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Genetic Analysis of Chromosomal Mutations in the Polysialic Acid Gene Cluster of *Escherichia coli* K1

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The *kps* gene cluster of *Escherichia coli* K1 encodes functions for sialic acid synthesis, activation, polymerization, and possibly translocation of polymer to the cell surface. The size and complexity of this membrane polysaccharide biosynthetic cluster have hindered genetic mapping and functional descriptions of the *kps* genes. To begin a detailed investigation of the polysialic acid synthetic mechanism, acapsular mutants were characterized to determine their probable defects in polymer synthesis. The mutants were tested for complementation with *kps* fragments subcloned from two separately isolated, functionally intact *kps* gene clusters. Complementation was assayed by immunological and biochemical methods and by sensitivity to the K1-specific bacteriophage K1F. The *kps* cluster consisted of a central 5.8-kilobase region that contained at least two genes coding for sialic acid synthetic enzymes, a gene encoding the sialic acid-activating enzyme, and a gene encoding the sialic acid polymerase. This biosynthetic region is flanked on one side by an approximately 2.8-kilobase region that contains a potential regulatory locus and at least one structural gene for a polypeptide that appears to function in polysialic acid assembly. Flanking the biosynthetic region on the opposite side is a 6- to 8.4-kilobase region that codes for at least three proteins which may also function in polymer assembly and possibly in translocating polymer to the outer cell surface. Results of transduction crosses supported these conclusions and indicated that some of the *kps* genes flanking the central biosynthetic region may not function directly in transporting polymer to the cell surface. The results also demonstrate that the map position and probable function of most of the *kps* cluster genes have been identified.

The polysialic acid capsule of *Escherichia coli* K1 is an unbranched homopolymer of 200 sialic acid residues in α-2,8-ketosidic linkage (19). Molecules with more than three α-2,8-linked internal sialic acid residues are rare in nature and, until recently, only were known in *E. coli* K1 and *Neisseria meningitidis* group B (26). Poly-α-2,8-sialic acid chains similar to the K1 antigen are now known to be widely distributed on the vertebrate cell adhesion molecule NCAM (20, 31). The rarity of sialic acids in bacteria and the association of polysialic acid capsules with virulence suggested that the bacterial polysaccharide may mimic polysialic acid moieties on mammalian host glycoconjugates (5). Repeated failures to develop effective vaccines for the α-2,8-linked capsule may thus be due to immune tolerance in the host (5). Through a better understanding of the genetics and biochemistry of capsule biosynthesis, it may be possible to develop alternative therapeutic approaches for treating infections caused by polysialic acid-encapsulated bacteria.

Polysialic acid synthesis in *E. coli* K1 has been one of the best-characterized capsular polysaccharide biosynthetic systems. The broad outlines of polysialic acid synthesis are understood at a biochemical level (26), and the genes necessary for its expression have been cloned (4, 23). At least 17 kilobases (kb) of DNA are required for polysialic acid capsule synthesis (23); this DNA is referred to as the *kps* gene cluster and encodes proteins which catalyze sialic acid synthesis, activation, and polymerization (4, 24, 25, 33). Interestingly, a large portion of the *kps* cluster has been assumed not to function directly in any of these processes. For example, a variety of *kps* mutations do not prevent polysaccharide synthesis but cause intracellular accumulation of polysialic acid, possibly in the periplasm or cytosol (3, 4, 16, 17, 22, 24). These results suggest that translocation of chemically different capsular polysaccharides to the outer surface may use similar mechanisms. Other similarities are the existence of 1,2-diacylglycerol phosphate esterified to the reducing end of several different K antigens (7, 21) and the apparent involvement of undecaprenyl phosphate and outer membrane porins in polysaccharide assembly mechanisms (14, 26; J. Foulds and W. Aaronson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, D-21).

The function and map position of most of the *kps* genes are not known in detail. We have approached this problem by constructing *E. coli* K-12–K1 hybrids that are amenable to both traditional genetics and recombinant DNA methodologies (32, 33). In this report, isolation and biochemical characterization of K1 acapsular mutants and the results of complementation and transduction analyses with these mutants are described. We also propose a unified nomenclature for *E. coli* capsular polysaccharide genes that is based on the existence of functionally homologous loci in otherwise structurally distinct biosynthetic gene clusters (16, 17). We suggest that shared loci retain the *kps* designation, with loci unique to a particular polysaccharide designated by a dominant structural feature of the polysaccharide. For example, K1 antigen is a sialic acid homopolymer and the designation *neu* (*N*-acetylneuraminic acid) is proposed for K1-specific biosynthetic genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and growth conditions. The derivations and properties of bacteria, plas-

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TABLE 1. Bacterial strains, plasmids, and phage

Strain or plasmid	Relevant genotype or properties	Source or reference
E. coli laboratory strains		
HB101	*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 leu*	Laboratory collection
PA360	*thi-1 leuB6 hisG1 serA1 argH1 thi-1 lacY1 gal-6 malA1 xyl-7 mtl-2 rpsL9 tonA2 supE44*	B. Bachmann
LE392	*hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	Laboratory collection
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	Laboratory collection
JK589	C600 (F^+ *zzf*::Tn*5*)	J. Konisky
E. coli K-12–K1		
EV1	PA360 (kps^+ $serA^+$ $malA^+$)[a]	33
EV3	EV1 *kps-44*	This study
EV4	EV1 *kpsT21*	33
EV5	EV1 *neuA22*	33
EV7	EV1 *neuA40*	This study
EV11	EV1 *kps-23*	33
EV13	EV1 *kps-24*	33
EV14	EV1 *kps-42*	This study
EV15	EV1 *kpsS25*	This study
EV19	EV1 *neu-41*	This study
EV24	EV1 *neuB25*	33
EV32	EV1 *neu-43*	This study
EV33	EV1 *neuA26*	This study
EV36	*galP23 rpsL9* ($argA^+$ rha^+ kps^+)	33
EV93	EV36 *kpsC30*::Tn*10*	This study
EV94	EV36 *kpsS31*::Tn*10*	This study
EV95	EV36 *kpsT32*::Tn*10*	This study
Plasmid		
pSX48	pHC79 with 38.5-kb kps^+ *Sau*3A insert from EV1	This study
pSX49	pHC79 with 40-kb kps^+ *Sau*3A insert from EV1	This study
pSX50	pBR329 with 10.5-kb *Eco*RI insert from pSX49; includes 370 base pairs of pHC79	This study
pSX51	*Bam*HI digestion of pSX50 followed by religation to give 2.7-kb *Eco*RI-*Bam*HI insert	This study
pSX52	pSX50 *kpsS45*::Tn*1000*	This study
pSX53	pSX50 *kpsS46*::Tn*1000*	This study
pSX54	pSX50 *kpsS47*::Tn*1000*	This study
pSX55	pSX50 *kpsS48*::Tn*1000*	This study
pSX56	pSX50 *kpsS49*::Tn*1000*	This study
pSX57	pSX50 *kpsS50*::Tn*1000*	This study
pSX58	pSX50 *kpsS51*::Tn*1000*	This study
pSX59	pSX51 *neuS1*::Tn*1000*	This study
pSX70	pSX51 *kpsS52*::Tn*1000*	This study
pGB124	pBR328 with 13.5-kb *Xho*I-*Eco*RI insert from K7 biosynthetic cluster	I. Roberts & G. Boulnois (16)
pSR23	pHC79 with 34-kb kps^+ *Sau*3A insert from RS218	24
pSR25	pHC79 with 5.5-kb *Bam*HI insert from pSR23	24
pSR26	pHC79 with 6.6-kb *Bam*HI insert from pSR23	24
pSR28	pHC79 with 11.6-kb *Bam*HI insert from pSR23	24
pSR34	pHC79 with 16.3-kb *Bam*HI-*Hin*dIII insert from pSR23	This study
pSR35	pBR322 with 3-kb *Hin*dIII insert from pSR23	28
pSR43	pBR325 with 10-kb *Hin*dIII inserts from pSR23	24
pSR46	pSR23::Tn*5* (kps^+)	24
pSR47	pSR23 *kpsT1*::Tn*5*	24
pSR49	pSR23 *kps-3*::Tn*5*	24
pSR51	pSR23 *kpsT5*::Tn*5*	24
pSR52	pSR23 *kps-6*::Tn*5*	24
pSR65	*Eco*RI fragment from pSR47 inserted into pACYC184; contains Tn*5*	This study
pSR67	*Eco*RI fragment from pSR49 inserted into pACYC184; contains Tn*5*	This study
pSR69	*Eco*RI fragment from pSR52 inserted into pACYC184; contains Tn*5*	This study
pSR74	*Eco*RI fragment from pSR51 inserted into pACYC184; contains Tn*5*	This study
pSR95	pACYC184 with 3.9-kb insert from pSR46; includes 1.2 kb of Tn*5*	22
Phage		
P1	*vir*	Laboratory collection
λ840	Tn*10* *del*4 HH104 (Tc^r)	34
K1F	*E. coli* K1-specific phage	31, 33

[a] Markers in parentheses are of non-K-12 origin (33).

mids, and phages used in this work are described in Table 1. Bacteria were propagated in Luria broth or on solid medium solidified with 1.5% agar, as described previously (32). When required, media were supplemented with tetracycline hydrochloride (10-μg/ml final concentration), ampicillin (100-μg/ml final concentration), and streptomycin sulfate (150-μg/ml final concentration). Unless otherwise indicated, all incubations were carried out at 37°C.

