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Genetic and Molecular Analyses of *Escherichia coli* K1 Antigen Genes

RICHARD P. SILVER,* WILLIE F. VANN, AND WENDY AARONSON

Office of Biologics, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland 20205

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The plasmid pSR23, composed of a 34-kilobase *E. coli* chromosomal fragment inserted into the *Bam*HI site of the pHC79 cosmid cloning vector, contains genes encoding biosynthesis of the K1 capsular polysaccharide. Deletions, subclones, and Tn*5* insertion mutants were used to localize the K1 genes on pSR23. The only deletion derivative of pSR23 that retained the K1 phenotype lacked a 2.7-kilobase *Eco*RI fragment. Subclones containing *Hin*dIII and *Eco*RI fragments of pSR23 did not produce K1. Cells harboring pSR27, a subclone containing a 23-kilobase *Bam*HI fragment, synthesized K1 that was not detectable extracellularly. Six acapsular Tn*5* insertion mutants of three phenotypic classes were observed. Class I mutants synthesized K1 only when *N*-acetylneuraminic acid (NANA) was provided in the medium. Reduced amounts of K1 were detectable in cell extracts of class II mutants. Class III mutants did not produce detectable K1 in either extracts or when cells were provided exogenous NANA. All mutants had sialyltransferase activity. Analysis in the *E. coli* minicell system of proteins expressed by derivatives of pSR23 identified a minimum of 12 polypeptides, ranging in size from 18,000 to 80,000 daltons, involved in K1 biosynthesis. The 16-kilobase coding capacity required for the proteins was located in three gene clusters designated A, B, and C. We propose that the A cluster contains a NANA operon of two genes that code for proteins with apparent molecular weights of 45,000 and 50,000. The A region also includes a 2-kilobase segment involved in regulation of K1 synthesis. The B region encoding five protein species appears responsible for the translocation of the polymer from its site of synthesis on the cytoplasmic membrane to the cell surface. The C region encodes four protein species. Since the three gene clusters appear to be coordinately regulated, we propose that they constitute a *kps* regulon.

The K1 capsular polysaccharide confers invasiveness to *Escherichia coli* (33). K1 strains account for more than 80% of *E. coli* cerebrospinal fluid isolates from neonatal meningitis, and it is the most common capsular type in neonatal septicemia without meningitis and in childhood pyelonephritis (17, 30).

The K1 polysaccharide (colominic acid) is an α2-8-linked homopolymer of *N*-acetylneuraminic acid (sialic acid) (3, 10). Biosynthesis of K1 involves at least four steps: (i) the synthesis of *N*-acetylneuraminic acid (NANA), (ii) activation of NANA to CMP-NANA, (iii) polymerization of NANA into a homopolymer by the sialyltransferase complex (STC), and (iv) translocation of sialyl polymers from the cytoplasmic membrane to the cell surface. In vitro studies of the STC reactions have shown that polymerization involves a lipid intermediate (39). NANA residues are added to the nonreducing end of the growing polymer (31). The STC reactions are uncoupled in membrane fractions of *E. coli* grown at 15°C. This STC does not polymerize CMP-NANA to K1 unless exogenous K1 polysaccharide is added (37). It has been postulated that membranes grown at 15°C contain active enzymes, but that synthesis or assembly of an endogenous acceptor is lacking (37).

An understanding of the molecular and genetic events controlling the synthesis and assembly of the K1 polysaccharide requires a knowledge of the structural and regulatory genes involved in its biosynthesis and how these genes are organized, controlled, and function. A genetic locus (*kpsA*) determining K1 antigen synthesis was mapped near *serA* on the *E. coli* chromosome (27). We reported the molecular cloning of genes encoding K1 biosynthesis from an O18:K1:H7 *E. coli* strain (36). A 40-kilobase (kb) K1 hybrid plasmid, designated pSR23, contains a 34-kb chromosomal fragment inserted into the *Bam*HI site of the pHC79 cosmid cloning vector. Subclones containing the 23-, 6.6-, and 5.0-kb *Bam*HI fragments of pSR23 did not produce K1. The structure of K1 purified from the *E. coli* K-12 host harboring pSR23 was the same as that isolated from wild-type strains (36). We studied the location and organization of the K1 genes on pSR23. Deletions and subclones of pSR23 were constructed, and Tn*5* insertion mutants were isolated and characterized. The *E. coli* minicell system was used to detect K1 gene products. We demonstrate that at least 12 proteins are involved in K1 biosynthesis. The DNA sequences encoding these proteins were found in three coordinately regulated gene clusters.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and growth conditions. *E. coli* HB101 (F⁻ *lacY galK thi thr pro hsr hsm recA rpsL*); C600 (F⁻ *thr leu thi supE lacY ton*A) (21), and the minicell-producing strain DS410 (*minA minB ara xyl mtl azi thi*) (11) served as bacterial hosts. RS218 is an O18:K1:H7 *E. coli* strain (35). The plasmids used are listed in Table 1. Bacteriophage used were λ *b*221 *c*I857 Tn*5* (5) and the K1-specific bacteriophage E (15). Bacterial cultures were grown at 37°C in L broth (LB) or on L agar (21). Brain heart infusion (BHI, Difco Laboratories, Detroit, Mich.) was used for growth of cells for DNA isolation. Cells containing plasmid pSR23 or its derivatives were grown in medium supplemented with ampicillin (Ap; 500 μg/ml in broth, 100 μg/ml in agar). When necessary, the medium was supplemented with 25 μg of kanamycin (Km) or chloramphenicol

