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Expression of the Capsular K5 Polysaccharide of *Escherichia coli*: Biochemical and Electron Microscopic Analyses of Mutants with Defects in Region 1 of the K5 Gene Cluster

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The gene cluster of the capsular K5 polysaccharide, a representative of group II capsular antigens of *Escherichia coli*, has been cloned previously, and three regions responsible for polymerization and surface expression have been defined (I. S. Roberts, R. Mountford, R. Hodge, K. B. Jann, and G. J. Boulnois, J. Bacteriol. 170:1305–1330, 1988). Region 1 has now been sequenced, and five open reading frames (*kpsEDUCS*) have been defined (C. Pazzani, C. Rosenow, G. J. Boulnois, D. Bronner, K. Jann, and I. S. Roberts, J. Bacteriol. 175:5978–5983, 1993). In this study, we characterized region 1 mutants by immunoelectron microscopy, membrane-associated polymerization activity, cytoplasmic CMP-2-keto-3-deoxyoctonate (KDO) synthetase activity, and chemical analysis of their K5 polysaccharides. Certain mutations within region 1 not only effected polysaccharide transport (lack of region 1 gene products) but also impaired the polymerization capacity of the respective membranes, reflected in reduced amounts of polysaccharide but not in its chain length. KDO and phosphatidic acid (phosphatidyl-KDO) substitution was found with extracellular and periplasmic polysaccharide and not with cytoplasmic polysaccharide. This and the fact that the K5 polysaccharide is formed in a *kpsU* mutant (defective in capsule-specific K-CMP-KDO synthetase) showed that CMP-KDO is engaged not in initiation of polymerization but in translocation of the polysaccharide.

The capsular antigens (K antigens) of *Escherichia coli* are acidic polysaccharides which, on the basis of microbiological, biochemical, and genetic findings, were divided into two groups (18). An important representative of group II is the K5 polysaccharide, the structure of which was determined as 4)-β-D-GlcA-(1,4)-α-D-GlcNAc-(1, (47). Group II capsular polysaccharides, which are characteristic of invasive *E. coli*, are not expressed at growth temperatures of 20°C and below (27), and they are present in strains belonging to many O groups. Their expression is determined by the chromosomal *kps* genes located close to the *serA* gene (28, 29, 48). The *kps* gene cluster consists of three regions. Central region 2 is type specific and encodes proteins involved in polymerization of the capsular polysaccharides, while regions 1 and 3 encode proteins involved in translocation of the polysaccharide across the cytoplasmic membrane and transport through the periplasm onto the cell surface (5, 6, 23, 40, 41, 43, 49).

Region 3 of *E. coli* K1 and K5 contains two genes, *kpsM* and *kpsT*, products of which are thought to constitute an ABC transporter system (32, 45) for translocation of the polysaccharide across the cytoplasmic membrane. Homologous systems have been identified in *Haemophilus influenzae* (20, 21) and *Neisseria meningitidis* (13, 14).

Several genes have been identified in region 1 of the *E. coli* K1 and K5 gene clusters (41, 44, 49), but little is known of the respective gene products and their functions in capsule expression. A 60-kilodalton periplasmic protein encoded by the *kpsD* gene in region 1 was described as necessary for the transport of a number of group II capsular polysaccharides through the periplasm (43). The existence of a similar protein in *E. coli* K5 has been proposed (34).

The nucleotide sequence of region 1 of the *E. coli* K5 capsule gene cluster has now been determined (34). On the basis of these data, a single transcriptional unit of five open reading frames, the products of which may participate in different stages of capsule expression, was suggested (34). Thus, within region 1, the *kpsU* gene that encodes a CMP-2-keto-3-deoxyoctonate (KDO) synthetase, the presence of which accounts for the elevated levels of CMP-KDO synthetase in group II strains, was located (10, 11). However, the role of this and other region 1-encoded proteins remained unclear. In this communication, we report biochemical and electron microscopic studies on recombinant *E. coli* with defined defects in region 1 genes.

MATERIALS AND METHODS

Chemicals. Alcian Blue 8GX, dithiothreitol, agar VII, agarose type II, ampicillin, chloramphenicol, KDO (ammonium salt), UDP-GlcA, and UDP-GlcNac were from Sigma (Deisenhofen, Germany); UDP-[U-^{14}C]GlcA was obtained from Amersham Buchler (Braunschweig, Germany); Rotiszint 22 was from Roth (Karlsruhe, Germany); acrylamide, *N,N*-methylenebisacrylamide, *N,N*-tetramethylenediamine, Epon 812, dodecyl-succinic anhydride, methylphthalic anhydride, 2,4,6-tri(dimethylaminoethyl)phenol, kanamycin, lysozyme, osmium tetroxide, uranyl acetate, and CTP were from Serva (Heidelberg, Germany); cetyltrimethylammonium bromide (CTAB), Tris, and EDTA, as well as all media for cultivation of bacteria, were from Merck (Darmstadt, Germany); the components of the Lowicryl K4M resin (triethylene glycol dimethyl acrylate, hydroxypropyl methacrylate, *n*-hexacyl methacrylate, and benzoin methyl-

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Reference or source	Serotype, genotype, and properties
Plasmids		
pGB110	40	Ap^r original $K5^+$ cosmid clone
pGB110::34	33	Ap^r Km^r $K5^+$ Tn*phoA* insertion upstream of region 3
pGB118	41	Ap^r $K5^+$ subclone of pGB110
pGB118::1	11	Ap^r $K5^-$ Tn*1000* insertion *kpsS* mutant
pPC1	This study	Cm^r K5 13.5-kb *Xho*I-*Sma*I fragment from pGB110::34 subcloned in pACYC184
pPC2	This study	Cm^r $K5^+$
pPC3	This study	Cm^r $K5^-$ 4.2-kb *Sph*I deletion of pPC2 deleting genes *kpsE*, *kpsD*, and *kpsU*
pPC7	This study	Cm^r $K5^-$ 0.6-kb *Sma*I-*Hpa*I deletion of pPC2 inactivating gene *kpsC*
pPC10	This study	Cm^r $K5^+$ insertion of oligonucleotide within gene *kpsU*
pHc4	33	Cm^r 1.2-kb *Hinc*II-*Eco*RV fragment that encodes *KpsS* subcloned in pACYC184
pHHP1	41	Ap^r 5.2-kb *Hind*III-*Hpa*I fragment that encodes KpsE, KpsD, and KpsU subcloned in pUC18
pPVSM2	33	Ap^r 1.3-kb *Pvu*II-*Sal*I fragment that encodes KpsU subcloned into pUC18
pRG1	14	Tet^r 4.2-kb *Eco*RI-*Hind*III fragment that encodes KdsB subcloned in pBR322
Strains		
2980	9, 10	*E. coli* O18:K5:H^-
20026	10	*E. coli* O10:K5:H4

ester) were from Chemische Werke Lowi (Waldkraiburg, Germany); gold-conjugated anti-mouse immunoglobulin antibody was from Janssen (Kaldenkirchen, Germany).

