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Thermoregulation of *kpsF*, the First Region 1 Gene in the *kps* Locus for Polysialic Acid Biosynthesis in *Escherichia coli* K1

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The *kps* locus for biosynthesis of the capsular polysialic acid virulence factor in *Escherichia coli* K1 contains at least two convergently transcribed operons, designated region 1 and regions 2 plus 3. On the basis of DNA sequence analysis, *kpsF* appeared to be a good candidate for the first gene of region 1 (M. J. Cieslewicz, S. M. Steenbergen, and E. R. Vimr, J. Bacteriol. 175:8018–8023, 1993). A preliminary indication that *kpsF* is required for capsule production is the capsule-negative phenotype of an *aphT* insertion in the chromosomal copy of *kpsF*. The present communication describes the isolation and phenotypic characterization of this mutant. Although transcription through *kpsF* was required for capsule production, complementation analysis failed to indicate a clear requirement for the KpsF polypeptide. However, since *E. coli* contains at least two other open reading frames that could code for homologs of KpsF, the apparent dispensability of KpsF remains provisional. DNA sequence analysis of 1,100 bp upstream from the *kpsF* translational start site did not reveal any open reading frames longer than 174 nucleotides, consistent with *kpsF* being the first gene of region 1. Since *kpsF* appeared to be the first gene of a region whose gene products are required for polysialic acid transport and because capsule production is known to be thermoregulated, primer extension analyses were carried out with total RNA isolated from cells grown at permissive (37°C) and nonpermissive (20°C) temperatures. The results revealed a potentially complex *kpsF* promoter-like region that was transcriptionally silent at the nonpermissive temperature, suggesting that thermoregulation of region 1 may be exerted through variations in *kpsF* expression. Additional evidence supporting this conclusion was obtained by demonstrating the effects of temperature on expression of the gene *kpsE*, immediately downstream of *kpsF*. Chloramphenicol acetyltransferase assays were carried out with constructs containing the *kpsF* 5′ untranslated region fused to a promoterless *cat* cassette, providing further evidence that *kpsF* is thermoregulated. Although the function of KpsF is unclear, primary structure analysis indicated two motifs commonly observed in regulatory proteins and homology with glucosamine synthase from *Rhizobium meliloti*.

Escherichia coli K1 is the leading cause of gram-negative neonatal meningitis in humans and a serotype frequently isolated from patients with urinary tract infections (17, 18, 27). One of the primary virulence factors associated with sepsis is the polysialic acid capsule, or K1 antigen, that both directly inhibits complement fixation and mimics the molecular structure of vertebrate polysialic acid (33, 36). Bacterial polysialic acids are homopolymers containing 50 to 200 α2,8-linked sialyl residues terminating at their reducing ends via esterification to phosphatidic acid, a modification which presumably anchors these chains to the outer membrane, thereby imparting a high net negative surface charge (4, 15, 41, 42). In addition to *E. coli* K1 and vertebrate animals, α2,8-linked polysialic acid is synthesized by group B meningococci, *Pasteurella haemolytica* A2, and *Moraxella nonliquefaciens* (36, 42), while group C *Neisseria meningitidis* produces an α2,9-linked capsule and *E. coli* K92 synthesizes a unique polysialic acid containing alternating α2,8 and α2,9 linkages (12, 21). We and others have been investigating the biosynthesis of polysialic acid in *E. coli* K1 and related systems (47). This knowledge is allowing us to better understand the molecular details of capsule synthesis, which may in turn suggest new avenues for therapeutic interventions.

A common theme from studies of many different exopolysaccharide biosynthetic systems is the clustering of genes encoding sugar-specific enzymes with genes coding for transport or modification functions (47). The *kps* locus for polysialic acid synthesis maps near *serA*, where the capsule locus is organized into three functional units: region 2 encodes the sugar-specific biosynthetic enzymes (sugar synthases, synthetases, and transferases) and is flanked on either side by region 1 or 3 that carries out the more general functions (see Fig. 1). Region 1 and 3 gene products are structurally as well as functionally homologous with those of other *kps* loci that direct synthesis of polysaccharides with subunits unrelated to sialic acid (6). Most of the known *kps* loci that have been mapped near *serA* direct synthesis of relatively low-molecular-mass, acidic, heat-labile polysaccharides collectively designated group or type II antigens to distinguish them from type I polysaccharides, with biosynthetic gene loci mapping near *his* (6). Type I polysaccharides are uncharged, heat-stable polymers containing generally more complex repeat units than type II molecules (6).