TABLE 1. Plasmids used

Strain	Genotype/comment	Source/reference
pHC79	*bla*$^+$ *tet*$^+$	16
pBR322	*bla*$^+$ *tet*$^+$	7
pBR325	*bla*$^+$ *tet*$^+$ *cat*$^+$	7
pSM1	IS*1* probe	E. Ohtsubo (22)
pSR23	34-kb *E. coli* fragment encoding *kps* genes cloned in pHC79	36
pSR26	6.6-kb *Bam*HI fragment of pSR23 in pHC79	36
pSR27	23-kb *Bam*HI fragment of pSR23 in pHC79	36
pSR27IS	Spontaneous IS*1* insertion into pSR27	This study
pSR28	5- and 6.6-kb *Bam*HI fragments of pSR23 in pHC79	This study
pSR35	3.3-kb *Hin*dIII fragment of pSR23 in pBR322	This study
pSR41	7.2-kb *Eco*RI fragment of pSR23 in pBR325	This study
pSR43	10-kb *Hin*dIII fragment of pSR23 in pBR325	This study
pSR44	2.7-kb *Eco*RI deletion of pSR23	This study
pSR45	pSR23::Tn*5* *kps*$^+$	This study
pSR46	pSR23::Tn*5* *kps*$^+$	This study
pSR47	pSR23 *kps-1*::Tn*5*	This study
pSR48	pSR23 *kps-2*::Tn*5*	This study
pSR49	pSR23 *kps-3*::Tn*5*	This study
pSR50	pSR23 *kps-4*::Tn*5*	This study
pSR51	pSR23 *kps-5*::Tn*5*	This study
pSR52	pSR23 *kps-6*::Tn*5*	This study
pSR57	24-kb *Eco*RI deletion of pSR23	This study
pSR70	pHC79::Tn*5*	This study

(Cm) or 10 μg of tetracycline (Tc) per ml. Stock cultures were kept as cell suspensions at −20°C in 30% glycerol–1% peptone.

Plasmid DNA techniques. Plasmid DNA was purified from cleared lysates by cesium chloride-ethidium bromide density gradient centrifugation (21). The alkaline extraction procedure of Portnoy et al. (29) was used for rapid screening of plasmids. Plasmid DNA isolated by this procedure was extracted with Tris-saturated (10 mM Tris, pH 8.0) phenol-chloroform (1:1, vol/vol). The DNA was precipitated from the aqueous phase by the addition of 0.5 volume of 7.5 M ammonium acetate and 2 volumes of cold ethanol. The tubes were placed in crushed dry ice for 5 min and centrifuged for 5 min. The precipitated DNA, vacuum dried and suspended in TE, was used for restriction endonuclease digestion and transformation.

Restriction endonucleases, obtained from Bethesda Research Laboratories (Rockville, Md.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.), were used as instructed by the manufacturers. Ligation reactions using T4 DNA ligase (New England Biolabs, Beverely, Mass.) and electrophoresis of DNA on vertical 0.7% agarose gels were as described previously (36). *Hin*dIII fragments of bacteriophage λ DNA served as molecular weight standards. The transformation method of Dagert and Ehrlich (9) was used.

Southern blotting and hybridization. DNA fragments in agarose gels were denatured in 0.5 M NaOH–1.5 M NaCl for 1 h, neutralized for 1 h in 0.5 M Tris–3.0 M NaCl (pH 7), and transferred from the gel to a nitrocellulose filter (21). Air-dried filters were baked for 2 h at 80°C under vacuum. Whole plasmid DNA was labeled in vitro by nick translation (21) with a translation kit (Bethesda Research Laboratories) and ^{32}P-labeled dATP and dGTP (Amersham Corp., Arlington Heights, Ill.). Heat-denatured (100°C, 5 min), ^{32}P-labeled probe DNA (10^7 cpm/μg) was hybridized with nitrocellulose blots as described by LeBlanc and Lee (20). Kodak X-Omat AR film was used for autoradiography.

Construction of deletions and subclones of pSR23. Plasmid DNA was partially digested with *Eco*RI or *Pst*I, the reaction was stopped by heating at 65°C for 5 min, and the mixture was extracted with phenol-chloroform. The DNA was precipitated with ammonium acetate and ethanol as described above, incubated overnight at −20°C, and collected by centrifugation at 4°C for 15 min. The pellet was dried under vacuum and suspended in sterile water. The DNA was ligated and transformed into strain C600. Plasmid DNA was isolated from Ap^r K1$^+$ and Ap^r K1$^-$ transformants by the rapid screening procedures described above and examined by agarose gel electrophoresis. *Eco*RI and *Hin*dIII restriction fragments of pSR23 were subcloned into pHC79, pBR322, or pBR325 (21).