Bacteria and cultivation. The plasmids and strains used are listed in Table 1. Strain LE392 was used as the host in all experiments. For preparation of membranes and cytosolic fractions and for electron microscopic analysis, the bacteria were grown in Merck Standard I medium containing, per liter, 15.6 g of peptone, 2.8 g of yeast extract, 5.6 g of NaCl, and 1.0 g of glucose (medium A). For isolation of the K5 polysaccharide, bacteria were grown in a medium containing, per liter, 3.57 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of sodium citrate heptahydrate, 1.0 g of $(NH_4)_2SO_4$, 20.0 g of Casamino Acids (Difco), 0.14 g of $MgSO_4 \cdot 7H_2O$, and 4.0 g of glucose (medium B). Plasmid-containing bacteria were grown in the presence of the appropriate antibiotic (Table 1).

Preparation of membranes and cytosol fractions. Bacteria were grown in medium A to the late logarithmic phase (optical density at 600 nm, 0.6 to 0, 7; 3 to 4 h at 37°C) and centrifuged (10,000 × *g*, 10 min, 4°C). The sediment was suspended in the same volume of 50 mM Tris (pH 8.0)–2 mM dithiothreitol–30 mM magnesium acetate (T buffer) and centrifuged (10,000 × *g*, 10 min, 4°C). The sediment of the centrifugation was resuspended in T buffer (approximately 1/20 of the original volume; approximate density, 10^{10} bacteria per ml). The bacteria were disrupted by three passages of the suspension through a French pressure cell (Aminco) at 75 kg/cm^2 (11,000 lb/in^2). The homogenate was centrifuged for 10 min at 10,000 × *g* and 4°C to remove large bacterial cell fragments and then for 60 min at 180,000 × *g* and 4°C. The sediment of the ultracentrifugation was suspended in T buffer to a final concentration of 3 to 5 mg of protein per ml. These membrane fractions (9) were kept in portions at −80°C. They were stable for several months. The supernatant of the ultracentrifugation was used as the cytosol fraction (10, 11).

Polymerization of the K5 polysaccharide in vitro. Membranes (200 to 250 μg of protein), UDP-[^{14}C]GlcA (50 μM, 50,000 cpm), UDP-GlcNAc (50 μM), and T buffer (50 mM) in a total volume of 100 μl were incubated at 37°C for 30 min. The reaction was stopped by addition of 100 μl of ice-cold 12% acetic acid, and the mixture was filtered through a cellulose acetate filter (450-nm pore size; Sartorius, Göttingen, Germany). The filters were washed twice with 12% acetic acid, and the retained radioactivity was determined with a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) with Rotiszint 22 as the scintillation cocktail.

Determination of CMP-KDO synthetase activity. The enzyme test was performed as described by Ray and Benedict (38). Briefly, samples (60 μl) of the cytosol fractions were added to a mixture containing KDO (2 mM), CTP (10 mM), and Tris acetate (pH 9.8, 200 mM) in a total volume of 100 μl. After 15 min at 30°C, the reaction was stopped by addition of 200 μl of ice-cold ethanol. Unreacted KDO was reduced with sodium borohydride, and excess reagent was destroyed at 4°C with phosphoric acid (final concentration, 4 N). By raising the temperature to 37°C, the CMP-KDO formed in the enzyme reaction was hydrolyzed. The KDO thus liberated was determined with thiobarbituric acid reagent (19, 50).

Preparation of spheroplasts. A bacterial suspension (medium A) was centrifuged (10,000 × *g*, 10 min at 4°C), and the sediment was suspended in 2 ml of phosphate-buffered saline (PBS) containing 20% sucrose to a cell density of about 2 × 10^{10}/ml. After addition of sodium azide (final concentration, 5 mM) and incubation at 37°C for 30 min, lysozyme (1 mg; Serva) and EDTA (final concentration, 10 mM) were added. The suspension was kept at 37°C for 1 h and then fixed with glutaraldehyde (final concentration, 2%) for 30 min at 37°C.

Sample preparations for electron microscopy. As described previously (22, 23), the bacteria were grown in medium A to a cell density of about 10^8/ml (optical density at 600 nm, 0.3; 2 to 3 h at 37°C) and then concentrated by centrifugation (10,000 × *g*, 10 min, 4°C) about 40-fold and sodium azide was added to a final concentration of 5 mM. For stabilization of extracellular polysaccharide, 50 μl of undiluted anti-K5-capsular monoclonal antibody (4, 22, 23) was added. After 30 to 60 min at 37°C, the bacteria were fixed with glutaraldehyde (final concentration, 2%) in PBS, centrifuged, and transferred to low-temperature-gelling agar (agar VII; Sigma) at 37°C. After solidification of the agar on ice, the samples were cut into small cubes (1 mm^3). The agar cubes containing the immobilized bacteria were fixed with glutaraldehyde (final concentration, 2%) in PBS for 1 h at room temperature.

Sample embedding in Epon. After fixation with osmium tetroxide (1% in PBS) for 1 h at room temperature, the agar cubes were dehydrated with ethanol (EtOH) at room temperature as follows: 70% EtOH for 10 min, 80% EtOH for 10 min, 90% EtOH for 15 min, 95% EtOH for 15 min, and 100% EtOH twice for 30 min each time. The agar cubes were

6incubated with xylene twice for 30 min each time and embedded and polymerized in Epon 812 (24).

Sample embedding in Lowicryl K4M. The progressive-lowering-of-temperature embedding technique was done as previously described (22, 23), with some modifications (3). The agar cubes were dehydrated with EtOH as follows: 30% EtOH at −10°C for 1 h, 50% EtOH at −20°C for 1 h, 70% EtOH at −35°C for 1 h, 90% EtOH at −35°C for 1 h, and 100% EtOH at −35°C twice for 1 h each time. To prevent detachment of bacteria from the surrounding resin in the ultrathin sections, uranyl acetate (0.5%) was added at the 70% EtOH step (1, 8).

Infiltration of the samples with increasing concentrations of Lowicryl K4M resin in 100% EtOH at −35°C was done as follows: Lowicryl K4M-EtOH (1:3) for 2 h, Lowicryl K4M-EtOH (1:1) overnight, Lowicryl K4M-EtOH (3:1) for 2 h, and Lowicryl K4M for 2 h, 4 h, and then overnight. The samples were transferred to gelatin beem capsules and polymerized by indirect UV irradiation (360 nm) for at least 2 days. During polymerization, the temperature was slowly raised to 4°C. Further hardening was achieved by direct UV irradiation at 4°C for 3 h when necessary.

Specimen preparations and electron microscopy. Epon-embedded samples were cut into ultrathin sections (50 to 100 nm). The sections were placed on Formvar-coated nickel grids and stained with uranyl acetate for 7 min and lead citrate for 5 min. Ultrathin sections from Lowicryl-embedded samples were labeled by the immunogold technique as follows (22, 23). To reduce unspecific background labeling, the grids were placed on drops of a solution of milk powder (1% in PBS) for 30 min and washed twice with PBS. They were transferred to a solution of the anti-K5 capsular monoclonal antibody (diluted 1:500 in PBS) and incubated at room temperature for 2 h. After being washed five times with PBS, the grids were transferred to a solution of gold particle (10-nm diameter)-conjugated anti-mouse immunoglobulin M antibody (diluted 1:100) and incubated for 1 h. The sections were then washed once with PBS and four times with distilled water; finally, they were stained for 10 min with uranyl acetate (2%).