Although a great deal is known about the biosynthetic steps leading to polysialic acid, there is little information about the mechanism of transmembrane transport (translocation) or the possible functions of most region 1 gene products (47). Even less is known about mechanisms of genetic or environmental regulation of capsule synthesis. However, it is known that unlike their type I cohorts, type II capsules are not produced at temperatures below about 25°C (5, 28), a phenotype representing environmental control of capsule production with clear implications for the host-pathogen interaction (47). We are aware of only two reports in the literature concerning possible thermoregulation of type II capsules: one, involving the cold

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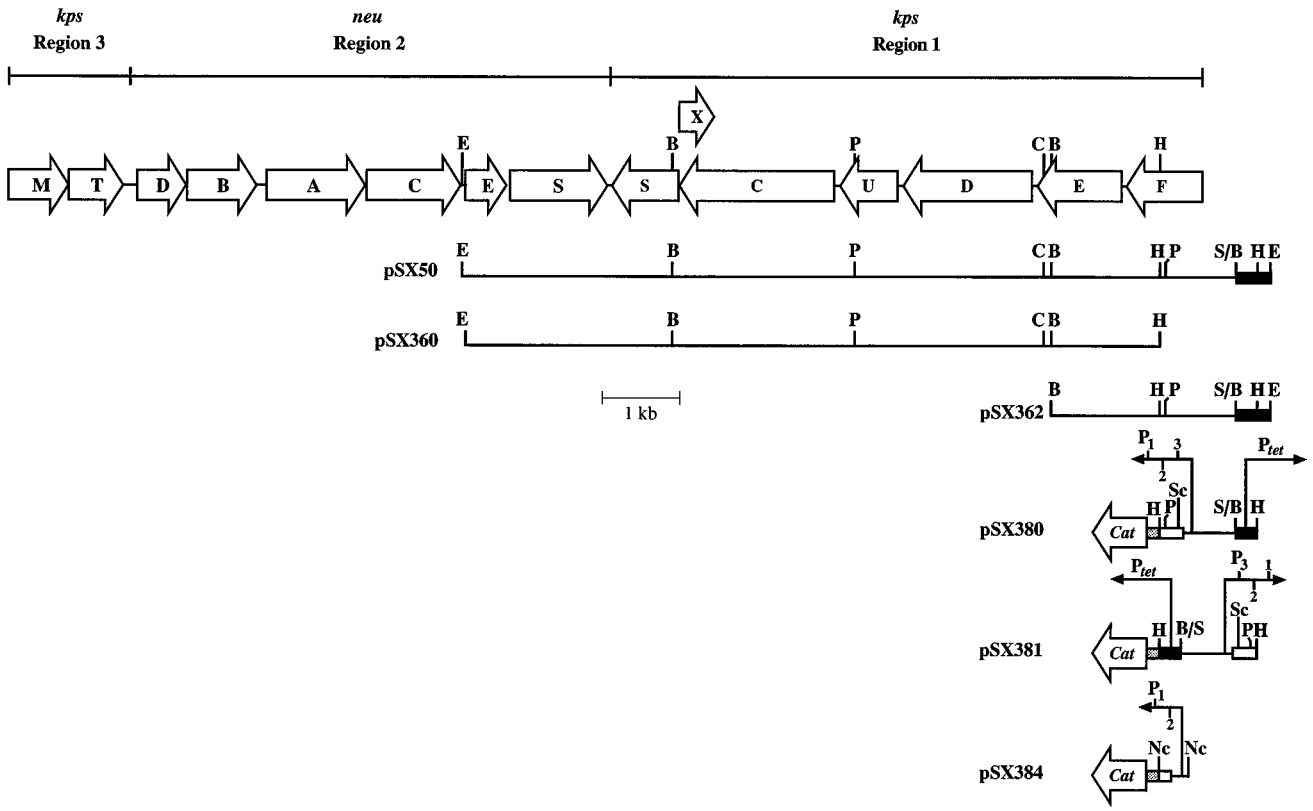

