide, and transports the polymer to a component in the outer membrane.

The isolation of polymer from the periplasm of mutant cells, however, need not imply the passage of polymer through the periplasm during transport to the cell surface. Our results do not rule out a model in which region 1 gene products are needed to connect export machinery of the inner membrane, presumably the KpsMT transporter, directly to a protein in the outer membrane, allowing the polymer to bypass the periplasmic space. This model predicts a large biosynthetic-translocation complex that would span both the inner and outer membranes. We envision that such a complex would generate a pore or channel through which the polymer passes to the surface of the bacterial cell. Indeed, many of the bacterial ABC exporters require additional proteins (accessory factors) to form a functional transport complex (10). Accessory factors seem to be required when secretion occurs through both membranes (10). The accessory factors are postulated to connect the inner and outer membranes and facilitate the export of substrates through both membranes (10). Deletion of the HlyD protein, an example of such a factor, results in the cytoplasmic accumulation of substrate (18). Interestingly, the phenotype of cells harboring mutations in some region 1 genes is the cytoplasmic accumulation of polymer (5). In contrast, cells harboring mutations in the *kpsD* gene are still able to transfer PSA to the periplasmic space, perhaps indicating that KpsD is not an integral part of a transport complex.

The observation that a functional porin in the *E. coli* outer membrane is required for capsule expression (14) is consistent with both views of polymer transport. Although any porin may be able to mediate capsule expression, the presence of a particular porin, protein K, is correlated with capsule expression in naturally occurring encapsulated *E. coli* (32, 51, 57).

FIG. 6. Transmission electron microscopy of ultrathin sections of RS2484 (A and a); the *kpsM* insertion mutant, RS2439 (B and b); and the wild-type control, EV36 (C and c). The electron-lucent bulges near the cell boundaries are accumulation sites of PSA (5, 35). The arrows in panels A, a, B, and b denote the outer and inner membranes delineating the periplasmic space noted by arrows in panels C and c. Bars in panels A, B, and C, 0.5 μm; bars in panels a, b, and c, 50 nm.

Protein K may be the natural component of the transport apparatus. Whitfield et al. (58) demonstrated a temporal correlation between the presence of protein K in the outer membrane and the expression of PSA on the cell surface.

Testing either model will require studying potential protein-protein interactions between the gene products of region 3 and region 1 with KpsD as well as defining any protein-polysaccharide interactions between KpsD and PSA. Since regions 1 and 3 are functionally interchangeable between *E. coli* K serotypes, KpsD and other *kps* gene products apparently possess the ability to transport a range of acidic polysaccharides. How chemically distinct polymers are recognized by the transport apparatus remains a mystery. Perhaps the occurrence of positively charged amino acid residues throughout KpsD is important for interaction with the negatively charged moieties of acidic polysaccharides.

We observed that the endogenous STA activity of the KpsD mutant strain, RS2276, was approximately 8% of that of the wild type. Exogenous activity was at wild-type levels, indicating that the STA enzyme encoded by *neuS* is not affected. A major theme that has emerged from the analysis of acapsular mutants in the *kps* gene cluster is that one cannot clearly separate synthetic and transport activities (46, 55). The phenotype of cells with mutations in region 1 or region 3 is the accumulation of intracellular polymer in vivo and a reduction of endogenous STA activity in vitro (55, 56). These observations imply that transport defects in some way affect polymer synthesis and strengthen the view that a thorough understanding of the structure and function of the biosynthetic apparatus is necessary for a fundamental understanding of transport processes. In particular, the nature of the endogenous sialyl acceptor within the cytoplasmic membrane needs to be elucidated, as does the nature of the interaction between the biosynthetic and transport machineries.

The mechanism by which a long, hydrophilic molecule such as PSA moves across the lipid bilayer is an interesting and challenging problem. Continued investigation into the specific functions of KpsD and other region 1 gene products, as well as the KpsMT transporter, will aid in understanding the expression of capsular polysaccharides in *E. coli* and other encapsulated bacteria possessing similar genetic systems. Given the poor prospect of developing vaccines based on purified K1 polysaccharide (13, 59), an understanding of the key reactions involved in the synthesis and export of the PSA capsule is needed to provide novel rationales for intervention in the disease process.

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