All samples were examined in a Philips 400 T/ST microscope with an accelerating energy of 80 kV.

Isolation of the K5 polysaccharide. For isolation of the extracellular K5 polysaccharide, bacteria from a 10-liter liquid culture (medium B) were centrifuged (10,000 × *g*, 10 min, 4°C), washed with PBS, and extracted twice for 30 min each time at 37°C with 50 mM Tris–5 mM EDTA (pH 7.3). CTAB (final concentration, 2%) was added to the combined extract, and the precipitated polysaccharide was dissolved in 0.25 M NaCl and purified with a sequence of precipitations with ethanol as previously described (46).

For isolation of the intracellular polysaccharide, bacteria were disintegrated by two passages through a French press (Aminco) at 75 kg/cm^2 (11,000 lb/in^2). The homogenate was centrifuged (100,000 × *g* for 1 h at 4°C), and the polysaccharide was obtained from the supernatant by precipitation with CTAB. After centrifugation (10,000 × *g* for 10 min at 20°C), the pellet was dissolved in 0.5 M NaCl and diluted to 0.25 M NaCl. The precipitated nucleic acids were removed by centrifugation (10,000 × *g* for 10 min at 20°C), and the supernatant was diluted to 0.06 M NaCl. The precipitated polysaccharide was collected by centrifugation (10,000 × *g* for 10 min at 20°C), dissolved in 0.25 M NaCl, and purified by repeated precipitation with ethanol (final concentration, 80%). The final precipitate was dissolved in distilled water and lyophilized.

For isolation of the periplasmic polysaccharide, bacteria grown in medium B were collected by centrifugation and the pellet (160 g) was washed by suspension in T buffer and centrifugation (2,000 × *g* for 10 min at 4°C). After the supernatant was decanted, the pellet was suspended in the rest of the supernatant. Chloroform (120 ml) was added, and after mixing, the suspension was incubated for 15 min at room temperature. Addition of 1.2 liters of ice-cold Tris buffer (1 mM, pH 8) released the periplasmic material. The mixture was centrifuged (10,000 × *g*, 20 min, 4°C), and the polysaccharide was obtained from the supernatant by precipitation with CTAB and purified as described above. To remove residual protein and nucleic acid, the polysaccharide was incubated with proteinase K and benzonase.

Polyacrylamide gel electrophoresis of polysaccharides. Polyacrylamide gel electrophoresis of polysaccharides was done as described by Pelkonen et al. (35), in 15% polyacrylamide gels at pH 8.5 (borate-EDTA), 4°C, and 12 V/cm for 1 h and, after application of the samples (300 μg), at 25 V/cm for 4 to 5 h. Bromphenol blue, bromcresol blue, and phenol red were used as tracking dyes. The gels were stained by the alcian blue-periodate-silver method (25).

Gel permeation chromatography. Samples of polysaccharide (5 mg) were dissolved in 0.2 M ammonium acetate (1 ml) and chromatographed on a Sephacryl S300 column (13 by 450 mm). Fractions of 1 ml were collected and analyzed for glucuronic acid with the carbazole reagent.

Immunoelectrophoresis. Immunoelectrophoresis was done as described by Ørskov et al. (26), on microscope slides coated with 1.3% agarose at pH 8.6 (Veronal buffer). After the electrophoretic run (7 V/cm, 2 h, 4°C), the polysaccharides were detected by immunoprecipitation with a K5-specific monoclonal antibody (4, 22, 23).

Detection of KDO at the reducing end of polysaccharide fractions. To remove phospholipid from the reducing end, the polysaccharide (5 to 10 mg) was heated in 0.01 M HCl for 20 min at 100°C. An aqueous solution of the polysaccharide thus treated (2 ml) was then treated with sodium borodeuteride (5 to 10 mg) overnight at 4°C. The solution was neutralized with acetic acid and evaporated three times with methanol. Subsequent methanolysis (12) was performed by addition of 2 ml of 0.5 M HCl in methanol and incubation for 16 h at 85°C. The reaction mixture was evaporated twice with methanol, and the residue was methylated with potassium-dimethyl sulfoxide-methyl iodide (36). After neutralization with acetic acid, the solution was passed through a cartridge of Sep-Pak$_{18}$. An eluate with water was discarded, and the fraction eluting with chloroform-methanol (3:2, by volume) was subjected to gas chromatography-mass spectrometry with a Hewlett-Packard 5988 instrument with a DB 5 capillary column (0.2 mm by 30 m) and helium as the carrier gas.

RESULTS

Construction of plasmids pPC3, pPC7, and pPC10. Both pGB110 and pGB118 are large plasmids with multiple restriction enzyme sites which carry flanking DNA in addition to the cloned K5 gene cluster (41). This complicates attempts to manipulate the K5 capsule genes and to generate defined mutations within specific genes. To overcome these problems, the entire K5 capsule gene cluster with a minimum of flanking DNA was subcloned. This exploited a pGB110 Tn*phoA* insertion mutant termed pGB110::34 which had an insertion 700 bp upstream of the *kpsM* gene (Fig. 1). This insertion had no effect on expression of the K5 capsule (33a).

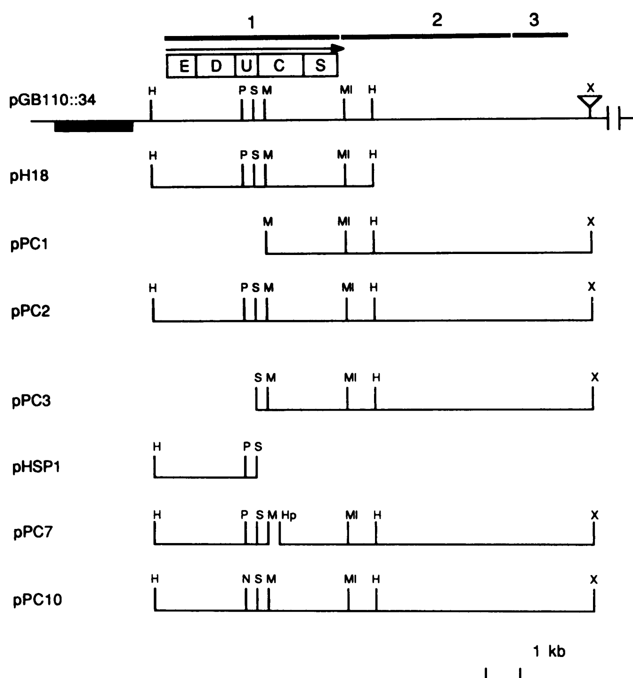

FIG. 1. Physical maps of recombinant plasmids carrying either the entire biosynthesis genes for the K5 antigen or subclones. Lines 1 to 3 refer to functional gene blocks involved in K5 antigen expression. Boxes E, D, U, C, and S are the five genes encoded in region 1, and the arrow denotes the direction of transcription. The closed box below the line refers to plasmid vector sequences, and the open triangle above the line denotes the site of a Tn*phoA* insertion. Restriction enzyme sites: H, *Hin*dIII; Hp, *Hpa*I, M, *Sma*I; MI, *Mlu*I; N, *Not*I; P, *Pst*I; S, *Sph*I; X, *Xho*I.