FIG. 1. Genetic organization of the K1 *kps* locus. Genes and their directions of transcription are indicated by open arrows. The three functional regions of *kps* described in the text are indicated above the arrows. The evidence for this genetic organization has been summarized previously by Vimr et al. (47), and is summarized in the text of the present report. *kpsX* (GenBank accession no. U35467) was identified as a translational MudK fusion (1). P_1, P_2, and P_3 are the putative promotors for *kpsF* described in the text. Solid black boxes denote vector sequences, hatched boxes denote pKK232-8 sequences, and open boxes denote *kpsF* translational sequences. The partial restriction endonuclease map of the pBR329-derived region 1 subclone pSX50 (46) is shown below the arrows. E, *Eco*RI; B, *Bam*HI; P, *Pst*I; C, *Cla*I; H, *Hin*dIII; Sc, *Sca*I; Nc, *Nco*I; S, *Sau*3A.

sensitivity of several K1 *kps* region 2 gene products, implies a posttranslational mechanism of control (24), and the other involves reduced activity at 18°C of the cytidine monophosphate-ketodeoxyoctonate synthetase encoded by *kpsU* (7). In this communication, we demonstrate that thermoregulation of region 1 is exerted at the transcriptional level. Our results thus represent the first functional characterization of a *kps* promoter region, providing the foundation for more detailed analysis of the mechanism of type II capsule thermoregulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Most of the mutants used in this study were derived from the prototrophic strain EV36 (*galP23 rpsL9 kps*$^+$), an *E. coli* K-12–K1 hybrid previously described by Vimr and Troy (48). Isolation and characterization of EV93 (*kpsC*::Tn*10*), EV94 (*kpsS*::Tn*10*), and EV95 (*kpsT*::Tn*10*) were previously described by Vimr et al. (46), who also describe the methods of phage P1 transduction used in the present study. The isolation and characterization of EV286 (EV36 *kpsF*::*aphT*) are described in Results. The isolation of EV243 (*kpsE*::MudJ) was previously described by Cieslewicz et al. (9). EV285 was constructed by transduction with EV243 as a P1 donor with EV35 as the recipient, selecting for Km^r carried on the MudJ element. EV35 is a *kps*$^+$ hybrid derivative of the K-12 strain CSH50 [*ara* Δ(*lac-pro*) Sm^r *thi*] (25), generally constructed by the same method as was used for EV36. HB101 [F$^-$ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Sm^r) *xyl-5 mtl-1 recA13*], DH5α [*endA1 hsdR17* (r_k^- m_k^-) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* (ϕ80*dlac*) Δ(*lacZ*)M15], and MC4100 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150* (Sm^r) *relA1 flbB5301 deoC1 ptsF25 rbsR*] were from our laboratory collection. Bacteria were routinely propagated with vigorous aeration in modified Luria broth (25) containing 0.5% NaCl or in the same medium in a recycling fermenter (8). Agar was used at 1.5% (wt/vol) for growth of bacteria on a solid medium. Propagation of bacteria at 20°C was to within ±1°C.

The suicide vector pGP704 (26) was kindly provided by Chris Whitfield (University of Guelph, Guelph, Ontario, Canada). Both pUC4K, the source of *aphT* (Km^r), and the promoterless *cat* plasmid pKK232-8 were from Pharmacia (Piscataway, N.J.). Lysis of cells from within, using pLysS, was previously described by Vimr (45). The construction of plasmids pSX360 and pSX362 was previously described by Cieslewicz et al. (9). Plasmid pSX384 was constructed by isolating the 400-bp *Nco*I fragment from pSX362 (9), blunt ending it with mung bean nuclease (Gibco/BRL, Grand Island, N.Y.), and ligating it to *Sma*I-cut pKK232-8. Other plasmids described in Results are shown schematically in Fig. 1. Appropriate plasmid markers were maintained by growth of strains harboring the various plasmids in the presence of a 100-μg/ml final concentration of ampicillin, 50 μg/ml for kanamycin or chloramphenicol, and 10 μg/ml for tetracycline.