Mutant isolation. Spontaneous K1F^r mutants derived from EV1 and EV36 (Table 1) were selected as described previously (33). Chromosomal Tn*10* insertions into the *kps* cluster were isolated by infecting liquid cultures of EV36 with a λ840 lysate as described before (32). After 30 min of adsorption, EGTA [ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid] was added to a final concentration of 10 mM and cultures were incubated for 1 h to permit expression of Tc^r. Cultures were incubated for an additional 4 to 7 h in the presence of tetracycline to allow phenotypic expression of the acapsular phenotype and enrichment of Tc^r bacteria. Mutants were selected by plating culture dilutions onto solid medium containing tetracycline and 10^7 infective K1F particles. After overnight incubation, Tc^r K1F^r colonies were purified and used as donors for P1-mediated backcross with recipient strain EV36. Isolates which gave 100% linkage of Tc^r (selected marker) with K1F^r (unselected marker) were saved as EV93, EV94, and EV95 (Table 1).

A positive selection was used to isolate Tn*1000* (8) insertions into *kps* genes carried on pSX50. Plasmid was transformed into the F^+ strain JK589 (Table 1), and the resulting donor was conjugated with EV15 or EV93 in liquid medium for 1 h without shaking. Transconjugates were selected by plating dilutions of the mating mix onto agar containing ampicillin and streptomycin as the counterselection. Selection plates were seeded with K1F so that the only viable exconjugates isolated had Tn*1000* inserted into *kpsS* on pSX50, when EV15 or EV94 was the recipients, or into *kpsC* on pSX50, when EV93 was the recipient.

Immunological procedures. Polyclonal horse antiserum containing precipitating immunoglobulin M antibodies specific for ≥10 poly-α-2,8-sialic acid residues was used in all procedures (31, 33). The halo test assays for extracellular K1 antigen produced by bacteria growing on antiserum agar, as described previously (33). Radial immunodiffusion was used to test for intracellular K1 antigen. Bacteria were grown in broth and lysates were prepared by sonication (32) or freeze-thaw lysozyme treatment (see below). Extracts were added to wells punched into 1% agarose containing 10% horse serum and Trizma-barbital electrophoresis buffer (Sigma Chemical Co., St. Louis, Mo.). Plates were incubated in a humidified chamber at 37°C for 3 to 12 h and then at 4°C for at least 3 h. A positive response is indicated by a sharp precipitin ring circumscribing the well. Rocket immunoelectrophoresis of cell extracts was carried out as described previously (31).

To screen transductants for intracellular antigen, a modification of the radial immunodiffusion procedure described above was used. Transductants were inoculated into 250 μl of medium contained in the depressions of 96-well microdilution plates and grown overnight without shaking. Bacteria were pelleted by centrifugation, washed once in 50 mM Tris hydrochloride (pH 7.6)–5 mM $MgCl_2$, and then suspended in 20 μl of the same buffer containing 20 mg of lysozyme per ml. Cells were lysed by three freeze-thaw cycles as described previously (30). Polysialic acid was detected in 20-μl samples by radial immunodiffusion. Positive and negative control strains were included in every test.

Electron microscopy. Antibody-stabilized and ruthenium red-counterstained polysialic acid capsules were visualized by transmission electron microscopy as described previously (35).

Sialyltransferase assays. Membrane pellets prepared by

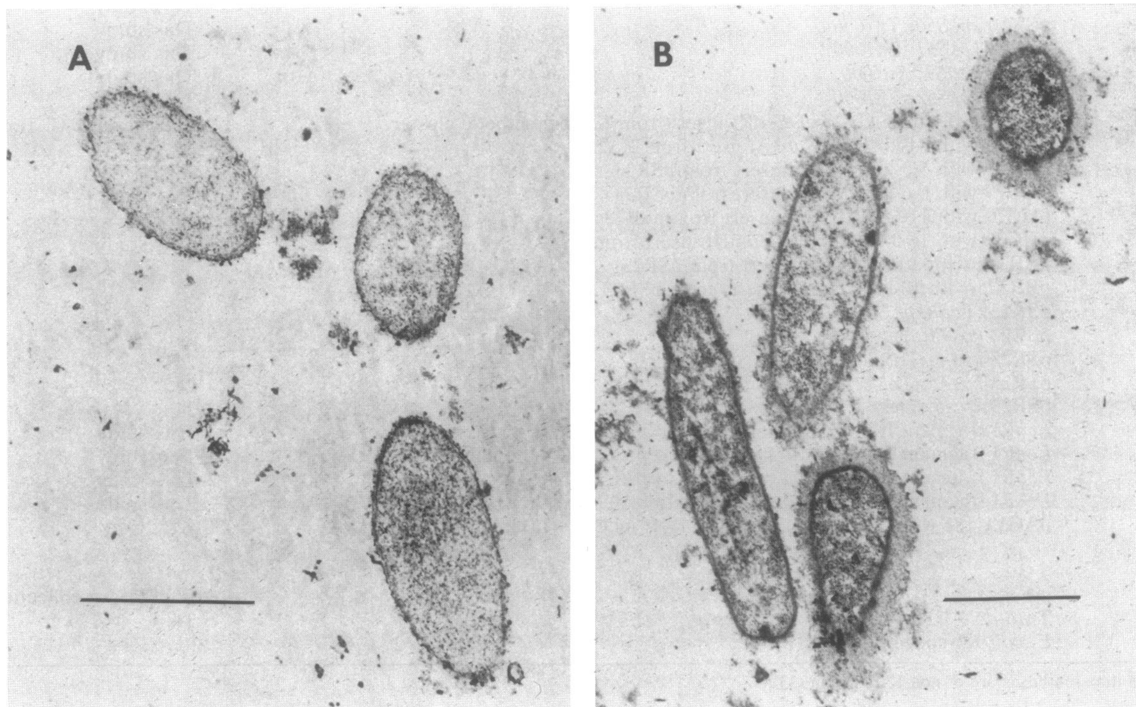

FIG. 1. Polysialic acid capsule morphology in *E. coli* harboring a cloned *kps* gene cluster. (A) HB101; (B) HB101(pSX48). Bar, 1 μm.

centrifuging sonic extracts were used as the source of enzyme. Buffers, fractionation conditions, substrates, and detection of products were described in a previous publication (33), with the following modifications. Membranes (300 to 600 μg of protein) were incubated at 33°C in a total volume of 120 μl, which contained 0.6 μCi of CMP-[^{14}C]sialic acid (7,000 dpm/nmol) and 900 μg of colominic acid, when exogenous sialyltransferase activity was measured. At 10-, 20-, 30-, 60-, and 90-min intervals, 20-μl samples were spotted onto Whatmann 3 MM filter paper and chromatographed as described before (33). Sialic acid transferred to endogenous membrane acceptors (endogenous sialyltransferase) or to endogenous plus exogenous acceptors (exogenous sialyltransferase) was quantitated by liquid scintillation spectrometry; initial rates were estimated graphically, and activity was expressed as nanomoles of sialic acid transferred per unit of time.

CMP-sialic acid synthetase assay. Activating enzyme was assayed in soluble extracts as described previously (33).