A 13.5-kb *Xho*I-*Sma*I fragment was subcloned from pGB110::34 into vector plasmid pACYC184 (7) to generate plasmid pPC1 (Fig. 1). The entire K5 capsule gene cluster was then regenerated by subcloning an 8.4-kb *Hin*dIII-*Mlu*I fragment from plasmid pH18 (31) into pPC1 cleaved with *Hin*dIII and *Mlu*I. This generated plasmid pPC2 (Fig. 1), which has a complete K5 capsule gene cluster. Subsequently, pPC3 was generated by digestion of pPC2 with restriction enzyme *Sph*1, which was followed by self-ligation. This resulted in deletion of the 4.2-kb *Sph*I fragment that encodes the KpsE, KpsD, and KpsU proteins (Fig. 1). As predicted, strain LE392(pPC3) was resistant to K5-specific bacteriophage (Table 1). To confirm that pPC3 was defective in the expression of *kpsE*, *kpsD*, and *kpsU* alone and had not acquired additional mutations during its construction, plasmid pHHP1 (Table 1) was introduced into strain LE392(pPC3). Plasmid pHHP1 was able to complement pPC3 and restore sensitivity to K5-specific bacteriophage (17).

Plasmid pPC7 was generated in two stages. First, a 0.6-kb deletion within the *kpsC* gene was created by digesting plasmid pH18 (Fig. 1) with restriction enzymes *Sma*1 and *Hpa*1, which was followed by self-ligation. Subsequently, pPC7 was generated by subcloning the 7.8-kb *Hin*dIII-*Mlu*I fragment. This generated pPC7, which has an entire K5 capsule gene cluster but a deletion within the *kpsC* gene (Fig. 1). To confirm that this deletion was not polar on expression of the *kpsS* gene, pPC7 was used to complement pGB118:1, which has a Tn*1000* insertion within the *kpsS* gene (41).

TABLE 2. Enzyme activities of *E. coli* K5 region 1 mutants

Strain	*kps* locus (loci) affected	Transferase sp act[a]	Transferase relative activity[a]	CMP-KDO synthetase activity[b]
LE392(pGB118)		14.4	100	850
LE392(pGB118::1)	*S*	0.3	2.3	680
LE392(pGB118::1/pHc4)		32.0	230	218
LE392(pPC10)	*U*	3.5	24.3	20
LE392(pPC10/pPVSM2)		13.1	91	282
LE392(pPC3)	*E*, *D*, *U*	4.3	30.7	25
LE392(pPC3/pHHP1)		11.8	82	790
LE392(pPC3/pPVSM2)	*E*, *D*	5.3	36.4	472
LE392(pGB110)		15.9	110	385
LE392(pGB110:pPC7)	*C*	0.27	1.8	350
LE392		0.001		35

[a] Membrane preparations were used for incubation with UDP-[^{14}C]GlcA and UDP-GlcNAc in the presence of 20 mM magnesium chloride. Values are expressed as nanomoles of GlcA incorporated per milligram of protein in 30 min.

[b] Cytosol fractions were used for incubation with CTP and KDO in the presence 20 mM magnesium chloride. Values are expressed as nanomoles of CMP-KDO formed per milligram of protein in 15 min.

Plasmid pPC7 was able to complement the mutation within pGB118:1 and restore K5 phage sensitivity (33b).

Construction of pPC10 was more complex. The first stage involved insertion of an oligonucleotide into the unique *Pst*I restriction site of plasmid pHSP1 (34) (Fig. 1). This *Pst*I site is within the *kpsU* gene (34). The 21-bp oligonucleotide had cohesive termini that were compatible with the *Pst*I site but would, upon ligation, abolish the *Pst*I site within pHSP1 and contained a unique *Not*I restriction enzyme site. In addition, the oligonucleotide had a translational stop codon in frame with the *kpsU* reading frame. Successful ligation of the oligonucleotide into pHSP1 was confirmed by abolition of the *Pst*I restriction enzyme site and appearance of a new *Not*I site. Subsequently, the 4.4-kb *Hin*dIII-*Sph*I fragment containing the inserted oligonucleotide was subcloned into pPC3 to generate plasmid pPC10 (Fig. 1). This plasmid therefore contains a complete K5 capsule gene cluster with an insertion in the *kpsU* gene. Interestingly, strain LE392(pPC10) was still sensitive to K5-specific bacteriophage (Table 1).

Enzyme activities. Membrane and cytosol fractions were prepared from LE392 harboring different plasmid constructs and assayed for in vitro polymerization of the K5 polysaccharide (9) and CMP-KDO synthetase activity (10, 11) (Table 2). Mutations in different genes within region 1 reduced transferase (polymerization) activity in vitro (Table 2). These effects were repaired by supplying the wild-type gene in *trans* (Table 2).

By using membranes from *E. coli* Le392(pGB118:1) (*kpsS*), we found that in the presence of the K5 polysaccharide as an exogenous acceptor, the synthesizing (chain-elongating) capacity increased from 2% of the wild-type activity (Table 2) to about 25%.

Electron microscopy. Electron microscopic analysis of thin sections from Epon-embedded bacteria showed that cells from wild-type *E. coli* O18:K5, as well as recombinant *E. coli* LE392(pGB110) and LE392(pGB118), were fully encapsulated (visible in about 80% of the cellular population). No capsule was detected in *E. coli* LE392 harboring plasmid pGB118::1 (*kpsS*), pPC7 (kpsC), or pPC3 (*kpsE kpsD kpsU*). *E. coli* LE392(pGB118::1) and LE392(pPC7) had areas of low electron density in their cytoplasm. This is shown in Fig.

2A with *E. coli* LE392(pGB118::1) as an example. In cells of strain LE392(pPC10) (*kpsU*) (about 80% of the population), large areas of the cytoplasm, in most cases close to the cytoplasmic membrane, appeared to be free of electron-dense material (Fig. 2B). About 20% of these cells also exhibited extracellular capsular material directly above the translucent areas.

Since membranes of the above-mentioned unencapsulated mutants showed some polymerization activity, we wanted to see where in the cells the capsular material was located. For this, immunoelectron microscopic analyses were performed on thin sections of Lowicryl K4M-embedded bacteria. In strains harboring plasmid pGB118::1 or pGB110(pPC7) (Fig. 2C with *kpsS* mutant *E. coli* LE392(pGB118::1) as an example), capsular material was located in cytoplasmic areas of low electron density. Cells of *kpsU* mutant *E. coli* LE392 (pPC10) also contained polysaccharide in such cytoplasmic areas, and some cells exhibited extracellular polysaccharide in addition (Fig. 2D). Cells of *kpsE kpsD kpsU* mutant strain LE392(pPC3) contained capsular material bound to the periplasmic sides of inner and outer membranes which could be better demonstrated after spheroplasting of the cells (Fig. 2E). The periplasmic space itself, as obtained by spheroplasting, contained little polysaccharide.