Isolation of plasmids carrying Tn*5* insertions. Insertions of the kanamycin transposon Tn*5* in pSR23 were obtained by using phage λ *b*221 *c*I857 Tn*5* (5). An overnight culture of strain C600(pSR23), grown at 37°C in LB supplemented with 0.2% maltose, was diluted 1:100 into fresh media and incubated at 32°C to an absorbance (550 nm) of 0.5. Cells (1.0 ml) were incubated with λ::Tn*5* (multiplicity of infection, 0.5) at 32°C for 60 min. The infected cells were diluted fivefold in LB and incubated for 90 min at 32°C to permit expression of kanamycin resistance. The culture was diluted fourfold in LB containing Ap and Km and incubated at 37°C. Plasmid DNA was isolated from 3.0 ml of the overnight culture by the rapid screening procedure described above. This DNA was used to transform strain C600 with selection for Ap^r Km^r transformants. Only one acapsular mutant, i.e., no halos surrounding a bacterial colony on antiserum agar, was selected from each λ::Tn*5*-infected culture. The location of Tn*5* insertions was determined by digesting plasmids with *Eco*RI, *Hin*dIII, *Bgl*II, and *Bam*HI and by double digest with *Eco*RI and *Xho*I. *Hin*dIII cuts the 5.7-kb Tn*5* element twice, 1.1 kb from each end; *Bgl*II cuts twice, 1.5 kb from each end; *Bam*HI cuts once, and *Xho*I cuts three times (2, 32). Tn*5* does not contain *Eco*RI sites, and pSR23 does not contain any *Xho*I sites.

Purification and labeling of minicells. Minicells were purified from 200-ml overnight LB cultures through two successive sucrose gradients as described previously (1). Purified minicells were suspended to an absorbance of 0.6 at 600 nm in M9 minimal medium (21) supplemented with 0.2% glucose, vitamin B1, and 10% methionine assay medium (Difco) and incubated at 37°C for 45 min. The cells were then incubated in the presence of [^{35}S]methionine (1,195 Ci/mmol, New England Nuclear Corp., Boston, Mass.) for 60 min and harvested by centrifugation. The labeled minicells were suspended in 100 μl of sample buffer (10 mM Tris [pH 6.8]–2% sodium dodecyl sulfate (SDS)–2% mercaptoethanol–10 mM $MgCl_2$–0.07% bromophenol blue), heated at 100°C for 5 min, and stored at −20°C.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed in a discontinuous buffer system, using 1.5-mm slab gels with a 10% acrylamide running gel and 4% acrylamide stacking gel (19). Samples of 5 to 25 μl (80,000 to 100,000 cpm) were electrophoresed at 50 V until bromophenol blue tracking dye entered the running gel and continued at a constant current of 30 mA for 3 h. The gels were fixed (10% trichloroacetic acid–10% acetic acid–10% methanol), dried under vacuum at 70°C, and autoradio-

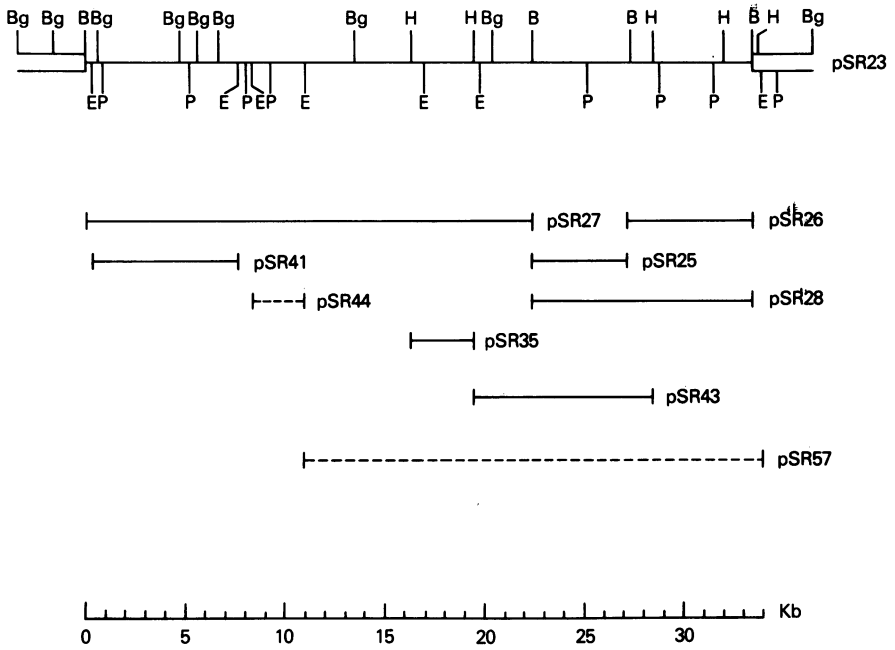

FIG. 1. Restriction endonuclease digestion map of pSR23. The central line represents the cloned DNA sequences in pSR23. The wider boxes at each end represent the pHC79 vector. The plasmid is linearized at the *Bgl*II site at 3.7 kb of pHC79 (16), and the length is expressed in 10^3 base pairs (kb). The solid lines beneath correspond to DNA segments which have been cloned separately into other vectors; broken lines correspond to DNA sequences which have been deleted. The restriction map was constructed from digest and double digests of pSR23 and its derivatives. Restriction enzyme sites are designated as follows: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; P, *Pst*I.

graphed with Kodak XAR-5 film. ^{14}C-labeled proteins (New England Nuclear) used as molecular weight standards were phosphorylase B, 97,400 (97.4K); bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; and cytochrome C, 12.3K.