Endo-N purification. Poly-α-2,8-sialic acid depolymerase (endoneuraminidase [Endo-N]) was purified from lysates of K1F grown on EV1 by a modification of a previously published procedure (9). Briefly, after concentration of the lysate by ammonium sulfate precipitation, the sample was fractionated on DEAE-Sephacel in 10 mM Tris hydrochloride, pH 7.6, with a 0 to 0.4 M linear sodium chloride gradient. Endo-N eluting at 0.15 M salt was concentrated by ammonium sulfate, dialyzed against 50 mM potassium phosphate, pH 7.2, and fractionated on a Sephacryl-300 column. Endo-N eluted near the void volume, and enzyme-positive fractions were again concentrated by salt precipitation, dialyzed against 10 mM sodium phosphate, pH 6.8, and batch adsorbed with hydroxyapatite. Endo-N does not adsorb under these conditions, while all other contaminating proteins bind and are removed by centrifugation. Approximately 1 mg of electrophoretically pure Endo-N is recovered from 6 liters of phage lysate. One unit of Endo-N degraded 1 μg of colominic acid (Sigma) in 1 min at 37°C (20).

Gene library construction and DNA manipulations. Cosmids pSX48 and pSX49 were isolated from a chromosomal library of EV1 fragments cloned into the *Bam*HI site of pHC79. Details of genomic DNA and plasmid isolation, DNA sizing, restriction endonuclease digestion, alkaline phosphatase treatment, ligation, in vitro packaging into lambda particles, and infection of HB101 are given in reference 11. Ap^r transductants were screened for production of the polysialic acid capsule by replica plating to selective medium seeded with K1F phage. Sensitive colonies were isolated at a frequency of 10^{-2} and were recovered from master plates for further characterization.

RESULTS

Polysialic acid capsule morphology in *E. coli* harboring a cloned *kps* gene cluster. It has been assumed that the capsules produced in *E. coli* K-12 hosts by cloned *kps* clusters are morphologically similar to capsules in natural K1 isolates from which the *kps* genes were originally isolated (23). To demonstrate the validity of this assumption experimentally, transmission electron microscopy was used to visualize antibody-stabilized capsules in *E. coli* HB101 (Table 1). HB101(pSX48) expressed a polysialic acid capsule that extended approximately 100 nm from the cell surface (Fig. 1B). This size capsule was previously identified in natural K1 isolates (35). The apparent capsule heterogeneity may represent real differences in the population or may be an artifact

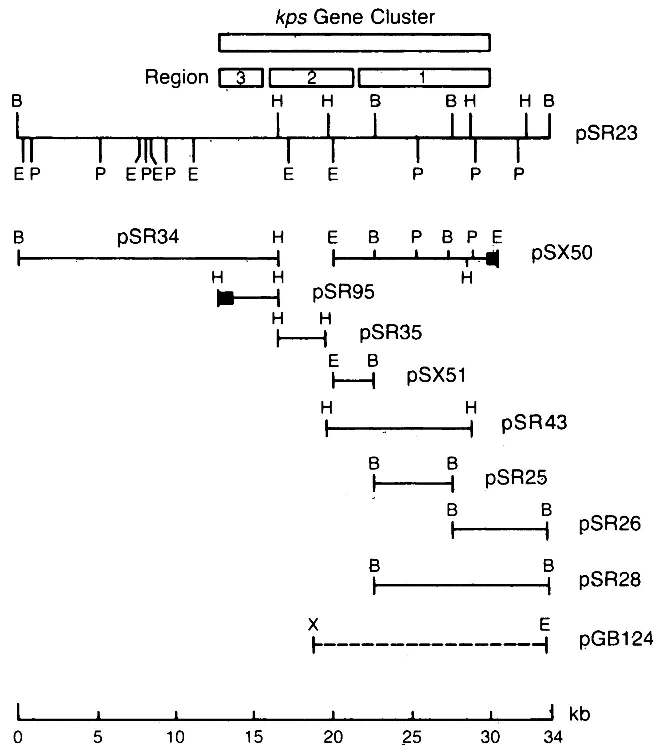

FIG. 2. Derivation of *kps* gene cluster subclones. A description of the *kps* cluster and its three functional regions (open boxes above pSR23) is given in the text. Plasmids designated by a pSR number are derived from pSR23; those with a pSX number were isolated from pSX49 (Table 1). Restriction sites were mapped in pSX50 and were identical to a corresponding region of pSR23, allowing placement of pSX50 and pSX51 as shown. In all other subclones, only the restriction sites used in ligating *kps* fragments to vector DNA (Table 1) are shown. A plasmid indicated by a dashed line was derived from the K7 polysaccharide gene cluster; its placement relative to pSR23 is based on its ability to complement region 1 mutations (16). Closed boxes indicate fragments of vector DNA (Table 1). Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I.

of sample preparation, since similar heterogeneity has also been observed in some natural K1 isolates (35). Capsules were missing in HB101 not containing plasmid (Fig. 1A). We conclude that cloned *kps* genes produce a capsule that is morphologically similar or identical to capsules produced by chromosomal *kps* genes in natural K1 isolates. Conclusions reached from analysis of *kps* genes expressed in laboratory strains of *E. coli* are thus likely to be relevant to clinically important *E. coli* K1.

Structure of the *kps* gene cluster. Figure 2 shows a restriction map of the K1 cosmid pSR23 (24). The region from 11 to 34 kb is nearly identical to a similar region in the restriction map of pKT274, a plasmid isolated by Echarti et al. (4) and shown to confer a K1$^+$ phenotype in laboratory strains of *E. coli*. The approximately 20-kb *kps* cluster in these cosmids also is present in pSX48 and pSX49 (E. Vimr, unpublished observations), cosmids which contain inserts from EV1 (Table 1). The similarity in restriction sites among these four plasmids makes it likely that the *kps* cluster in *E. coli* K1 strains is highly conserved. This conclusion is consistent with genetic evidence that the region contains information for polysialic acid capsule synthesis (4, 24).

The boxes over pSR23 in Fig. 2 indicate three functional regions of the *kps* cluster, as suggested by Timmis et al. (25).

TABLE 2. Phenotypes of *E. coli* K1 acapsular mutants

Strain	Relevant genotype	Sialyltransferase sp act (nmol/h per mg of membrane protein)[a]		CMP-sialic acid synthetase sp act (nmol/h per mg of soluble protein)[a]	Polysialic acid (radial immunodiffusion)	Proposed biochemical or genetic defect
		Exogenous	Endogenous			
PA360	Null	<0.1[b]	<0.1[b]	<0.1[b]	−	*kps* cluster not present
EV1	Wild type	18.6 ± 4.3	5.2 ± 2.1	19.1 ± 1.3	+	None
EV5	*neuA22*	14.5 ± 5.2	9.2 ± 3.8	0.9 ± 0.2	−	CMP-sialic acid synthetase
EV7	*neuA40*	16.3	6.4	0.7	−	CMP-sialic acid synthetase
EV33	*neuA26*	20.1	13.7	0.2	−	CMP-sialic acid synthetase
EV19	*neu-41*	7.9	2.5	10.7	−	Sialic acid synthesis
EV24	*neuB25*	11.2	3.7	6.7	−	Sialic acid synthesis
EV32	*neu-43*	24.1	18.0	10.8	−	Sialic acid synthesis
EV3	*kps-44*	23.7	<0.5	10.0	+	K1 assembly or translocation
EV4	*kpsT21*	10.3 ± 2.5	<0.5[c]	18.8	+	K1 assembly
EV11	*kps-23*	3.0 ± 0.8	<0.5[c]	27.7 ± 2.8	+	Sialyltransferase
EV14	*kps-42*	26.6	1.9	7.4	+	K1 assembly or translocation
EV15	*kpsS25*	18.1 ± 4.8	<0.5[c]	22.7	+	K1 assembly or translocation
EV93[d]	*kpsC30*::Tn*10*	36.6 ± 6.7	<0.5[c]	ND[e]	+	K1 assembly or translocation
EV94[d]	*kpsS31*::Tn*10*	42.8 ± 5.2	<0.5[c]	ND	+	K1 assembly
EV95[d]	*kpsT32*::Tn*10*	33.5 ± 6.3	<0.5[c]	ND	+	K1 assembly
EV13	*kps-24*	2.4 ± 0.6	<0.5[c]	0.1[b]	−	Regulatory gene mutation

[a] ± SEM, where n = two to four separate experiments. Other values are from a single experiment.
[b] Values were not significantly different from zero.
[c] Values were significantly different from zero.
[d] Mutations are in EV36 background (Table 1).
[e] ND, Not done.

Regions 1 and 3 are thought to function in polysialic acid membrane translocation and postpolymerization processing, respectively (3, 25). Region 2 is believed to encode enzymes for sialic acid synthesis, activation, and polymerization (3, 25). These three regions are made up of 17 kb of DNA in pSR23 and currently define the *kps* gene cluster (Fig. 2).