Complementation of strains bearing pGB118::1 (*kpsS*) or pPC3 (*kpsE kpsD kpsU*) with plasmids pHC4 (*kpsS* insert) and pHHP1 (*kpsE kpsD kpsU* insert), respectively, resulted in complementation and in fully encapsulated bacteria (data not shown). To complement the *kpsU* defect in *E. coli* LE392(pPC10), two plasmids were available, pRG1, containing the *kdsB* gene for CMP-KDO synthetase, which is operative in LPS synthesis (15), and pPVSM2, which encodes the KpsU protein CMP-KDO synthetase operative in capsular expression (10, 11, 33). Whereas after complementation with pPVSM2, the bacteria expressed the K5 capsule normally (Fig. 2F), complementation with pRG1 resulted in disappearance of the cytoplasmic areas of low electron density. However, the bacteria produced only an incomplete (patchy) capsule (data not shown).

Characterization of polysaccharides. The K5 polysaccharide was isolated from *E. coli* LE392(pGB118) by extraction of the bacteria with EDTA-Tris, from LE392(pPC3) (*kpsE kpsD kpsU*) by release with chloroform (2), and from LE392(pGB118::1) (*kpsS*) and LE392(pPC10) (*kpsU*) by CTAB precipitation (46) of the cytoplasmic fractions after French press disintegration. For comparison of their molecular sizes, the different preparations were subjected to polyacrylamide gel electrophoresis without sodium dodecyl sulfate (35). They had the same size as wild-type polysaccharide isolated from *E. coli* O18:K5. This was also borne out by column chromatography on Sephacryl S300 (Fig. 3). For comparison, Fig. 3 shows the elution profile of the K5 polysaccharide from *E. coli* O10:K5, which was known to be smaller and more heterogeneous than that from *E. coli* O18:K5 (unpublished data). The results showed that polymerization in the mutants was impaired with respect to amount but not with respect to chain length.

The polysaccharide preparations isolated from the capsule of wild-type *E. coli* K5 and encapsulated LE392(pGB118) and that isolated from the periplasm of *kpsE kpsD kpsU* mutant *E. coli* LE392(pPC3) consist of an electrophoretically fastmoving population and an electrophoretically slowly moving population (Fig. 4). In keeping with previous reports (16, 42), this is taken as an indication for lipid substitution of the part of the molecular population. The intracellular polysaccharide from strains LE392(pGB118::1) (*kpsS*) and LE392(pPC7) (*kpsC*) consisted only of the fast-moving (presumably lipid-free) form. This indicated that passage of the polysaccharide through the cytoplasmic membrane may be associated with lipid substitution. The polysaccharide preparations from strains LE392(pGB118), LE392(pPC3), LE392 (pGB118::1), and LE392(pPC10) were analyzed for fatty acids. Whereas no fatty acids were present in the preparation from *E. coli* LE392(pGB118::1) and only trace amounts were present in the *E. coli* LE392(pPC10) preparation, that from *E. coli* LE392(pGB118) contained 0.4% fatty acids (mainly C_{16}) and that from *E. coli* LE392(pPC3) contained 0.8% fatty acids. This corresponds to average phospholipid substitutions of 0.5 and 1.0%, respectively. None of the preparations contained the typical lipid A constituent β-hydroxymyristic acid, indicating that the polysaccharide preparations were essentially free of lipopolysaccharide (LPS).

Removal of the lipid substitution and subsequent chemical analysis had revealed KDO as the reducing sugar of capsular polysaccharides, specifically, the K5 polysaccharide (9, 42). To analyze for the presence of reducing KDO, purified polysaccharides were reduced with sodium borodeuteride and subjected to methanolysis (12). Subsequent methylation converted the sugar components to the methyl glycosides of permethylated sugar constituents. This sequence of reactions converted terminal KDO to the methyl ester of 3-deoxy-2,4,5,6,7,8-hexa-hydroxymethyl-octonic acid. In gas-liquid chromatography–mass spectroscopy, peaks were screened for mass fragments characteristic of this component. Signals corresponding to these fragments were detected in peaks from the polysaccharides of *E. coli* LE392(pGB118) and LE392(pCP3) (*kpsE kpsD kpsU*) but not in those from polysaccharides of *E. coli* LE392(pGB118::1) (*kpsS*). Thus, the substitution of polysaccharides with lipid seems to coincide with the appearance of KDO as a reducing end.

DISCUSSION

In this communication, we report on the effects of mutations in region 1 genes *kpsS*, *kpsC*, and *kpsU*, as well as those in *kpsE* and *kpsD* (Fig. 1), on expression of the *E. coli* K5 capsular polysaccharide. The findings obtained with the different mutants (Fig. 5) are discussed below in terms of possible functions for these region 1-encoded proteins.

The *kpsS* and *kpsC* genes. Mutation in *kpsS* had an impact on polymerization activity, as well as on translocation. Membranes of *E. coli* LE392(pGB118::1) had drastically reduced polymerization activity. The fact that exogenous polysaccharide could be used as an acceptor in vitro indicated that the *kpsS* mutation did not severely affect the sugar transferases. Electron microscopy with the immunogold technique revealed the polysaccharide within the cytoplasm, indicating that the *kpsS* mutation affected primarily transport rather than polymerization. The polysaccharide isolated from the cytoplasm had a high molecular weight, comparable to that of the polysaccharide isolated from the capsule of wild-type *E. coli* K5, but did not contain KDO and phospholipid. Although the chain length of the polymerization product is independent of the transport process, the efficiency of synthesis of the K5 polysaccharide seems to be regulated by the efficiency with which it is transported out of the cell. In this process, the *kpsS* gene product apparently plays an important role. A regulatory effect of *KpsS*, as a nonpolymerase component of a transferase, on the synthesis of polysialic acid in *E. coli* K1 has been discussed (49).