Immunological procedures. Halos were observed around bacterial colonies on antiserum agar (24) after overnight incubation at 37°C and 4 to 8 h in a refrigerator. Cell extracts for immunodiffusion and rocket immunoelectrophoresis were prepared from 200-ml cultures. The cells were harvested by centrifugation and suspended in 5.0 ml of water. The cell suspension was extracted with 5.0 ml of buffered phenol at 0°C. The aqueous phase containing K1 polysaccharide was extracted with ether, bubbled with nitrogen, lyophilized, and suspended in distilled water to 5 mg/ml.

Immunodiffusion was performed in 0.9% agarose in phosphate-buffered saline. K1 polysaccharide was quantitated by rocket immunoelectrophoresis (40). Gel slides contained 10% agarose (ME, SeaKem Marine Colloids, Rockport, Maine) in 0.05 M Veronal acetate buffer (pH 8.6) and 6.25% horse 46 group B meningococcal antiserum. The gels were electrophoresed at 4°C for 4 h at 2.5 V/cm^2. The peak height of the rockets was plotted against a standard curve of purified K1 polysaccharide.

Sialic acid determination. Cells from 200-ml broth cultures were extracted in 0.5 M formic acid at 80°C for 1 h and centrifuged. The supernatant was extracted with petroleum ether, passed through Dowex 50 × 8 H^+, and lyophilized. Sialic acid was extracted from this residue by mild methanolysis (0.1 M HCl-methanol, 1 h, 80°C). The resulting methyl ester methyl ketosides were trimethylsilylated with trimethylsilylmidazole and analyzed by gas-liquid chromatography. Gas chromatography (Perkin-Elmer Sigma 3B) was performed in a 3% SE-30 column (2 mm by 2 m) programmed from 150 to 240°C at 8°C per min.

Sialyltransferase activity. Membrane fractions and EDTA-permeabilized cells were prepared as described previously (18). Sialyltransferase activity was determined by paper chromatography (38).

RESULTS

Construction of subclones and deletions of pSR23. Subclones and deletion derivatives of pSR23 (Fig. 1) were isolated to locate the K1 genes. Digestion with *Eco*RI generated fragments of 15, 7.2, 6.4, 5.6, and 2.7 kb (Fig. 2,

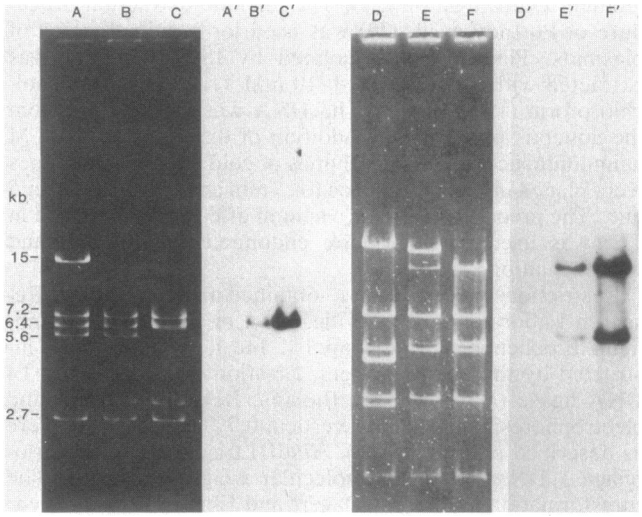

FIG. 2. Electrophoresis in 0.7% agarose gels of restriction endonuclease digests of plasmid DNA and autoradiograms of DNA patterns obtained after hybridization with ^{32}P-labeled pSM1 DNA. *Eco*RI digest of pSR23 (lane A), pSR27 (lane B), and pSR27IS (lane C); *Pst*I digest of pSR23 (lane D), pSR27 (lane E), and pSR27IS (lane F). Lanes A′ through F′ are autoradiograms of the corresponding lanes after hybridization.

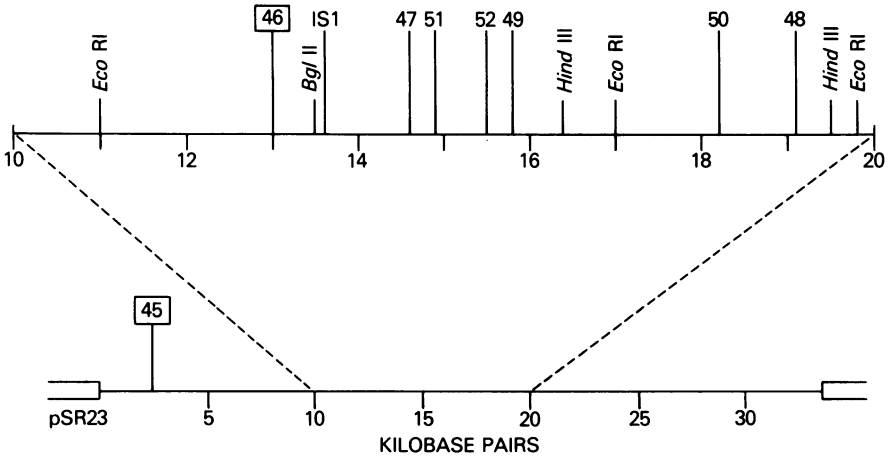

FIG. 3. Tn*5* insertions in pSR23. An enlargement of the region between 10 and 20 kb on pSR23 (Fig. 1) is shown on the top line. Tn*5* insertions into pSR23 were isolated by infecting a C600 host harboring pSR23 with λ::Tn*5*. Mutation numbers correspond to plasmid designations (Table 1). Mutation numbers enclosed in boxes are $K1^+$. The location of the IS1 insertion in pSR27 is also shown.