Plasmids shown below pSR23 in Fig. 2 represent subclones of either pSR23 or pSX49 (Table 1). The dashed line represents pGB124 which carries region 1 genes from the K7 polysaccharide gene cluster (16) that encodes synthesis of an *N*-acetylmannosaminuronic-glucose capsule (27). None of the subclones confers a K1$^+$ phenotype when transformed into *E. coli* K1 null strains, consistent with the previous conclusion that multiple functions are required for polysialic acid expression (23). With the exception of the genes encoding CMP-sialic acid synthetase (28) and a 60-kilodalton (kDa) periplasmic protein (22), none of the other genes in the *kps* cluster has been identified conclusively.

Characterization of *kps* and *neu* chromosomal mutations. To perform a detailed functional investigation of the *kps* cluster, we carried out complementation analyses of chromosomal mutations with the plasmids shown in Fig. 2. Table 2 summarizes relevant phenotypes of the mutants used as recipients for this investigation. The characterized mutants fall into several categories based on phenotypic comparison with EV1 and its null parent, PA360. Mutants represented by EV5 are defective in CMP-sialic acid synthetase and cannot activate sialic acid for participation in polymerization reactions in vivo. Another category of mutants, represented by EV24, has no obvious defects in activation or polymerization functions in vitro but fails to express polysialic acid in vivo (Table 2). These mutants appear to have defects in sialic acid synthesis. This conclusion is supported by observations that EV24 growing in batch culture becomes K1F^s in the presence of exogenously added sialic acid (data not shown). A broad category of mutants was recognized based on defects in endogenous sialic acid polymerization (Table 2). These mutants have essentially wild-type sialyltransferase activity when supplied with exogenous colominic acid acceptors, but have low or barely detectable endogenous activity (Table 2). One additional mutant, EV13, appeared to have a pleiotropic defect, as reported previously (33). Unlike mutants in the first two categories, polymerization-defective mutants still produced immunoreactive polysialic acid (Table 2). Polymeric material is apparently not present at the cell surface in these mutants, which explains their K1F^r phenotype. Rocket immunoelectrophoresis of soluble extracts prepared from the polymerization-defective mutants confirmed the intracellular localization of polysialic acid (E. Vimr and F. Troy, unpublished observations). Taken together, these results indicate that the complex phenotypes of these strains are due to mutations in distinct loci within the *kps* cluster. The mutants include strains with spontaneous chromosomal mutations as well as those generated by insertion mutagenesis (Table 1).

Map position of *kpsS*. To construct a detailed fine-structure map of the *kps* cluster, we began by digesting pSX49 to completion with *Eco*RI and randomly ligating fragments into pBR329 for transformation into EV15 (*kpsS25*). Ap^r transformants were selected on agar plates containing anti-polysialic acid antibodies. Figure 3 shows the results obtained for a positive complementation response. The evident precipitin halo around transformed EV15 is a strong indication of cell surface polysaccharide. Two halo-positive subclones were isolated; as expected, both were K1F^s, indicating that the polysialic acid capsules detected immunologically served as K1F phage-specific receptors. The pSX49 subclones in these isolates contained identical 10.5-kb *Eco*RI inserts, as shown for pSX50 in Fig. 2. This plasmid has the potential to carry over half the genetic information encoded by *kps* (Fig. 2), yet pSX50 transformed into strains EV4, EV5, EV11, EV13, and EV24 did not yield halo-positive bacteria, demonstrating that the mutations in these strains (Table 2) may not map within the 10.5-kb *Eco*RI fragment carried on pSX50.

To further define the map position of *kpsS*, we transformed EV15 with plasmid subclones shown in Fig. 2. The

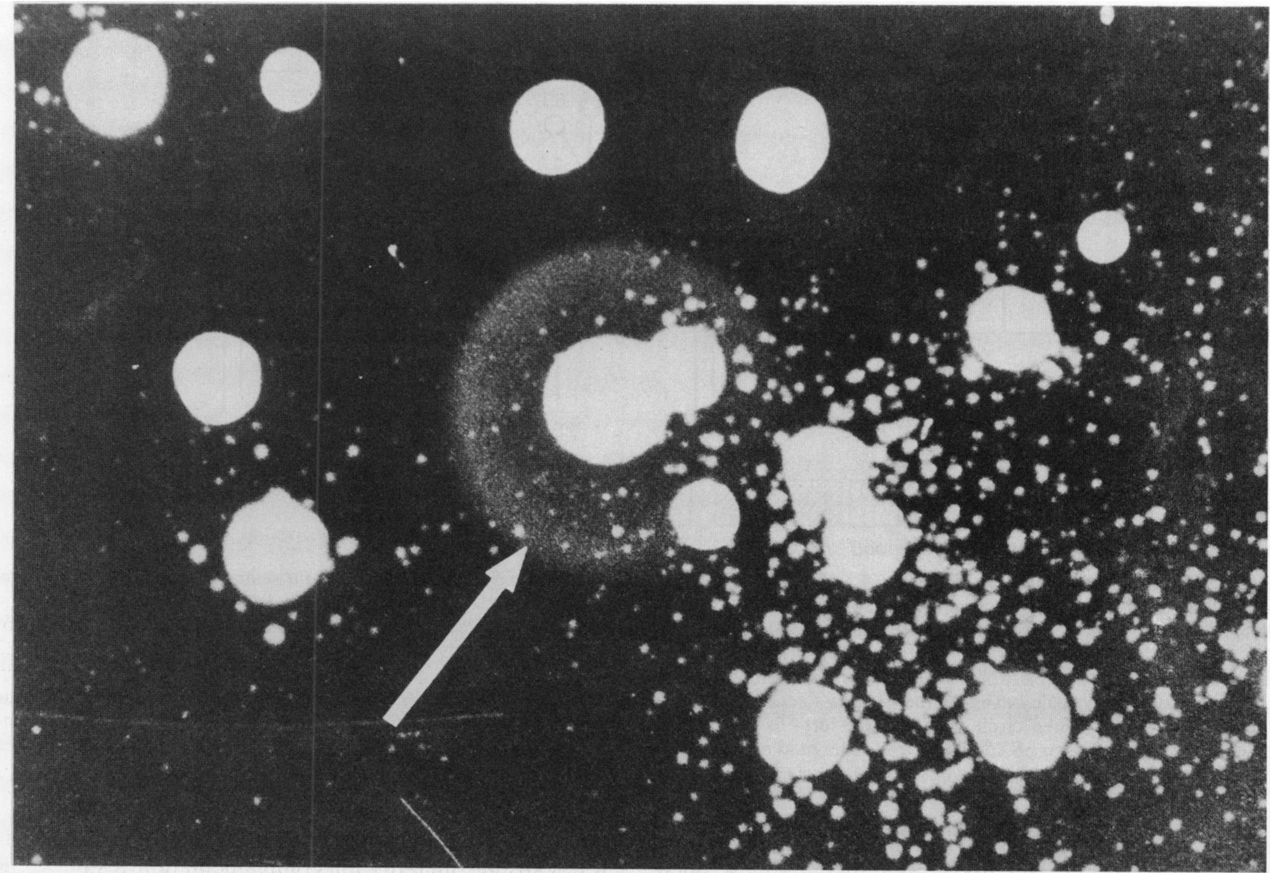

FIG. 3. Illustration of halo test scoring in vivo complementation. Strain EV15 was transformed with plasmids harboring random *Eco*RI fragments from pSX49 by selecting Ap^r. Arrow identifies periphery of the precipitin halo that is indicative of polysialic acid capsule production. Small colonies are Ap^s satellites; large Ap^r colonies show halo-negative phenotype of EV15. Magnification, ×2.5.

results of this complementation analysis are shown in Table 3. A 2.7-kb *Eco*RI-*Bam*HI fragment subcloned from pSX50 to yield pSX51 (Fig. 2) also complemented EV15 (Table 3), indicating that *kpsS25* mapped in *kps* region 1. In addition to complementation by pSX50 and pSX51, EV15 was complemented by pSR25, pSR28, and pSR43 (Table 3). However, pSR25 and pSR28 did not yield K1F^s isolates. This pattern of "pseudocomplementation" was observed with other plasmid-mutant pairs shown in Table 3 (asterisks). It is not clear whether these plasmids lead to increased cell death, with subsequent release of immunoreactive material, or whether expression of certain polypeptides encoded by high-copy-number plasmids causes polysialic acid leakage. That strains with the pseudocomplementation phenotype were K1F^r indicated that the polysialic acid detected by halo formation was incapable of acting as a phage receptor and for this reason may not be cell associated. A complementation pattern similar to that of EV15 was observed with EV94 (Table 3), suggesting that *kpsS31*::Tn*10* and *kpsS25* are allelic mutations. On the basis of these complementation results, we concluded that *kpsS* mapped in the 2.7-kb *Eco*RI-*Bam*HI fragment carried on pSX50.