Immunological procedures. Intracellular polysialic acid was detected by radial immunodiffusion against H.46 antipolysialic acid antiserum (46). Immunoelectrophoresis was carried out as previously described by Bronner et al. (7) with a Multiphor II apparatus (Pharmacia). Samples for immunoelectrophoresis were prepared by inoculating single colonies of strains harboring pLysS into 40 ml of Luria broth plus chloramphenicol and growing the cultures overnight at 37°C. Cells were harvested by centrifugation and washed with an equal volume of water before being concentrated 40-fold in 100 mM Tris, pH 8.5. Cells were frozen at −20°C and allowed to lyse by thawing at room temperature. To minimize physical trapping of polysialic acid in the lysates after the thaw cycle, 10 μl of DNase (20 mg/ml; Sigma) was added for 1 h at room temperature to reduce viscosity; this was followed by the addition of 2 μl of protease K (30 mg/ml; Sigma) before loading 15 μl of the treated samples for electrophoresis. Where indicated, samples were treated prior to protease K digestion either with a mixture of phospholipases A2, C, and D (Sigma) by diluting a stock containing 5 U of each phospholipase per ml sixfold with sample and incubating for 1 h at 37°C to release phospholipids (14) or by adding an excess of purified phage K1F endo-*N*-acylneuraminidase specific for cleaving α2,8-linked polysialic acid (31).

RNA analyses. Total cellular RNA was prepared according to the method of Reddy et al. (32). Cultures pregrown at 20°C overnight were diluted in fresh rich medium plus the appropriate antibiotic and grown at either 37 or 20°C to an A_{600} of 0.6. Five micrograms of RNA plus 10^4 to 10^5 cpm of ^{32}P-labeled primer were

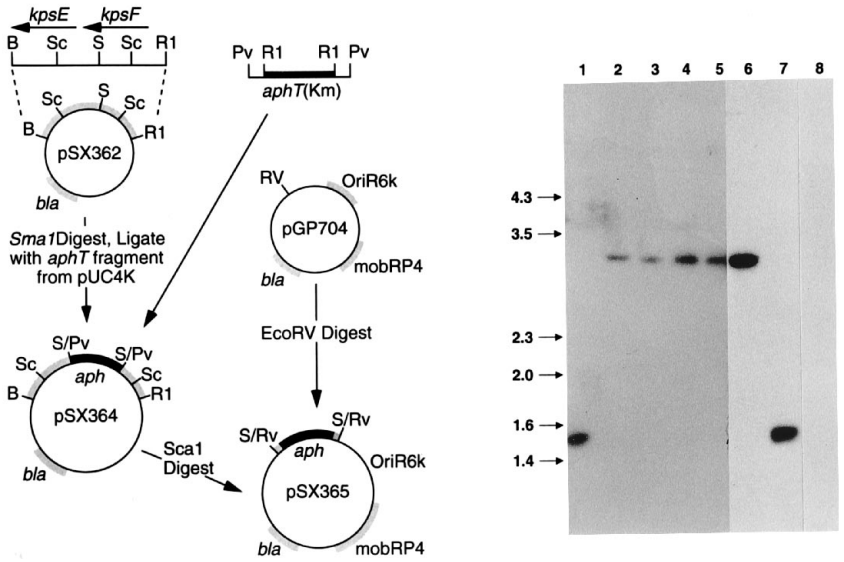

FIG. 2. Construction of *kpsF*::*aphT* and verification of chromosomal insertion. (Left) Construction of the pGP704-derived suicide replacement vector pSX365. The *Bam*HI-*Eco*RI fragment isolated from pSX50 (Fig. 1) contains the intact *kpsF* open reading frame plus a portion of the *kpsE* gene truncated at the *Bam*HI site; arrows indicate transcription directions. B, *Bam*HI; Sc, *Sca*I; S, *Sma*I; R1, *Eco*RI; Pv, *Pvu*II; Rv, *Eco*RV. (Right) After insertion of *aphT* into EV36 (wild type) by homologous recombination and transduction into the fresh wild-type background, chromosomal DNA from EV36 (lane 1) or four independent recombinants (lanes 2 to 5) was digested with *Sca*I and hybridized against the *Sca*I *kps* fragment from pSX362. Lanes 6 to 8, *Sca*I-digested pSX364, pSX362, or HB101 chromosomal DNA, respectively, probed with the same *Sca*I fragment as noted above. Numbers on the left indicate sizes (in kilobases) of selected lambda *Hin*dIII-*Eco*RI fragments.