lane A), and digestion with *Hin*dIII generated fragments of 21, 10, 3.3, and 2.4 kb (data not shown). Subclones containing each fragment, with the exception of the 5.6-kb *Eco*RI fragment (Fig. 1, 11 to 17 kb coordinates; Fig. 2, lane A) were isolated. None of the subclones produced K1, as determined by halos on antiserum agar plates. The only deletion plasmid that remained $K1^+$ was pSR44 (Fig. 1). pSR44 differs from pSR23 by deletion of a 2.7-kb *Eco*RI fragment (Fig. 1, 8.4 to 11 kb coordinates). All other transformants were acapsular and contained plasmids with deletions resulting from excision of more than one DNA fragment. All of the acapsular deletion plasmids lacked a common fragment between 11 and 20 kb (Fig. 1).

Polymer synthesis by cells harboring subclone pSR27. The plasmid pSR27 contains the 23-kb *Bam*HI fragment of pSR23 (Fig. 1). Cells harboring pSR27 did not produce capsule but had STC activity. The STC activity of pSR27 (1.62 nmol/h per mg of protein) was comparable to that observed with extracts from RS218 or HB101(pSR23). No other deletion derivative or subclone of pSR23 had detectable STC activity in the presence or absence of exogenous colominic acid. Cells harboring pSR27, however, grew slowly and were difficult to maintain, even when grown in medium containing 500 μg of ampicillin (Ap) per ml. In contrast, the parent plasmid, pSR23, although unstable, could be maintained in cells grown in broth culture or on agar plates containing Ap (36).

The presence of polysaccharide in whole cell suspensions and either EDTA-treated or sonicated cells of HB101(pSR27) was tested in double immunodiffusion experiments. No precipitin lines were observed between untreated bacterial cell suspensions and the antiserum. In contrast, a single precipitin line, showing identity with K1, was observed with bacterial cells that had been sonicated or treated with EDTA (data not shown). These observations indicated that cells carrying pSR27 synthesize polysaccharide that is not transported outside the cell. The data also indicated that genetic information within the 11-kb fragment, deleted in pSR27 (Fig. 1), is required for transport of the polysaccharide to the cell surface.

A stable derivative of pSR27 was isolated after transformation of *E. coli* C600 with purified pSR27 DNA. This derivative, pSR27IS, could be maintained in the absence of antibiotic selection. However, STC activity in this transformant was sharply reduced and detectable only at high substrate concentrations (fivefold the normal level) or by the addition of exogenous K1. Purified pSR27IS DNA contained an 0.8-kb insertion in the *Eco*RI fragment (Fig. 2, lane c). The length of the insertion, its spontaneous origin, and the observation that it carried one *Pst*I site (Fig. 2, lane F) and one *Bal*I site (data not shown) suggested that the insertion element IS*1* (23) had inserted into pSR27. This was confirmed by Southern blot hybridization with an IS*1* plasmid probe, pSM1 (22). The ^{32}P-labeled probe hybridized to the single *Eco*RI fragment (Fig. 2, lane C′) and to the two *Pst*I fragments (Fig. 2, lane F′) of pSR27IS containing the insertion, but not to pSR23 (Fig. 2, lanes A′ and D′). The pSR27 DNA preparation also contained small amounts of pSR27IS DNA (Fig. 2, lanes B′, E, and E′). The IS*1* insertion mapped at coordinate 13.6 kb of pSR23.

Isolation and characterization of Tn*5* insertion mutants of pSR23. To define more precisely the location of the K1 genes, Tn*5* insertion mutations of pSR23 were isolated (Fig. 3) and characterized (Table 2). Six independently isolated acapsular mutants clustered within a 5-kb region of pSR23 (Fig. 3). Two $K1^+$ Tn*5* insertions were also isolated (Fig. 3).

The production of extracellular K1 by cells carrying insertion mutations was examined by sensitivity to the K1-specific bacteriophage, E. In these experiments, the K1 phage were added to actively growing cells and phage sensitivity was detected by cell lysis. None of the strains carrying mutant plasmids were sensitive to the K1 phage. However, after growth for 30 min in medium containing 100 μg of NANA per ml, cells harboring *kps-4* and *kps-2* showed marked sensitivity to K1 phage (data not shown). These data suggest that *kps-4* and *kps-2* are defective in NANA synthesis. CMP-NANA synthetase and subsequent biosynthetic steps were expressed in these mutants since capsule was made after the addition of NANA.