To define more precisely the map position of *kpsS*, we used a positive selection for Tn*1000* insertions into pSX50

TABLE 3. Complementation of *E. coli* K1 acapsular mutants transformed with DNA fragments from K1 and K7 gene clusters

Mutant	Complementation[a] by:									
	pSR95	pSR35	pSR43	pSR28	pSR25	pSR26	pSX50	pSX51	pSR34	pGB124
EV4	+	−	−	−			−		−	
EV5	−	+	−	−			−		−	
EV11	−	−	−	−			−		−	−
EV13	+	−	−	−			−		−	
EV15	−	−	+	+*	+*	−	+	+	−	+
EV24	−	−	−	−			−		−	
EV93	−	−	+	+	+*	+*	+	−	−	+
EV94	−	−	+	−			+	+	−	+
EV95	+	−	−	−			−	−	−	−

[a] +, Polysialic acid was present on the cell surface and reacted with antipolysialic acid antibodies in a halo test and cells were sensitive to K1F. −, Cells were halo negative and K1F^r. * Cells were halo positive but K1F^r. A blank space indicates that the transformation was not performed.

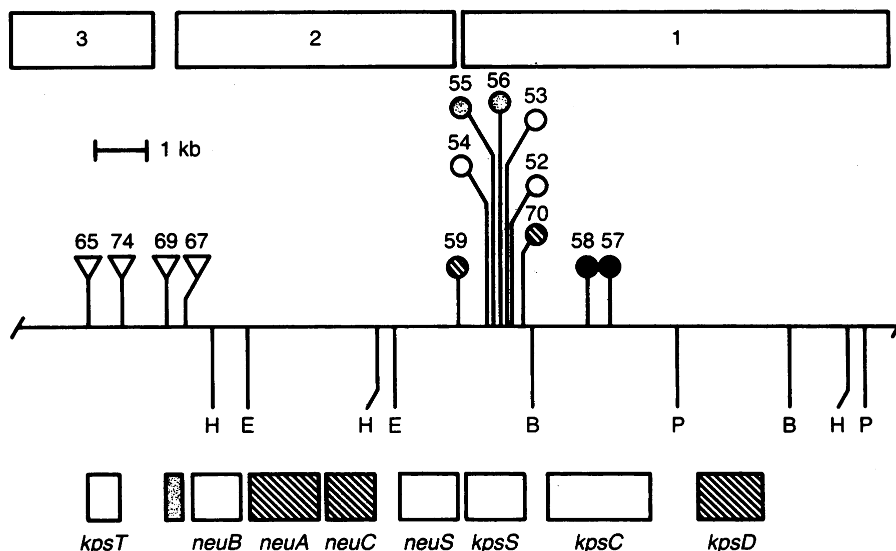

FIG. 4. Genetic organization of *E. coli* K1 polysialic acid gene cluster. The *kps* cluster and its partial restriction map are shown, with functional regions indicated by boxes 1, 2, and 3. Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I. Triangles indicate Tn*5* insertions (pSR numbers shown) which minimally define *kpsT* and an undesignated genetic locus (stippled box). Gene loci indicated by hatched boxes are drawn to scale based on previously published information (*neuA* and *neuC* [formerly *neuS*; 22a], reference 28; *kpsD*, reference 22a). Circles represent Tn*1000* insertions (pSX numbers shown). Closed circles were isolated in a cross between pSX50 and EV93 and define *kpsC* whose size is drawn to scale based on the 77-kDa size of its encoded polypeptide (22a). Hatched circles represent random Tn*1000* insertions into pSX51 (see text). Open circles represent insertions selected in a cross between pSX50 and EV15, and stippled circles represent those selected in a cross between pSX50 and EV94. The map position of *neuB* is based on transduction data (Table 6). The indicated sizes of *neuB*, *neuS*, and *kpsS* are approximations based on the relative map positions of these genes to other markers and Tn*1000* insertions.

that specifically blocked complementation (see Materials and Methods). The Tn*1000* insertions shown in Fig. 4 (open circles) were isolated in a cross between pSX50 and EV15. Transformation of these pSX50::Tn*1000* plasmids into EV94 failed to complement this mutant, further indicating that EV15 and EV94 contain alleles of *kpsS*. Additional evidence for this conclusion was obtained by mapping Tn*1000* insertions into pSX50 from a cross with EV94 (Fig. 4, stippled circles). These results demonstrated that EV15 and EV94 define a gene near coordinate 22 kb on the pSR23 restriction map (Fig. 4). Since both mutants were complemented by pGB124 (Table 3), the mutations in these strains define a region 1 gene that is apparently conserved among different polysaccharide gene clusters. Therefore, the *kps* designation is used instead of "*neu*."

Map position of *neuA*. Strain EV5 (*neuA22*) contains a defect in CMP-sialic acid synthetase (Table 2) (33) and was only complemented by pSR35 (Table 3). This result was expected from previous results (28) and confirmed that *neuA* mapped to the 3.3-kb *Hin*dIII insert of pSR35 (Fig. 4). Based on phenotypic similarity of EV5 to EV7 and EV33 (Table 2), we conclude that these mutants contain defects in the sialic acid-activating enzyme, CMP-sialic acid synthetase, encoded by *neuA*.

Map position of *kpsT*. pSR95 complemented the acapsular mutants EV4, EV13, and EV95 (Table 3), suggesting that the *kps* mutations in these strains map in the 2.7-kb *Hin*dIII fragment carried on pSR95 (Fig. 2). Evidence that the pleiotropic phenotype of EV13 (Table 2) is distinct from the phenotype caused by *kpsT* mutations in EV4 and EV95 is given below.

A set of Tn*5* insertions maps in the 6-kb *Eco*RI fragment of pSR23 (Fig. 4, triangles). Isolation and characterization of these insertions has been described previously (24). *Eco*RI fragments bearing the various insertion mutations were subcloned into pACYC184 (Table 1) and transformed into K1 acapsular mutants for complementation testing. pSR65 and pSR74 did not complement EV4 or EV95 (Table 4), in contrast to pSR67 and pSR69 which gave halo-positive and K1F[s] transformants. These results map *kpsT* near coordinate 14 kb of pSR23 and define the minimum size of this gene (Fig. 4). We have used the *kps* designation because of the phenotypes of EV4 and EV95 (Table 2) and the map position of *kpsT* within region 3 of the cluster.

As originally isolated in pSR23 (Table 1), the Tn*5* insertions shown in Fig. 4 yielded two phenotypic classes of acapsular mutants (24). Strains harboring pSR23 derivatives which carried the Tn*5* insertions shown for pSR65 and pSR74 (Fig. 4) still produced intracellular polymer (24), results consistent with the phenotype of *kpsT* mutants (Table 2). In contrast, strains harboring pSR23 with the Tn*5* insertions shown for pSR67 and pSR69 (Fig. 4) neither produced intracellular polymer nor were complemented by exogenous sialic acid (24). That EV24 (*neuB25*) was complemented by all subclones tested in Table 4 and by exogenous addition of sialic acid to the medium (see text above) suggested that another gene, distinct from *kpsT* and *neuB*, maps within the 6-kb *Eco*RI fragment of pSR23. The phenotype and map position of Tn*5* insertions within this potential locus are

TABLE 4. Complementation of *kpsT* and *neuB* chromosomal mutations

Strain	Relevant genotype	Complementation[a] by:			
		pSR65	pSR74	pSR67	pSR69
EV4	*kpsT21*	−	−	+	+
EV95	*kpsT32*::Tn*10*	−	−	+	+
EV24	*neuB25*	+	+	+	+

[a] Symbols are explained in footnote *a*, Table 3.

consistent with it being a region 2 gene and thus specific to K1 antigen biosynthesis. However, we have refrained (Fig. 4) from assigning a gene designation for this locus until additional information becomes available.