denatured at 95°C for 10 min, annealed at 65°C for 15 min, and chilled on ice for 5 min before adding avian myeloblastosis virus reverse transcriptase (U.S. Biochemicals, Cleveland, Ohio). Primers (100 ng) were labeled with [γ-^{32}P]ATP (7,000 Ci/mmol; ICN, Irvine, Calif.) and T4 polynucleotide kinase as specified by the manufacturer (GIBCO/BRL). Extension conditions, sample preparation, electrophoresis, and autoradiographic detection were as previously described by Triezenberg (40). Standard DNA sequencing reactions with the T7 polymerase 2.0 from U.S. Biochemicals were carried out with denatured double-stranded template and the same primers used for the extensions. The primers are described in the appropriate figure legends.

For hybridization analysis of *kpsE* mRNA, serial twofold dilutions from 5 to 0.6125 μg of total RNA, isolated as described above, were applied to anylon membrane with a slot blot apparatus and hybridized against a random-primer-labeled *Cla*I-*Bam*HI probe specific for *kpsE* (Fig. 1).

Automated DNA sequence analysis. The DNA sequence on both strands was determined at the Genetic Engineering Facility of the University of Illinois by using Amplitaq with Dye-Terminator Chemistry in a PTC-100 Thermocyler (MJ Research, Watertown, Mass.) and then analyzed in an Applied Biosystems 373A Automated DNA Sequencer with stretch upgrade (38). Overlapping DNA sequences were compiled by using AssemblyLign (International Biotechnologies Inc., New Haven, Conn.), and nucleotide searches were conducted by using the BLAST program (2).

Chloramphenicol acetyltransferase assay. Cultures pregrown at 20°C were diluted 1,000 times into fresh Luria broth and grown to an A_{600} of 0.6 to 0.7 at 37 or 20°C. ^{14}C-labeled chloramphenicol (D-threo-[dichloroacetyl-1-^{14}C] CAT assay grade, 55 mCi/mmol; ARC, St. Louis, Mo.) was added to 10-fold-concentrated cells in fresh medium to a final specific activity of 0.09 μCi/μmol. At timed intervals during incubation at 30°C, 1-ml aliquots were removed for extraction three times with equal volumes of ethyl acetate. The pooled extracts were separated on Silica Gel 60 thin-layer chromatography plates as described previously by Shaw (35). The plates were exposed for 72 h against Fuji RX film and quantitated with a PDI scanner and RFLPrint software.

Transmission electron microscopy. Strains grown overnight at 37°C on rich agar medium were layered with modified Karnovsky's reagent. Four to six colonies were picked and collected in a glass vial which was then incubated in a Goldstar Turntable microwave oven, as previously described by Login and Dvorak (22). Fixed samples were stained with uranyl acetate and lead citrate, then ultrathin sectioned, and examined with a JEOL 100CX electron microscope at ×30,000, ×32,000, and ×100,000 magnifications.

Nucleotide sequence accession numbers. The sequence (both strands) of *kpsF* has been deposited as an update to our previous GenBank submission under accession no. L19929. The sequence of *kpsX* has been deposited with GenBank under accession no. U35467.

RESULTS

Isolation of *kpsF*::*aphT*. On the basis of DNA sequence analysis, we previously suggested that *kpsF* was a good candidate for the first gene of region 1 (9). To establish the requirement of *kpsF* for capsule production, an *aphT* cartridge was inserted into *kpsF* carried on pSX362, yielding pSX364 (Fig. 2). The *Sca*I fragment containing *aphT* was next ligated with the *Eco*RV-digested suicide vector pGP704, to produce pSX365 (Fig. 2). After conjugative transfer of pSX365 into EV36, selecting Km^r, followed by screening for Ap^s, four independent exconjugants that were likely to be the products of double recombination events were used as phage P1 donors for backcrosses into EV36. Several transductants from each cross were tested and found to accumulate intracellular polysialic acid after immunodiffusion of soluble cell extracts (data not shown), suggesting a defect in capsule translocation in these strains.