The presence of K1 was examined in phenol extracts of cells carrying the mutant plasmids. Immunodiffusion of cell extracts against horse 46 group B meningococcal antiserum shows a line of identity with purified K1 polysaccharide and extracts of cells harboring *kps-1* and *kps-5* (Fig. 4A, wells 3 and 5). No polysaccharide was detected in extracts of cells carrying other insertion mutations. The amount of polysac-

TABLE 2. Characteristics of cells carrying pSR23::Tn5 insertions

Plasmid[a]	Genotype	Capsule[b]	Effect of sialic acid[c]	Polysaccharide[d]	Sialic acid[e]	Sialyltransferase activity[f]
pSR45	*kps*$^{+g}$	+	ND	+	+	ND
pSR46	*kps*$^{+}$	+	ND	+	+	69.8
pSR47	*kps-1*	−	−	+	+	21
pSR51	*kps-5*	−	−	+	+	17
pSR52	*kps-6*	−	−	−	−	25.8
pSR49	*kps-3*	−	−	−	−	18
pSR50	*kps-4*	−	+	−	−	107
pSR48	*kps-2*	−	+	−	−	19.8

[a] In C600 host; plasmid designations correspond to mutant numbers in Fig. 3.
[b] Halos on antiserum agar and K1 phage sensitivity.
[c] Sensitivity to K1 phage after the addition of 100 μg of sialic acid per ml. ND, Not done.
[d] Immunodiffusion and rocket electrophoresis.
[e] Gas chromatography.
[f] Percentage of RS218 STC activity.
[g] We propose that *kps*, not *kpsA* (27), be used to designate loci involved in synthesis of acidic polysaccharide capsular (k) antigens.

charide produced by *kps-1* and *kps-5*, determined by rocket immunoelectrophoresis (data not shown), was 5- to 10-fold less than that produced by cells carrying pSR23::Tn5 *kps*$^{+}$ plasmids (Fig. 4A, wells 4 and 6). The K1 produced in cells harboring *kps-1* and *kps-5* was either not secreted or was below the level of detection of antiserum agar and K1-specific bacteriophage.

NANA was assayed in hot acid extracts of whole cells by gas chromatography (Fig. 5). In agreement with the results of the immunodiffusion experiments, NANA was detected in extracts of cells harboring *kps-1* and *kps-5*, but not in extracts of cells carrying other insertion mutations.

Sialyltransferase activity was not correlated with the presence of K1 in cell extracts. All insertion mutants possessed an active STC (Table 3). STC activity was present in cells harboring *kps-4* at levels equal to enzymatic activity of wild type, whereas cells containing other insertions possessed less than 25% activity (Table 2).

Polypeptides involved in expression of K1 polysaccharide. pSR23 derivatives were transferred to strain DS410, and plasmid-encoded protein synthesis was examined in purified minicells (Table 3). Gel electrophoresis of extracts from ^{35}S-labeled minicells containing pSR23::Tn5 kps^{+} (Fig. 6, lane B) showed that 12 proteins, designated P1 to P12 (apparent range of masses, 18 to 80 kilodaltons [kd]), were expressed in addition to the polypeptides encoded by the vector and transposon Tn5 (Fig. 6, lane A). The 12 proteins were not expressed in detectable amounts in minicells harboring insertion mutation *kps-1* (Fig. 6, lane C), implying a role for all

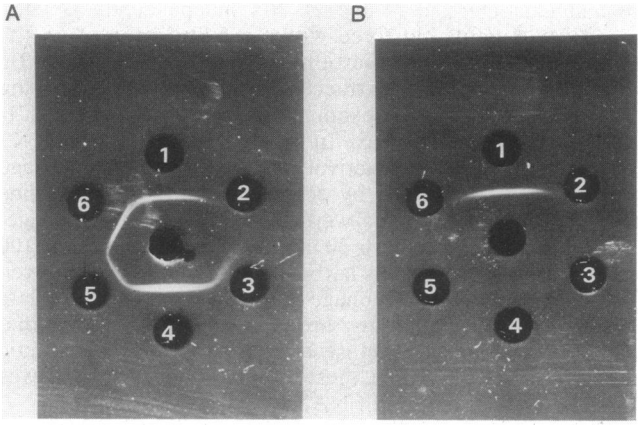

FIG. 4. Double immunodiffusion with horse 46 group B meningococcal globulin (center wells) and extracts (25 μg) from cells harboring pSR23::Tn5 insertions. In both panel A and panel B, well 1 contains purified K1 capsular polysaccharide (0.5 μg) and well 2 contains an extract of the C600 host. (A) Extracts from C600 containing (3) pSR47, (4) pSR45, (5) pSR51, (6) pSR46. (B) Extracts from C600 containing (3) pSR48, (4) pSR49, (5) pSR50, (6) pSR52.

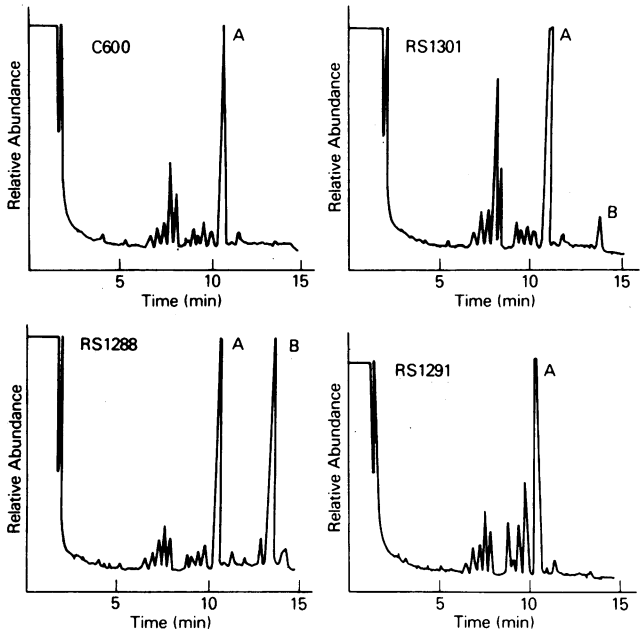

FIG. 5. Gas chromatographic determinations of sialic acid in mild acid extracts of *E. coli* harboring pSR23::Tn5 insertions. The methyl ester methyl ketosides prepared from formic acid extracts of cells were trimethylsilylated and analyzed by gas-liquid chromatography. Sialic acid methyl ester methyl ketoside (B) eluted with a relative retention time of R = 1.28 relative to inositol (A). RS1288, RS1301, and RS1291 are the C600 host carrying pSR45, pSR51, and pSR49, respectively. An extract from the plasmid-free C600 host is also shown.