The complementation results summarized in Table 3 were not free of potential ambiguity. For example, pSR34 overlaps pSR95 (Fig. 2) and should have complemented EV4, EV13, and EV95. At present, it is impossible to conclude whether the failure of pSR34 to complement the defects in these strains is related to the presumably non-*kps* flanking DNA sequences on pSR34 or simply to vagaries in complementation of chromosomal mutations by high-copy-number plasmids. Two other mutants, EV11 and EV24, also were not complemented by any of the plasmids tested in Table 3.

Map position of *kpsC*. The complementation pattern for EV93 (*kpsC30*::Tn*10*) shown in Table 3 suggests that *kpsC* maps within the 5-kb *Bam*HI fragment carried on pSX50 (Fig. 2). This conclusion was consistent with observations that EV93 was complemented by the pSX50::Tn*1000* plasmids which defined *kpsS* (Fig. 4, open and stippled circles). We next selected insertions into pSX50 which failed to complement EV93 and which were subsequently mapped near coordinates 24 and 25 kb of pSR23 (Fig. 4, closed circles). The gene mapped by these results was designated *kps*, since it too was complemented by pGB124 (Table 3). The map position of *kpsC* is distinct from that of *kpsD*, a gene previously mapped to region 1 of the cluster which codes for a periplasmic protein required for production of cell surface polysaccharide (22).

Biochemical characterization of HB101(pSX51) and mapping *neuS*. The previous results document that mutations in EV4, EV15, EV93, EV94, and EV95 cause a similar biochemical phenotype in *E. coli* polysialic acid synthesis; that is, diminution of endogenous sialyltransferase in vitro with retention of at least a low level of polysialic acid synthesis in vivo. Most or all of the polymer synthesized by these mutants in vivo is not present at the cell surface. Thus, while the precise biochemical defects in these mutants are not known (Table 2), it seemed likely that positive complementation responses (Table 3) would require restoration of endogenous sialyltransferase. This prediction was tested by measuring sialyltransferase in membrane fractions of EV4 (pSR95), EV15 (pSX50 and pSX51), EV93 (pSX50), EV95 (pSR95), and EV94 (pSX50). With no exceptions, each of the plasmids restored endogenous activity to a wild-type level (data not shown). The same results were obtained when pGB124 was expressed in EV15 or EV93, demonstrating that complementation by heterologous DNA was identical to complementation by homologous *kps* sequences and further supporting the functional homology of region 1 genes that are found in different polysaccharide gene clusters (4, 16, 17, 22). As expected from the results in Table 2, exogenous sialyltransferase was not dependent upon, nor was it augmented by, plasmid presence. The results also indicate that failure to express polymer at the cell surface in vivo is correlated with the in vitro phenotype.

As controls for the in vitro assays, we transformed *E. coli* HB101 with the plasmids described above and measured endogenous and exogenous sialyltransferase in membranes of the resulting transformants. *E. coli* K1 null strains are presumed to lack *kps* DNA sequences (33), and since multiple functions appear to be necessary for detection of endogenous sialyltransferase, we observed no endogenous activity in HB101 harboring these plasmids. However, both HB101(pSX50) and HB101(pSX51) had detectable exogenous activity. Since pSX51 is derived from pSX50, we

TABLE 5. Exogenous sialyltransferase activity by *neuS*

Strain	Relevant *kps* genotype	Exogenous sialyltransferase activity ([^{14}C]sialic acid cpm transferred to colominic acid ± SEM)[a]	
		− Endo-N	+ Endo-N
HB101	Null	19 ± 2	19 ± 1.5
HB101(pSX51)	*neuS*$^+$ *kpsS*$^+$	1,059 ± 157	19 ± 2
LE392(pSX51)	*neuS*$^+$ *kpsS*$^+$	1,721 ± 162	ND
C600(pSX51)	*neuS*$^+$ *kpsS*$^+$	1,755 ± 289	ND
HB101(pSX59)	*neuS1*::Tn*1000* *kpsS*$^+$	19 ± 2	ND
HB101(pSX70)	*neuS*$^+$ *kpsS52*::Tn*1000*	1,030 ± 226	ND

[a] Reaction mixtures contained 250 μg of membrane protein in a total volume of 15 μl. After 30 min of incubation at 33°C, 7.5-μl samples were developed chromatographically for quantitation of sialyltransferase, as described in Materials and Methods (− Endo-N) or treated with 10 U of Endo-N for 30 min prior to chromatography (+ Endo-N). Results of three to four separate experiments are given. ND, Not done.

concluded that the 2.7-kb insert in pSX51 contains a functional sialyltransferase gene. This gene must be different from *kpsS*, which is also present on pSX51, because a Tn*10* insertion into *kpsS* retained exogenous activity (EV94, Table 2). Further evidence that the sialyltransferase gene is distinct from *kpsS* was suggested by complementation of EV15 (*kpsS25*) with pGB124 (Table 3) and by the observation that pGB124 expressed in HB101 had no detectable exogenous activity. From these results we may infer that a gene, designated *neuS*, maps toward the *Eco*RI site of pSX51 (Fig. 4) and that expression of pSX51 in HB101 leads to a membrane activity that is capable of transferring sialosyl residues from CMP-sialic acid to exogenous colominic acid acceptors.

To demonstrate that the sialyltransferase was synthesizing authentic polysialic acid, the product of the in vitro reaction was subjected to digestion with Endo-N. HB101(pSX51) added 1,059 cpm to colominic acid acceptors (Table 5), while the activity in HB101 not transformed with pSX51 was at a background of 19 cpm. The activity conferred upon HB101 by pSX51 was not strain specific, since similar activities were observed when the plasmid was transformed into *E. coli* laboratory strains C600 and LE392 (Table 5). Significantly, all of the radioactivity in HB101(pSX51) was sensitive to Endo-N digestion (Table 5), demonstrating that sialosyl units were transferred to polysialic acid chains with a minimum of 5 to 10 sialic acid residues, the minimum substrate for Endo-N (31). Further evidence consistent with *neuS* encoding sialyltransferase was obtained by isolating and mapping random Tn*1000* insertions into pSX51. One mutation was mapped as shown in Fig. 4 (cross-hatched circles) and was found to eliminate sialyltransferase when pSX59 was transformed into HB101 (Table 5). Another random Tn*1000* insertion in pSX51 was isolated which had no effect on sialyltransferase (pSX70) and which mapped in *kpsS* (Table 5; Fig. 4, cross-hatched circles). These results provide additional evidence that *kpsS* and *neuS* are distinct loci carried on pSX51. Taken together, the results strongly imply that *neuS* encodes sialyltransferase. The extent of sialosyl addition by the strains tested in Table 5 was 2 to 5% of wild-type activity. The relatively low activity of strains bearing pSX51, compared with wild type, may indicate that sialyltransferase functions in association with other *kps* gene products which are lacking in *E. coli* K1 null strains. Sialyltransferase expression from *neuS* in pSX51 might also be limiting at either the transcriptional or the translational

TABLE 6. Transduction mapping of *neuB*

Strain (relevant genotype)		% Cotransduction (no. scored)[a]	Approx distance (kb) between selected and unselected markers[b]
Donor	Recipient		
EV93 (*kpsC30*::Tn*10*)	EV24 (*neuB25*)	66 (30)	10.5
EV95 (*kpsT32*::Tn*10*)	EV24	92 (32)	1.5

[a] In each cross, Tc[r] transductants were streaked to selective medium, and single colonies were inoculated into wells of a microdilution plate for assay of intracellular polysialic acid as described in Materials and Methods.

[b] Physical distance was calculated by the formulas of Kemper (10) and Wu (36), with the arithmetic average shown. Each method gave values that varied 1.5 kb or less from the average.

TABLE 7. Transduction analysis of *kps* region 1 and 3 genes[a]

Strain (relevant genotype)		% Cotransduction (no. scored)	Approx distance (kb) between selected and unselected markers
Donor	Recipient		
EV94 (*kpsS32*::Tn*10*)	EV13 (*kps-24*)	43 (58)	21.5
EV95 (*kpsT32*::Tn*10*)	EV13	88 (59)	3.1
EV94	EV15 (*kpsS25*)	100 (12)	<1
EV95	EV15	60 (12)	13
EV94	EV11 (*kps-23*)	100 (30)	<1
EV95	EV11	73 (30)	8.1

[a] See footnotes to Table 6 for details of transduction analysis.

level, which could explain why EV11 (*ksp-23*) was not complemented by pSX50 (Table 3).