Southern hybridization analysis confirmed that the *kpsF* mutant phenotype was not associated with chromosomal rearrangements, since DNA isolated from each of the transductants hybridized to a *kps*-specific probe and yielded signals that were 1.5 kb larger than the wild-type signal (Fig. 2), as predicted from the known size of the *aphT* cartridge. The failure of this probe to hybridize against strain HB101 genomic DNA (Fig. 2, lane 8) is consistent with the absence of the *kps* locus in *E. coli* K-12 (44) and, more specifically, with the absence of *kpsF* from this genome.

To complete the initial characterization of the gene, the entire DNA sequence on both strands was determined from the translational start site of the gene to the 5′ *Eco*RI site in pSX362 (Fig. 1). These data were deposited with GenBank as described above. Computer-assisted analyses of the new sequence data did not reveal any additional open reading frames on either strand that could encode polypeptides larger than 58 amino acids, results consistent with *kpsF* being the first gene of

region 1. These data confirmed that the portion of pSX50 derived from pHC79 in the original *kps*$^+$ cosmid clone, pSX49 (46), contained the *tet* promoter from the cosmid vector (Fig. 1). Computer searches of recently deposited data from the *E. coli* genome sequencing project (GenBank accession no. U28377), in the region containing *serA*, did not reveal any nucleotide homology with any known *E. coli* sequences, indicating that the *kps*–K-12 junction was not included in the original cloning of *kps* in pSX49. However, a 116-nucleotide segment near the end of the pSX362 *kps* insert sequence is 58% identical with IS*542* from *Pseudomonas atlantica*, which functions in the reversible inactivation of extracellular polysaccharide expression (GenBank accession no. M24471 [3]). Although we and others have speculated that *kps* loci are products of horizontal gene transfer (47), none of the sequences flanking *kps* having any similarity to mobile genetic elements have been identified until now.

To further analyze the *kpsF*::*aphT* phenotype, EV286 was plated in the presence of the polysialic acid-specific bacteriophage K1F. K1F is a lytic bacteriophage that when plated in the presence of K1 wild-type cells produces large plaques (10). Detectable plaques are not present in strains bearing *kpsE*::MudJ, *kpsS*::Tn*10*, *kpsC*::Tn*10*, or *kpsT*::Tn*10* mutations. However, when EV286 was plated in the presence of K1F, the phage efficiency of plating was unaffected but the diameters of plaques were 5 to 10% of those of plaques found on wild-type indicator cells. Although there are several possible conclusions, we suggest that the small-plaque phenotype of the *kpsF* mutant reflects a defect in capsular polysaccharide export or assembly at the cell surface. When taken together with the detection of intracellular polysialic acid described above, the phenotype of the *kpsF* mutant supports the conclusions that either KpsF facilitates capsule export or *kpsF* is in the same transcriptional unit as region 1 genes, since mutants with defects in region 1 also accumulate intracellular polysialic acid (46). Note that these scenarios are not mutually exclusive.

Complementation analysis of *kpsF*::*aphT*. Transformation of pSX360 (Fig. 1), a pSX50 subclone harboring a truncated *kpsF* into EV286 (*kpsF*::*aphT*), resulted in a K1F-sensitive phenotype (results not shown). While this plasmid is missing the natural *kps* region 1 promoter sequences, it may provide enough of the downstream gene products because of expression driven by plasmid promoters. (This was confirmed by showing that the same plasmid could complement a *kpsE*::MudJ mutant [9].) The reciprocal experiment, introducing a plasmid, pSX362, which expresses wild-type *kpsF* (Fig. 1) but does not carry the rest of the region, fails to complement. Therefore, there is an obligatory requirement of transcription beyond *kpsF* for capsule production. However, we previously reported that the GutQ polypeptide encoded by the last gene of the glucitol utilization operon in *E. coli* was 49% identical with KpsF, suggesting the possibility of functional overlap (9). This possibility was strengthened by detecting a potential second homolog of KpsF that could be encoded by *orf328* (GenBank accession no. U29579), which maps near *rpoN* at 70 map units. Any decision about the dispensability of KpsF is thus provisional on the potential functional overlap between KpsF homologs.