TABLE 3. Expression of ^{35}S-labeled, plasmid-coded proteins in minicells

Plasmid in minicells:	Proteins synthesized[a]:											
	P1 (80)	P2 (77)	P3 (73)	P4 (69)	P5 (60)	P6 (50)	P7 (45)	P8 (43)	P9 (40)	P10 (37)	P11 (19)	P12 (18)
Tn*5* insertions												
pSR46	+	+	+	+	+	+	+	+	+	+	+	+
pSR47	−	−	−	−	−	−	−	−	−	−	−	−
pSR51	+	−	+	+	−	−	−	−	−	−	+	+
pSR49	+	+	+	+	+	+	+	+	+	+	+	+
pSR52	+	+	+	+	+	+	+	+	+	+	+	+
pSR50	+	+	+	+	+	+	−	+	+	+	+	+
pSR48	+	+	+	+	+	+	−[b]	+	+	+	+	+
Subclones												
pSR35	−	−	−	−	−	+	+	−	−	−	−	−
pSR28	+	+	−	−	+	−	−	−	+	+	−	−
pSR26	+	−	−	−	−	−	−	−	−	+	−	−
pSR43	−	+	−	−	+	−	−	−	+	−	−	−
pSR41	−	−	+	+	−	−	−	−	−	−	−	−
pSR57	−	−	+	+	−	−	−	−	−	−	+	+

[a] Molecular weight ($\times 10^3$) is indicated in parentheses. Symbols indicate the presence (+) or absence (−) of proteins.
[b] Additional protein band of 35 kd.

12 proteins in K1 antigen synthesis. Minicells carrying the *kps-5* insertion mutation, located only 0.3 kb from the site of *kps-1* insertion, expressed P1, P3, P4, P11, and P12 (Fig. 6, lane D). No significant differences were detected between the proteins synthesized by cells harboring mutations *kps-3* or *kps-6*, and *kps*$^+$ (Fig. 6, lanes E and F).

Minicells harboring *kps-4* or *kps-2*, mutants in NANA synthesis, did not synthesize P7, a 45-kd protein (Fig. 6, lanes G and H). A new protein species with an apparent mass of 35 kd was observed in minicells harboring *kps-2* (Fig. 6, lane H). P7 was expressed in minicells harboring pSR35 (data not shown). pSR35 is a subclone containing the 3.3-kb *Hin*dIII fragment or pSR23 (Fig. 3, 16.2 to 19.5 kb coordinates), and includes the region that accommodates the *kps-4* and *kps-2* insertions. Minicells harboring pSR35 also expressed P6, a 50-kd protein (data not shown). P6 and P7 account for approximately 80% of the coding capacity of the DNA cloned in pSR35. We could assign an approximate location to the genes encoding P6 and P7 on the physical map of pSR23; P6 was located between 16.5 and 17.8 kb, and the 45-kdal species was found between 18 and 19.3 kb (Fig. 3).

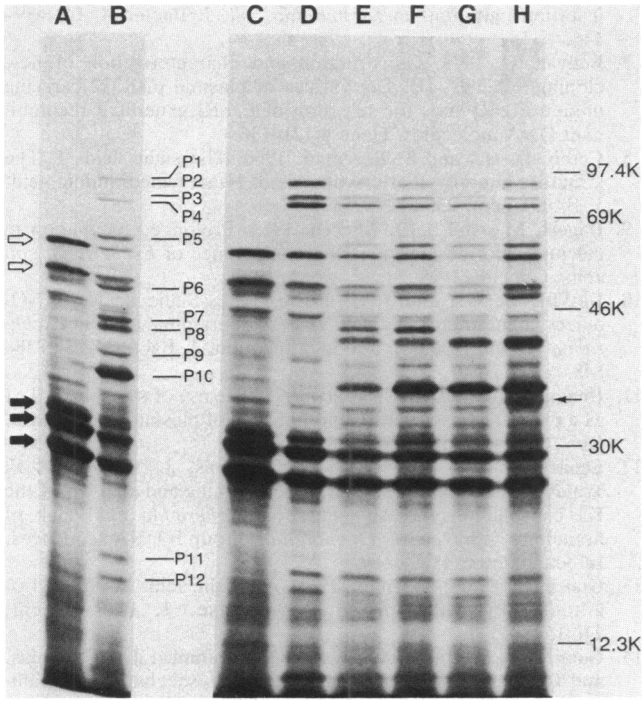

FIG. 6. Autoradiogram of [^{35}S]methionine-labeled proteins in minicells harboring pSR23::Tn*5* insertions. Samples were analyzed in a 10% SDS-polyacrylamide gel. Transposon Tn*5* coded proteins are indicated by open arrows on the left; closed arrows on the left indicate vector-encoded β-lactamase proteins. The molecular weight standards are indicated on the right. Minicell samples were from strain DS410 containing: lane (A), pSR70, (B), pSR46; (C), pSR47; (D), pSR51; (E), pSR52; (F), pSR49; (G), pSR50; (H), pSR48; the arrow on the right indicates truncated protein.