Transduction mapping of *neuB*. Since EV24 (*neuB25*) was complemented by the pSR23 subclones shown in Table 4, but not by any of the plasmids tested in Table 3, we tentatively concluded that *neuB* spans the *Hin*dIII site at coordinate 16.5 kb of pSR23 (Fig. 2). To map the *neuB* mutation by transduction, P1 phage were grown on EV93 and EV95 and used to transduce EV24 to Tc[r]. Transductants were then screened for the presence of immunoreactive polysialic acid as unselected marker (Table 6). EV24 does not synthesize polysialic acid due to a defect in sialic acid synthesis (Table 2), in contrast to donor strains EV93 and EV95 which produce immunoreactive polymer intracellularly. The levels of polysialic acid produced by these mutants were estimated by rocket immunoelectrophoresis at 50% of the wild-type strain, EV1 (52 μg of polysialic acid per mg of protein [33]). These observations suggested that the defect in EV24 would be epistatic to the donor Tn*10* insertions. Thus, any transductant that did not receive wild-type *neuB* from the donor would be phenotypically identical to EV24. As predicted from the map positions of *kpsC* and *kpsT* (Fig. 4) and from the suspected location of *neuB* (Table 3 and 4), the highest frequency of cotransduction was obtained with EV95 (*kspT32*::Tn*10*) donor (Table 6). By converting linkage data to physical distance (Table 6), we estimated that *neuB* mapped within 9 to 12 kb of *kpsC30*::Tn*10* and 1 to 2 kb of *kpsT32*::Tn*10*, results that are most consistent with *neuB* spanning the *Hin*dIII site at 16.5 kb in pSR23 (Fig. 4).

Transduction analysis of region 1 and 3 genes. To provide independent confirmation of region 1 and 3 gene order that was obtained by complementation analysis (Fig. 4), we carried out the series of transduction crosses shown in Table 7.

The pleiotropic mutation in EV13 (*kps-24*) prevented polysialic acid synthesis (Table 2) and could therefore be mapped by an extension of procedures described for mapping *neuB*. P1 phage grown on EV94 and EV95 were used to transduce EV13 to Tc[r]. Transductants were then assayed for intracellular polysialic acid as the unselected marker. The simplest interpretation of the results shown for these crosses is that *kps-24* maps to the leftmost position of region 3 (Table 7). The cross with EV94 donor generally supported this interpretation (Table 7); however, it is likely that the calculated physical distance between *kps-24* and *kpsS31*::Tn*10* overestimates the actual separation of these two mutations. It is apparent from these results that the pleiotropic phenotype of EV13 is not due to simple polarity, because the *kpsC30*::Tn*10* insertion in EV95, mapping to the region 2 side of *kps-24* (Fig. 4), did not have a pleiotropic phenotype (Table 2). We believe it is more likely that *kps-24* defines a regulatory gene controlling at least sialic acid activation and polymerization functions, as suggested by the results shown in Table 2. Work is in progress to define further the nature of this potential regulatory locus.

We next crossed the same donor phage preparations with EV15 (*kpsS25*) and EV11 (*kps-23*), selecting Tc[r]. As expected for a cross between allelic mutations and from the number of transductants scored, 100% linkage was observed when EV15 was transduced with phage from EV94 (Table 7). The same result was obtained with EV11 as the recipient (Table 7), suggesting that *kps-23* maps within or very near to *kpsS25* and may also be allelic. However, EV11 was not complemented by pGB124 (Table 3), suggesting that *kps-23* may map in *neuS* (Fig. 4), consistent with the EV11 phenotype (Table 2; see reference 33). When EV15 or EV11 was crossed with phage grown on EV95, approximately 70% linkage was observed (Table 7). This linkage was expected given the physical distance between *kpsT* and *kpsS* (Fig. 4). However, because both donor and recipient strains produce intracellular polymer (Table 2), we did not expect double mutants to have a polymer-negative phenotype. As described below, the result that strains with simultaneous defects in *kpsT* and *kpsS*, or in *kpsT* and *kps-23*, fail to produce intracellular polymer strongly suggests that at least some *kps* genes flanking biosynthetic region 2 may function in polysaccharide assembly rather than in translocation, as previously suspected.

DISCUSSION

General implications of the results to capsular polysaccharide assembly mechanisms. Synthesis of the polysialic acid capsule by *E. coli* K1 represents two problems of outstanding significance in biology. One is to explain how a long hydrophilic and negatively charged polymer synthesized at the inner membrane can efficiently traverse at least one lipid bilayer and be stably maintained at the cell periphery. Based on morphologic evidence (1), a possible solution to this problem suggested that extrusion of polymers was through membrane porelike structures that could represent adhesion zones in bacteria (2). Presumably, elongation could be coupled with extrusion through a limited number of adhesions between inner and outer membranes. For the case of polysialic acid synthesis, extrusion coupled with synthesis is conceptually unappealing because nascent chains are elongated by sialosyl additions to nonreducing termini (19, 26). Since the available evidence indicates an inner membrane

localization for polysialic acid polymerase (sialyltransferase complex) (29), it is unlikely that polymer extrusion could be coupled with its synthesis or that sialosyl oligomers could be transported to the cell surface for assembly. If extrusion is uncoupled from synthesis, then polysaccharide transport functions may exist to facilitate the membrane translocation event. Recent work by Silver et al. (22) and Roberts et al. (16, 17) indicated that DNA sequences mapping near the biosynthetic region 2 genes for polysialic acid and other capsular polysaccharides in *E. coli* are required for polysaccharide cell surface production. These flanking sequences encode polypeptides that could function in polysaccharide translocation, leading to the suggestion that structurally diverse polysaccharides may use homologous polypeptides in their translocation mechanism (17). Evidence for this suggestion is based on hybridization between flanking regions of different polysaccharide biosynthetic loci (17). Further support for common translocation functions was indicated by complementation of region 1 mutations by wild-type DNA from several structurally distinct biosynthetic clusters (16, 17). Our results demonstrating complementation of *kpsS* and *kpsC* by pGB124 (Table 3) extend these observations by showing that chromosomal mutations are complemented in *trans* by DNA from a K7 polysaccharide gene cluster. However, the mechanism to explain how the functionally homologous polypeptides encoded by these regions could facilitate translocation of structurally diverse membrane polysaccharides is made no clearer by these observations.

Another unsolved problem in K-antigen synthesis is the polymerization mechanism itself. The lack of information on this mechanism will limit a solution to the more general problem of polysaccharide translocation. In the polysialic acid system, it appears necessary to define the molecular structure of the glycosyltransferase complex which carries out polysialic acid assembly in vivo.

Unlike lipopolysaccharide O-antigen chains, proteins, fatty acids, and any other polymers synthesized by monomer or repeat unit additions to a chemically activated end, sialosyl additions to nascent polysialic acid involve growth at the nonreducing end of the molecule potentially distal to biosynthetic enzyme (19). Therefore, at this level of synthesis, polysialic acid assembly more closely resembles biosynthesis of lipopolysaccharide core oligosaccharide or glycogen. It has been suggested that the mechanism used by O antigen may reflect the more complex requirements for polymer assembly in a membrane-associated system (15). Presumably, enzymes which catalyze transfer to the reducing end of nascent chains would not be required to maintain contact with nonreducing termini. We have suggested that sialyltransferase may be a processive enzyme catalyzing transfer of sialosyl residues to the nonreducing termini of nascent chains in which the reducing termini remain anchored to endogenous membrane acceptors (E. R. Vimr, B. L. Bassler, and F. A. Troy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K-152,). This hypothesis suggests that both ends of a growing chain will be constrained near the synthetic apparatus and is therefore incompatible with extrusion being coupled with synthesis. In this communication, we have presented results of genetic and biochemical experiments which strongly implicate multiple gene loci in the polysialic acid assembly mechanism. The data indicate the existence of a single sialyltransferase gene, designated *neuS*; however, the phenotypes of several other mutants defined by genes *kpsT*, *kpsD*, *kpsS*, and *kpsC* may argue for sialyltransferase functioning within a multicomponent set of membrane proteins. Definitive evidence that some of these proteins participate in polysialic acid assembly, or in directing polymer translocation to the outer surface, as suggested by Timmis et al. (25), will require further experimentation.