The phenotype exhibited by *kpsC* mutant *E. coli*

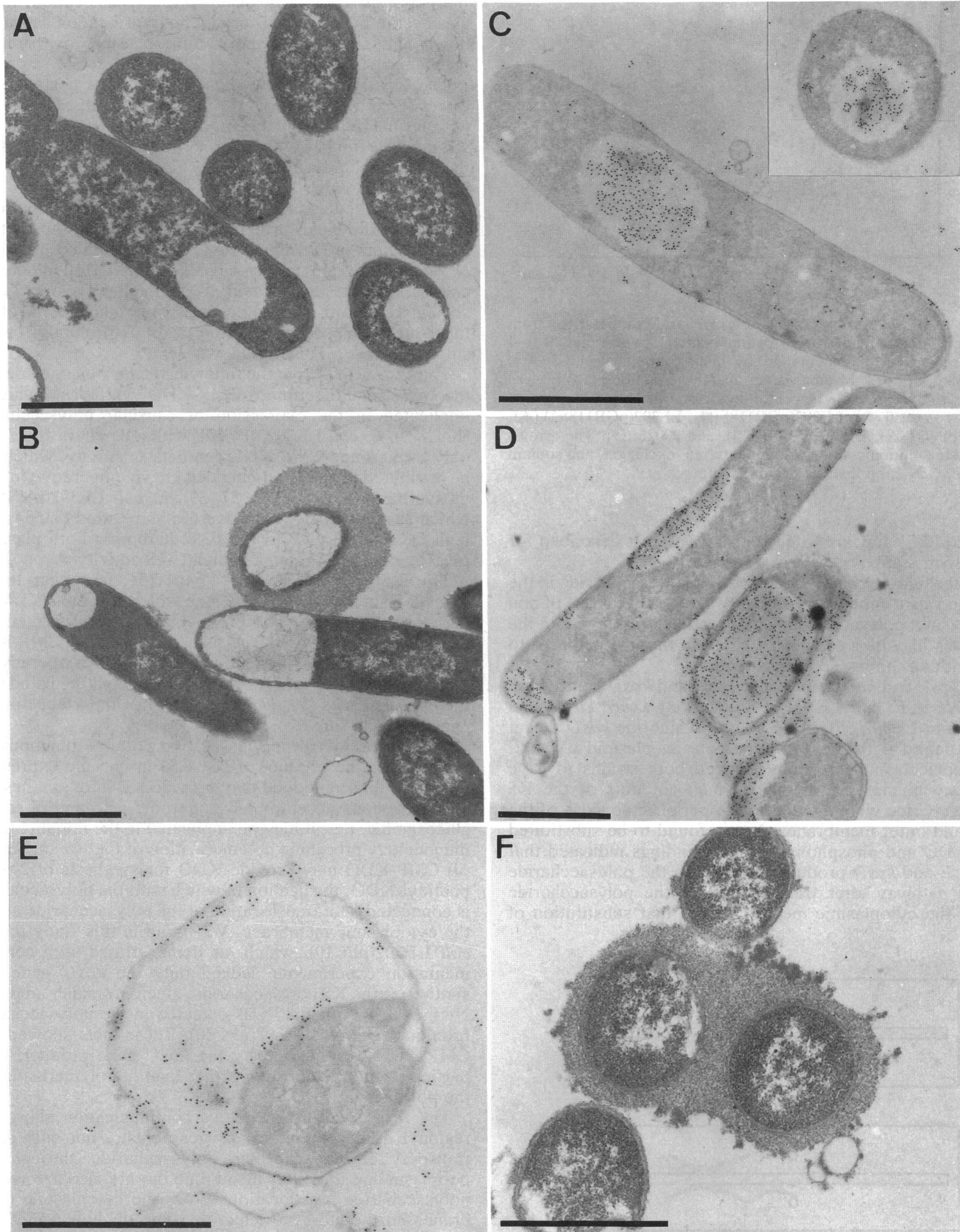

FIG. 2. Electron micrographs of ultrathin sections from region 1 mutants obtained from *E. coli* LE392(pGB118) and LE392(pGB110). Panels: A, *E. coli* LE392(pGB118::1) treated with monoclonal anti-K5 antibody and embedded in Epon; B, *E. coli* LE392(pPC10) treated as described for panel A; C, thin sections of *E. coli* LE392(pGB118::1) embedded in Lowicryl K4M and labeled with the immunogold technique by using monoclonal anti-K5 antibody (the insert shows a cross-section); D, *E. coli* LE392(pPC10) treated as described for panel C; E, *E. coli* LE392(pPC3) spheroplasted and then treated as described for panel C; F, *E. coli* LE392(pPC10/pRG1) treated as described for panel A. For details, see the text. Bars, 100 nm.

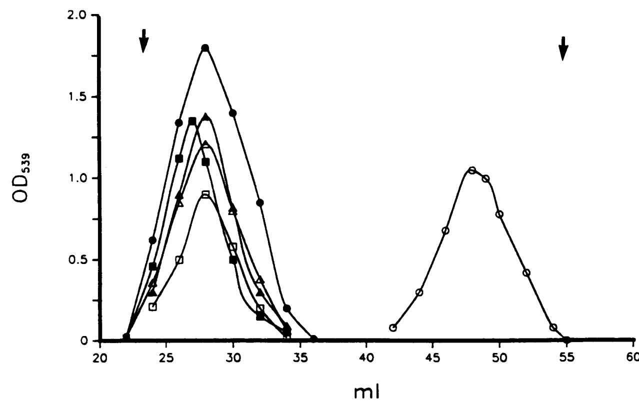

FIG. 3. Elution profiles of K5 polysaccharide preparations from wild-type K5 strains and from recombinants. The samples (3 to 4 mg) were applied to a column (1.3 by 45 cm) of Sephacryl S-300HR and eluted with 0.2 M ammonium acetate. Fractions (1 ml) were tested with the carbazole reagent. Polysaccharide preparations were from *E. coli* 2980 (●), LE392(pGB118) (■), LE392(pGB118::1) (▲), LE392(pPC3) (△), LE392(pPC10) (□), and 20026 (○). The arrows indicate the elution volumes of blue dextran (exclusion) and sodium chloride. OD_{539}, optical density of 539 nm.

LE392(pPC7) appears to be the same as that described for the *kpsS* mutant.

Intracellular accumulation of the K5 polysaccharide in the *kpsS* and *kpsC* mutants is probably caused by release of non translocated polysaccharide and its sequestration within the cell. This may be a salvage mechanism that the cell uses to deal with an otherwise toxic situation.

The *kpsE* and *kpsD* genes. The *kpsE* and *kpsD* genes were defective in *E. coli* LE392(pPC3) (lacking *kpsE*, *kpsD*, and *kpsU*) and LE392(pPC3/pVSM2), in which the *kpsU* defect was repaired with the wild-type gene in plasmid pVMS2. Immunoelectron microscopy showed both strains to have the same phenotype, in which small amounts of the K5 polysaccharide were located to the periplasmic sides of the inner and outer membranes. It was found to be substituted with KDO and phospholipid. These findings indicated that the *kpsE* and *kpsD* products function in the polysaccharide export pathway after translocation of the polysaccharide across the cytoplasmic membrane and that substitution of

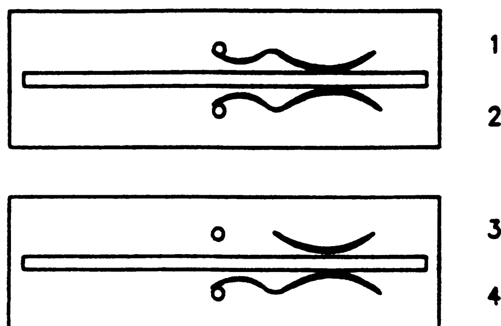

FIG. 4. Immunoelectrophoretic patterns of capsular material from *E. coli* O18:K5 (pattern 1), K5-expressing recombinant *E. coli* LE392(pGB118) (pattern 2), and expression mutants (region 1 mutants) *E. coli* LE392(pGB118::1) and LE392(pPC7) (pattern 3) and *E. coli* LE392(pPC3) (pattern 4). Monoclonal antibody was used for the immunoprecipitations.

the polysaccharide must occur during translocation. In the absence of the *kpsE-kpsD* products, translocation was tuned down and so was polymerization, possibly by feedback mechanisms.