Phenotypic characterization of EV286. To determine the intracellular compartment wherein polysialic acid accumulates in the *kpsF*::*aphT* mutant, EV286 was ultrathin sectioned for morphological examination by transmission electron microscopy. In contrast to the control wild-type strain, EV36 (Fig. 3A), EV286 (Fig. 3B) contained large, electron-transparent cytoplasmic lacunae caused by the accumulation of unexported polysaccharides (7, 30). To demonstrate the variation in phenotypes associated with region 1 mutations, electron microscopy was carried out on strains with defects in *kpsT*, *kpsS*, *kpsC*, or *kpsE* (Fig. 4A to D, respectively). We chose to include the region 3 *kpsT* null mutant in this analysis because Pavelka et al. (29) had previously characterized the lacunae in a K1 *kpsT* point mutant. Consistent with their result, our *kpsT* mutant accumulates relatively small aggregates peripherally distributed at the cytoplasmic surface of the inner membrane. Therefore, a null mutation in *kpsT* confers the same phenotype as a point mutation in the region of *kpsT* predicted to code for the ATP-binding site. In contrast, but similar to the *kpsF* mutant, other region 1 mutants with defects in *kpsS* or *kpsE* produced fewer but much larger cytoplasmic aggregates that were not obviously associated with the inner membrane (Fig. 4B and D). While the lacunae in the *kpsT* mutant may represent biosynthetic foci containing abortively transported polysialic acid, the phenotype is not exclusive to *kpsT* mutants, because similar peripheral aggregates were also detected in the *kpsC* mutant (Fig. 4C). The phenotype of the *kpsF*::*aphT* mutant is fully consistent with that expected of a region 1 polar defect and thus with *kpsF* being part of a region 1 operon.

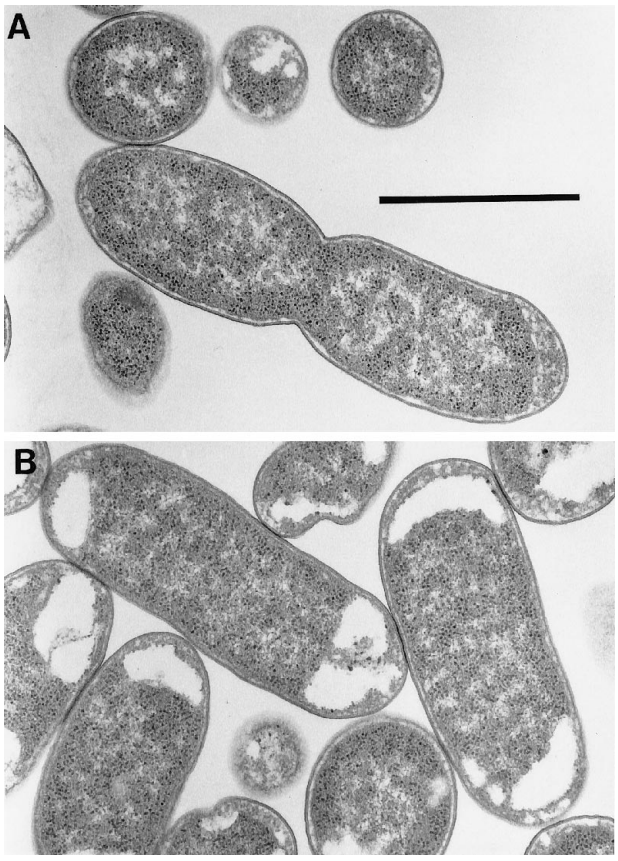

FIG. 3. Accumulation of intracellular polysialic acid by EV286 (*kpsF*::*aphT*). Transmission electron microscopy of ultrathin wild-type and *kpsF*::*aphT* strains EV36 (A) and EV286 (B), respectively. Note electron-transparent lacunae in EV286 and the absence of "holes" in the wild type. Wild-type capsule is not detected by this procedure because the polysaccharides were not stabilized by cross-linking prior to fixation. Bar, 1 μm.