The locations of genes encoding for other protein species were identified from deletions and subclones of pSR23 (Fig. 1). Plasmids pSR43, pSR26, and pSR28, subclones derived from the right end of pSR23 (between kb coordinates 20 and 34; Fig. 1), synthesized P1, P2, P5, P9, and P10 (data not shown). Minicells carrying pSR28 expressed all five species. P1 and P10 were made in minicells carrying pSR26. Cells harboring pSR43 synthesized P2, P5, P9, and a protein of 45 kd which we interpret as being a truncated derivative of P1. From these results, we could localize the 7.8-kb fragment needed to accommodate the genes encoding the five proteins approximately between the 24- and 32-kb coordinates (Fig. 1).

Minicells harboring pSR41 and pSR57, which contain regions derived from the left end of pSR23 (0 to 10 kb, Fig. 1), expressed P3, P4, P11, and P12 (data not shown). pSR57 synthesized all four proteins, whereas pSR41 expressed only P3 and P4.

DISCUSSION

Expression of the K1 capsular polysaccharide is a complex process involving synthesis, activation, and polymerization of NANA and translocation of K1 to the cell surface. Our results indicate that 12 proteins, P1 to P12, are involved in these events. The 16 kb of DNA required to encode P1 to 12 are not contiguous but are found in three gene clusters that we designate A, B, and C, located within the 11 to 20, 24 to 32, and 0 to 8 kb coordinates, respectively, on the physical map of pSR23. Echarti et al. (12) recently reported that a 15-

kb region, divided into at least two gene clusters, encodes functions for the synthesis and assembly of the K1 polysaccharide.

Three distinct phenotypic classes of insertion mutations were observed within the A region. Class I mutations (*kps-4* and *kps-2*) synthesized capsular polysaccharides only when NANA was provided in the medium. Class II mutations (*kps-1* and *kps-5*) produced low levels of polysaccharide detectable in cell extracts. K1 was not detectable in extracts of cells harboring class III mutations (*kps-6* and *kps-3*); nor was K1 synthesized when cells were provided exogenous NANA.

We propose that the A region includes a NANA operon of at least two proteins, P6 and P7. We interpret the 35-kd polypeptide observed in cells harboring *kps-2* to be a truncated derivative of P7. No detectable shortened peptide was seen in cells containing *kps-4*, suggesting that the direction of transcription of the gene encoding P7 was from left to right on the physical map of pSR23 (Fig. 3). Both P6 and P7 were expressed in minicells harboring insertion mutation *kps-3* (Fig. 6, lane F). Since Tn*5* insertions are strongly polar (6), the results imply that transcription of the genes encoding P6 and P7 initiates from a promoter located distal to the site of insertion of *kps-3*. The biosynthetic steps leading to NANA have not been elucidated in *E. coli*, but as few as two unique enzymes may be sufficient. An epimerase catalyzing the conversion of *N*-acetylglucosamine to *N*-acetylmannosamine has been detected in the K1 strain designated K235 (13). A NANA-aldolase, which condenses pyruvate and *N*-acetylmannosamine to sialic acid, has also been found in *E. coli* K235 (8). The A region also includes a segment of approximately 2 kb which appears important in the regulation of K1 synthesis. Insertions within this region effect polymerization of NANA as well as expression of the 12 proteins located in the three gene clusters. These data suggest that the three regions are coordinately regulated and may constitute a single unit of regulation, the *kps* regulon.

The mechanism for the translocation of capsular polysaccharide from its site of synthesis on the cytoplasmic membrane to the cell surface is not known. It has been postulated that transfer to the outer membrane occurs at discrete sites of contact between the inner and outer membranes (4). Our data suggest that the B region, missing in pSR27, is involved in the translocation process. Five protein species have been identified within the B cluster and may be involved in the stabilization, transport, or assembly of the polysaccharide.

Whereas the A and B gene clusters may be part of a large contiguous DNA fragment associated with K1 biosynthesis, the A and C regions are separated by a sequence, deleted in pSR44, that does not affect K1 expression. The function associated with the C region, encoding proteins with apparent masses of 73, 69, 19, and 18 kd, are unknown. A 20-kd protein was postulated to be involved in the synthesis or assembly of the endogenous acceptor required for K1 synthesis (37).

Among the serologically defined *E. coli* surface antigens, there are 103 K antigens (26). Genetic data (25) and recent hybridization experiments (12) support the concept that steps in the biosynthesis of distinct K antigens may be shared. In addition, transfer of the K1 genes to a mutant K100 strain restored K100 synthesis in the recipient (R. P. Silver and W. Aaronson, unpublished data). Common reactions could include synthesis of endogenous acceptor, involvement of the lipid intermediate, or the translocation process. A major outer membrane protein, K, has been correlated with capsular polysaccharide production in most encapsulated strains of *E. coli* (28). Also, several distinct capsular polysaccharide antigens of *E. coli* contain the same lipid moiety bound to the polysaccharide (14, 34). The separation of capsular genes into distinct regions suggests a genetic basis for common biosynthetic steps among strains of *E. coli* synthesizing different capsular polysaccharides.

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