The KpsD protein of *E. coli* K1 has been postulated to direct the transport of the polysaccharide across the periplasm (43). It will be interesting to determine which structural features of the different polysaccharides (possibly the phospholipid substituent or charge distribution) are recognized by *kpsD*.

The predicted amino acid sequence and derived hydropathy plot of the KpsE protein (34) are related to those of the BexC protein of *H. influenzae* (21) and the CitrB protein of *N. meningitidis* (13), which have been implicated in polysaccharide export and located to the cell envelope. Their sequences permit them to be considered constituents of the inner membrane. A relationship to the K protein of encapsulated *E. coli* (31) is not known.

The *kpsU* gene. From the following facts, we conclude that the *kpsU* gene determines CMP-KDO synthetase, which is implicated in the expression of capsular polysaccharide (K-CKS). *E. coli* LE392(pPC10), with a defect in *kpsU*, has only background CMP-KDO synthetase activity, which can be attributed to the *kdsB*-encoded enzyme operative in LPS biosynthesis (L-CKS) (30, 37, 39). *E. coli* LE392(pVSM2), which has only *kpsU* as an insert in its plasmid (33, 34), has high CKS activity. *E. coli* LE392 harboring both plasmids pPC10 and pVSM2 also has high CKS activity.

The sequence of the *kpsU* gene (34) is similar to but distinct from that of the *kdsB* gene which determines L-CKS (15). The respective gene products have been isolated and characterized (15, 41a). The fact that a second CMP-KDO synthetase (K-CKS), in addition to the L-CKS operative in LPS biosynthesis (30, 37, 39), is encoded and regulated with the capsule genes (10, 11) underlines the importance of this enzyme in capsule expression.

We had previously discussed two possible functions for CMP-KDO, the product of K-CKS, in capsule expression (9–11): (i) this compound may provide KDO for synthesis of an endogenous acceptor for glucuronic acid and *N*-acetylglucosamine, i.e., it may be implicated in the initial reaction immediately preceding polymerization of the K5 antigen, or (ii) CMP-KDO may provide KDO for synthesis of 2-phosphatidyl-KDO, the ligation of which with the polysaccharide is connected with translocation of the polysaccharide across the cytoplasmic membrane. We found in this study that *E. coli* LE392(pPC10), which, as demonstrated with complementation experiments, lacked only the *kpsU* gene, did synthesize the K5 polysaccharide, albeit in smaller amounts. Neither KDO nor lipid was detected in the polysaccharide fractions of strain LE392(pPC10). These facts showed that CMP-KDO, synthesized by the *kpsU* gene product, must participate in translocation rather than in polymerization of the polysaccharide.

The results described in this communication show that region 1 genes of the K5 *kps* gene cluster not only direct transport of the capsular polysaccharide through the periplasm and the outer membrane but are also involved in polysaccharide translocation across the cytoplasmic membrane. Furthermore, a defect in some of these genes also affects the efficiency of the polymerase complex. It is obvious that expression of the capsular polysaccharide is a much more complex process than hitherto assumed, with polymerizing and translocating proteins probably forming an intricate complex. For further insight into these mechanisms of capsule expression, isolation and characterization of the

	Region 1 Genes					Enzymes		Location	Polysaccharide		
						TF[a]	K-CKS[b]	EM[c]	IE[d]	KDO	Lipid
pGB118, pGB110	E	D	U	C	S	100	800	CPS	s,f	+	+
pGB118::1	E	D	U	C		2.3	680	CLD	f	-	-
pPC7	E	D	U		S	2.2	150	CLD	f	n.d.	n.d.
pPC10	E	D		C	S	25	20	(CPS)+CLD	(s),f	-	-
pPC3				C	S	30	25	PP	s,f	+	+
pPC3/pPVSM2			U	C	S	36	472	PP	n.d.	n.d.	n.d.

FIG. 5. Influence of mutations in region 1 genes of the plasmid-borne *kps* gene clusters in *E. coli* LE392 on enzyme activities, cellular location of the polysaccharide, and polysaccharide substitution with KDO and lipid. a, relative activity of glycosyltransferases as measured by incorporation of radioactivity in the incubation of membranes with UDP-[^{14}C]GlcA and GlcNAc; b, activities of CMP-KDO synthetase as measured by the formation of CMP-KDO from CTP and KDO in cytosol preparations (expressed as nanomoles of CMP-KDO formed per milligram of protein in 15 min); c, electron microscopic (EM) observation (CPS, capsular material; PP, periplasmic material; CLD, material in cytoplasmic areas of low electron density); d, immunoelectrophoretic patterns (s, slowly migrating polysaccharide population; f, fast-migrating polysaccharide population). n.d., not done.

various expression proteins are necessary. Attempts to this end are being made in our laboratories.

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REFERENCES

1. **Acetarin, J. D., E. Carlemalm, and W. Villiger.** 1986. Developments of new Lowicryl[R] resins for embedding biological specimens at even lower temperature. J. Microsc. **143:**81–88.
2. **Ames, G. F. L., C. Prody, and S. Kustu.** 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. **160:**1181–1183.
3. **Armbruster, B. L., E. Carlemalm, R. Chiovetti, R. M. Garavito, J. A. Hobot, E. Kellenberger, and W. Villiger.** 1982. Specimen preparation for electron microscopy using low temperature embedding resin. J. Microsc. **126:**77–85.
4. **Bitter-Suermann, D., I. Goergen, and M. Frosch.** 1986. Monoclonal antibodies to weak immunogenic *Escherichia coli* and meningococcal capsular polysaccharides, p. 395–396. *In* D. L. Lark (ed.), Protein carbohydrate interactions in biological systems; the molecular biology of microbial pathogenicity. Academic Press, Inc. (London), Ltd., London.
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. **Chang, A. Y. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
8. **Erickson, P. A., D. H. Anderson, and S. K. Fisher.** 1987. Use of uranyl-acetate en block to improve tissue perservation and labelling for post-embedding immunoelectron microscopy. J. Electron Microsc. Tech. **5:**303–314.
9. **Finke, A., D. Bronner, A. V. Nikolaev, B. Jann, and K. Jann.** 1991. Biosynthesis of the *Escherichia coli* K5 polysaccharide, a representative of group II capsular polysaccharides: polymerization in vitro and characterization of the product. J. Bacteriol. **173:**4088–4094.
10. **Finke, A., B. Jann, and K. Jann.** 1990. CMP-KDO synthetase activity in *Escherichia coli* expressing capsular polysaccharides. FEMS Microbiol. Lett. **69:**129–134.
11. **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.
12. **Fournet, B., G. Strecker, Y. Leroy, and J. Montreuil.** 1981. Gas-liquid chromatography and mass spectrometry of methylated and acetylated methyl glycosides. Application to the structural analysis of glycoprotein glycans. Anal. Biochem. **116:**489–502.
13. **Frosch, M., U. Edwards, K. Bousset, B. Krauße, and C. Weisgerber.** 1991. Evidence for a common molecular origin of capsular gene loci in gram-negative bacteria expressing group II capsular polysaccharides. Mol. Microbiol. **5:**1251–1263.
14. **Frosch, M., D. Muller, K. Bousset, and A. Muller.** 1992. Conserved outer membrane protein of *Neisseria meningitidis* involved in capsule expression. Infect. Immun. **60:**798–803.
15. **Goldman, R. C., and W. E. Kohlbrenner.** 1985. Molecular cloning of the structural gene coding for CTP:CMP-3-deoxy-*manno*-octulosonate cytidylyltransferase from *Escherichia coli* K-12. J. Bacteriol. **163:**256–261.
16. **Gotschlich, E. C., B. A. Frazer, O. Nishimura, J. B. Robbins, and T. Y. Liu.** 1981. Lipid on capsular polysaccharides of gram-negative bacteria. J. Biol. Chem. **256:**8915–8920.
17. **Gupta, D. S., B. Jann, G. Schmidt, J. R. Golecki, I. Ørskov, F. Ørskov, and K. Jann.** 1982. Coliphage K5, specific for *E. coli* exhibiting the capsular K5 antigen. FEMS Microbiol. Lett. **14:**75–78.
18. **Jann, B., and K. Jann.** 1990. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. Curr. Top. Microbiol. Immunol. **150:**19–42.
19. **Karkhanis, Y. D., J. Y. Zeltner, J. J. Jackson, and D. J. Carlo.** 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram negative bacteria. Anal. Biochem. **85:**595–601.
20. **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.