Implications of the results to *kps* gene cluster organization and function. In previous studies on *kps* gene organization, it was suggested that the *kps* cluster contained a central region (region 2) that encoded functions for sialic acid metabolism and which was flanked on either side by genes thought to function in postpolymerization processing (region 3) (3) and polymer translocation (region 1) (3, 25). One phenotype of region 2 mutations is the absence of polymeric material in vivo, in contrast to mutations in regions 1 and 3 which prevent polymer cell surface expression but do not block polymer synthesis (3, 22). The previous mapping of *neuA* to region 2 (28) and results in this communication extend these observations by identifying and mapping additional region 2 genes whose products function in sialic acid synthesis and polymerization. The results summarized in Fig. 4 demonstrate that all of the region 2 sialic acid-specific genes may now be defined in terms of their map position and probable biochemical function. Results shown in Table 2 also confirm that mutations in regions 1 and 3 cause intracellular accumulation of polysialic acid.

Region 2 gene order, reading left to right on pSR23, is *neuB* (sialic acid synthesis), *neuA* (sialic acid activation), and *neuS* (sialic acid polymerization), suggesting a genetic structural organization for a potential polysialic acid biosynthetic operon (Fig. 4). Another region 2 gene mapping between *neuA* and *neuS* and coding for a 45-kDa polypeptide (*neuC*) has also been identified and appears to function in sialic acid synthesis (24). This conclusion is supported by observations that strains bearing Tn*5* insertions in this gene are complemented by exogenous sialic acid (24), results similar to the observations with EV24 (*neuB25*) (see text). Boulnois et al. (3) observed that Tn*1000* insertions into pKT274 in a region of the *kps* cluster which should encode the 45-kDa protein were not complemented by exogenous sialic acid. These workers did report complementation by sialic acid addition of an insertion in pKT274 which mapped at the same position as *neuB* (3). A suggestion (3) that the region of pSR23 coding for the 45-kDa protein may be altered compared with pKT274 does not seem likely given the similarity in restriction maps of these two K1$^+$ plasmids (Fig. 2). An explanation for this apparent discrepancy is presently unavailable.

The possibility that more than one gene is required for sialic acid synthesis seems likely given the proposed bacterial pathway (33). However, previous suggestions that sialic acid aldolase could function in this pathway (3, 4, 24, 25) are untenable because *nanA* (aldolase-deficient) strains produce cell surface polysialic acid (32). Thus, we envision *neuB* or *neuC* to code for the sialic acid synthase which condenses *N*-acetylmannosamine and phosphoenolpyruvate (33). At least one other gene product would then be required to convert *N*-acetylglucosamine to *N*-acetylmannosamine as a synthase substrate in this pathway (6, 33). Preliminary unpublished experiments to detect condensing activity in extracts of EV24 support the idea that *neuB* may encode synthase. Interestingly, sialic acid synthase in mammals uses *N*-acetylmannosamine-6-phosphate rather than the unphosphorylated sugar that is apparently used by bacteria for sialic acid synthesis (33). Hence, sialic acid synthase may be an attractive target for therapeutic agents that could specifically block sialic and polysialic acid synthesis in a variety of

microbial pathogens without affecting host sialic acid metabolism.

The functions of region 1 and 3 genes are less clear. Our results show that *kpsT* and a potential regulatory locus defined by the defect in EV13 (Tables 2 to 4 and 7) map to region 3, while *kpsC*, *kpsS*, and *kpsD* map in region 1 (Fig. 4). Region 1 genes *kpsS* and *kpsC* were complemented by pGB124 (Table 3), results consistent with those of Roberts et al. (16, 17), who showed that region 1 genes from different polysaccharide gene clusters were freely exchangeable. Roberts et al. (16, 17) concluded that region 1 genes functioned in translocating polymer to the outer membrane and that the observed complementation of region 1 genes by heterologous DNA indicated common translocation mechanisms among the acidic K antigens. They further concluded, based on observing an altered electrophoretic migration of intracellular polysialic acid in region 3 mutants (3), that this region functioned in phospholipid attachment to or removal from polymeric reducing termini. However, the apparently altered molecule has not been characterized; thus, it is impossible to make any definite conclusion about the function of region 3 genes. Another phenotype of *kps* mutations in regions 1 and 3 is the greatly diminished endogenous sialyltransferase activity in vitro (Table 2). We can think of no compelling reason why a putative translocation defect, caused by a region 1 mutation, for example, would have this phenotype. It remains possible that some region 1 genes do not function directly in a translocation mechanism.

Further evidence that not all region 1 and 3 genes function as suggested previously (3, 4, 25) is based on results of transductions between donor strain EV95 and recipient strain EV15 (Table 7). The *kpsT32*::Tn*10* region 3 mutation in EV95 and the region 1 *kpsS25* mutation in EV15 both have accumulation of intracellular polysialic acid as a phenotype (Table 2). However, transductants were obtained which failed to synthesize detectable polysaccharide (Table 7). We believe that these polymer-negative transductants are double mutants carrying both donor and recipient mutations. The interpretation that region 3 genes function in postpolymerization processing and region 1 genes function in polymer translocation further suggests that such double mutants should still produce intracellular polymer, since the functions affected by these mutations would occur after polysaccharide synthesis. However, if *kpsT* and *kpsS* gene products function as nonpolymerase components of a glycosyltransferase complex, then double mutants might be expected to have an absolute block in polymer synthesis. If *kps-23* in EV11 is in fact an allele of *neuS*, as suggested by the results in Table 7, then the failure of *kpsT kps-23* double mutants to synthesize detectable polymer is further evidence that sialyltransferase functions as a complex. The observed complementation of some region 1 and 3 genes by heterologous K-antigen DNA could mean that K-antigen assembly requires common components that are not directly related to the type of sugar synthesis, activation, or polymerization activities encoded by region 2 genes. This interpretation suggests that there may be mechanistic steps common to the assembly of chemically diverse capsular polysaccharides, but that these reactions need not have a direct role in translocating polymers to the cell surface. Experiments are now in progress to determine the membrane orientation of region 1 and 3 gene products by isolating protein fusions to alkaline phosphatase and beta-galactosidase. These approaches will facilitate attempts to localize *kps* gene products that appear to be in low abundance (E. Vimr and S. Steenbergen, unpublished observations).

If some region 1 genes do not function in polymer translocation, what other functions would be consistent with previous observations? Since many of the acidic K-antigen gene clusters apparently map near *serA* on the *E. coli* chromosome (13, 33), and because of the likely involvement of endogenous acceptor (18, 26), phospholipid (21), undecaprenyl phosphate (12), and porins (14, 26) in the assembly process, it is possible that region 1 and 3 gene products participate in common reactions of K-antigen assembly. For example, if transfer of nascent chains to phospholipid were the terminal event in acidic polysaccharide assembly, then mutants with defects in this transferase process might be expected to accumulate soluble antigen. This is precisely a phenotype of region 1 and 3 mutations (Table 2) (16, 17, 25). That heterologous DNA from other K-antigen gene clusters is able to complement these defects is not inconsistent with this suggestion. Therefore, failure to observe polymer expression at the cell surface in region 1 mutants may not indicate a defect in polymer translocation per se, but simply some defect in the assembly mechanism. Thus, mutants with an intracellular polysialic acid phenotype vary from 10 to 50% of wild-type antigen detected by quantitative rocket immunoelectrophoresis (31; Vimr and Troy, unpublished observations), differences not detected by immunodiffusion or immunoelectrophoresis. These observations further imply that detection of antigenic material in an intracellular compartment is not necessarily evidence for a translocation defect and is insufficient in itself to make conclusions about defects in assembly or translocation processes. The possibility that sialyltransferase may function in complex with other *kps* gene products will complicate attempts to distinguish between assembly or translocation defects. The use of high-copy-number plasmids and insertion mutagenesis in many of these studies may also complicate analysis. We believe that construction of hybrid *E. coli* strains amenable to traditional genetic approaches (32, 33), as used in this study, will be important for further progress on the *kps* gene functions pertinent to regulatory, assembly, and translocation mechanisms.

The results we present demonstrate that we can now account for >50% of the *kps* cluster coding capacity (Fig. 4). Assuming that the remaining DNA codes for polypeptides with an average size of 50 kDa, then no more than five genes may await identification. If additional loci exist, then they must also map in regions 1 and 3 or be unlinked to the *kps* cluster as it is currently defined. We expect that at least some of these genes, if they exist, could also function in polymer assembly. Further progress in understanding the synthesis and regulation of polysialic acid may depend on identifying endogenous acceptor and determining at which stage phospholipid is added to polymer-reducing termini. Direct biochemical assays must then be devised to distinguish between assembly and translocation defects. We suggest that the eventual resolution of the polysialic acid assembly mechanism will have general significance to a variety of membrane polysaccharide biosynthetic systems and to the specific mechanism of polysaccharide membrane translocation.

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