21. **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.
22. **Kröncke, K.-D., G. Boulnois, I. Roberts, D. Bitter-Suermann, J. R. Golecki, B. Jann, and K. Jann.** 1990a. Expression of the *Escherichia coli* K5 capsular antigen: immunoelectron microscopic and biochemical studies with recombinant *E. coli*. J. Bacteriol. **172:**1085–1091.
23. **Kröncke, K.-D., J. R. Golecki, and K. Jann.** 1990b. Further electron microscopic studies on the expression of *Escherichia coli* group II capsules. J. Bacteriol. **172:**3469–3472.
24. **Luft, J. H.** 1961. Improvements in epoxy embedding methods. J. Biophys. Biochem. Cytol. **9:**409–414.
25. **Min, H., and M. K. Cowman.** 1986. Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution. Anal. Biochem. **155:**275–285.
26. **Ørskov, F., I. Ørskov, B. Jann, and K. Jann.** 1971. Immunoelectrophoretic patterns of extracts from all *Escherichia coli* O and K antigen test strains: correlation with pathogenicity. Acta Pathol. Microbiol. Scand. **79:**142–152.
27. **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.
28. **Ørskov, I., and K. Nyman.** 1974. Genetic mapping of the antigenic determinants of two polysaccharide K antigens, K10 and K54, in *Escherichia coli*. J. Bacteriol. **120:**43–51.
29. **Ørskov, I., F. Ørskov, B. Jann, and K. Jann.** 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. **41:**667–710.
30. **Osborn, M. J., P. D. Rick, and N. S. Rasmussen.** 1980. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Translocation and integration of incomplete mutant lipid A into the outer membrane. J. Biol. Chem. **255:**4246–4251.
31. **Paakkanen, J., E. C. Gotschlich, and P. H. Mäkelä.** 1979. Protein K: a new major outer membrane protein found in encapsulated *Escherichia coli*. J. Bacteriol. **139:**835–841.
32. **Pavelka, M. S., Jr., L. F. Wright, and R. P. Silver.** 1991. Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. J. Bacteriol. **173:**4603–4610.
33. **Pazzani, C.** 1992. Ph.D. thesis. Molecular and genetic analysis of the *Escherichia coli* K5 capsule gene cluster. University of Leicester, Leicester, United Kingdom.

33a. **Pazzani, C., G. J. Boulnois, and I. S. Roberts.** Unpublished data.

33b. **Pazzani, C., and I. S. Roberts.** Unpublished data.

34. **Pazzani, C., C. Rosenow, G. J. Boulnois, D. Bronner, K. Jann, and I. S. Roberts.** 1993. Molecular analysis of region 1 of *Escherichia coli* K5 antigen gene cluster: a region encoding proteins involved in cell surface expression of capsular polysaccharide. J. Bacteriol. **175:**5978–5983.
35. **Pelkonen, S., J. Hayrinen, and J. Finne.** 1988. Polyacrylamide gel electrophoresis of the capsular polysaccharides of *Escherichia coli* K1 and other bacteria. J. Bacteriol. **170:**2646–2653.
36. **Phillips, L. R., and B. A. Frazer.** 1981. Methylation of carbohydrates with dimsyl potassium in dimethyl sulfoxide. Carbohydr. Res. **90:**149–152.
37. **Raetz, C. R. H., S. Purcell, M. V. Meyer, N. Quereshi, and K. Takeyama.** 1985. Isolation and characterization of eight lipid A precursors from a KDO deficient mutant of *Salmonella typhimurium*. J. Biol. Chem. **260:**16080–16088.
38. **Ray, P. H., and C. D. Benedict.** 1982. CTP:CMP-3-deoxy-D-manno-octulosonate cytosyl transferase (CMP-KDO-synthetase). Methods Enzymol. **83:**535–540.
39. **Rick, P. D., and D. A. Young.** 1982. Isolation and characterization of a temperature sensitive lethal mutant of *Salmonella typhimurium* that is conditionally defective in 3-deoxy-D-manno-octulosonate-8-phosphate synthesis. J. Bacteriol. **150:**447–455.
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 polysaccharides in *Escherichia coli*. J. Bacteriol. **168:**1228–1233.
41. **Roberts, I. S., R. Mountford, R. Hodge, K. 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., et al.** Unpublished data.

42. **Schmidt, M. A., and K. Jann.** 1982. Phospholipid substitution of capsular (K) polysaccharide antigens from *Escherichia coli* causing extraintestinal infections. FEMS Microbiol. Lett. **14:**69–74.
43. **Silver, R. P., W. Aaronson, and W. F. Vann.** 1987. Translocation of capsular polysaccharides in pathogenic strains of *Escherichia coli* requires a 60-kilodalton periplasmic protein. J. Bacteriol. **169:**5489–5495.
44. **Silver, R. P., W. F. Vann, and W. Aaronson.** 1984. Genetic and molecular analyses of *Escherichia coli* K1 antigen genes. J. Bacteriol. **157:**568–575.
45. **Smith, A. N., G. J. Boulnois, and I. S. Roberts.** 1990. Molecular analysis of the *Escherichia coli* K5 *kps* locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. Mol. Microbiol. **4:**1863–1869.
46. **Vann, W. F., and K. Jann.** 1979. Structure and serological specificity of the K13-antigenic polysaccharide (K13 antigen) of urinary tract-infective *Escherichia coli*. Infect. Immun. **25:**85–92.
47. **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.
48. **Vimr, E. R.** 1991. Map position and genomic organization of the *kps* cluster for polysialic acid synthesis in *Escherichia coli* K1. J. Bacteriol. **173:**1335–1338.
49. **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.
50. **Weissbach, A., and J. Hurwitz.** 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. **234:**705–713.