Since the lipidated form of polysialic acid aggregates in vitro (15), it was reasonable to hypothesize that the intracellular polysialic acids detected in Fig. 3 and 4 might also be modified, because it was otherwise hard to imagine a physical explanation for how negatively charged, water-soluble polysialic acid

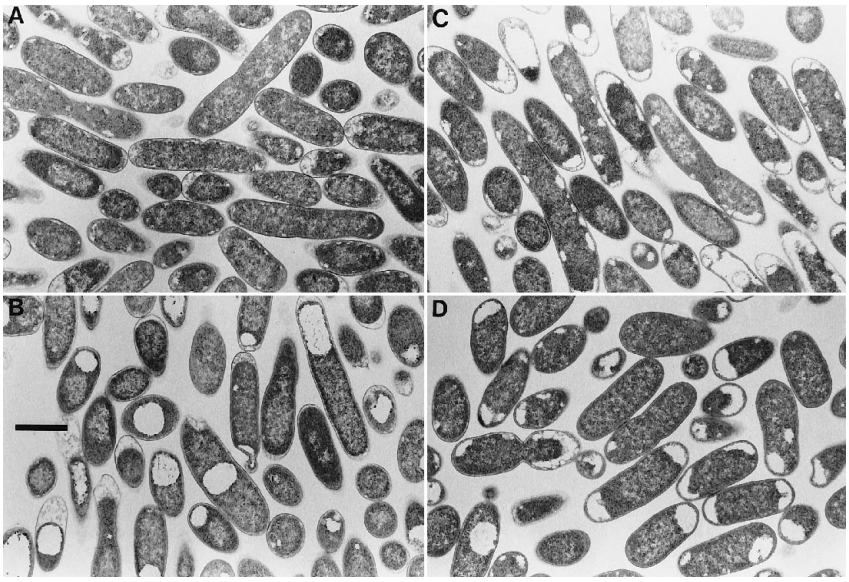

FIG. 4. Phenotype of region 1 or 3 mutants. Mutants EV95 (*kpsT*::Tn*10*) (A), EV94 (*kpsS*::Tn*10*) (B), EV93 (*kpsC*::Tn*10*) (C), and EV243 (*kpsE*::MudJ) (D) were prepared for electron microscopy as described in the legend to Fig. 3. Bar, 1 μm.

chains could accumulate as electron-transparent aggregates. Therefore, to further characterize the polysialic acids synthesized by the *kpsF*::*aphT* mutant, strains harboring pLysS were lysed for immunoelectrophoretic analysis of the resulting whole-cell extracts. The wild-type strain EV36, as well as each of the mutants examined, produces two populations of polysialic acid: a rapidly migrating fraction and one remaining near the origin that can be removed by predigestion with phospholipases (Fig. 5, lanes A and A^+). Although differing in intensity and, perhaps, relative amounts, a phospholipase-sensitive arc is clearly identified in each region 1 or region 3 mutant tested (Fig. 5, lanes B to F). We assume that the gentle handling of cell lysates and the use of DNase-protein digestion are critical features of our procedure, since previous researchers investigating a related *E. coli* K5 system failed to detect the low-mobility arcs in some region 1 or 3 mutants (7, 20). Control experiments showed that DH5α, a K-12 strain lacking the *kps* locus, produces neither population of immunoreactive material and that pretreating samples of K1 wild type and mutants with endo-*N*-acylneuraminidase (31) abrogates precipitin arcs, thus confirming that the immunoreactive material is polysialic acid (data not shown). We conclude that neither the region 3 gene product of *kpsT* nor those of region 1 are obligatory for phospholipid addition and that formation of intracellular lacunae may be a lipid-dependent phenomenon. Determining the enzymology of polysialic acid lipidation or whether this modification has any role beyond its obvious potential function as an outer membrane anchor obviously requires further investigation.

Transcriptional regulation of *kpsF*. To further define the function of *kpsF*, the putative transcriptional start site was investigated by primer extension with two different *kpsF*-specific oligonucleotides (Fig. 6). Extension of primer 1 gave three potential start sites, each preceded by a reasonable σ^{70} promoter consensus sequence (at least three of six identities in putative −35 and −10 regions, plus a spacing of 16 to 18 nucleotides between these regions). Two of these apparent start sites were detected by using primer 2, providing strong independent confirmation that the signals were not artifacts of contaminating RNase or reverse transcriptase pause sites. Failure to detect the most proximal start site with primer 2 may be due to the proximity of this primer's 3′ end to the message 5′ terminus, resulting in the potentially labeled product migrating in the region of the gel containing high background levels. Definitive evidence that *kpsF* has more than one promoter will require S1 analysis or use of another appropriate mapping

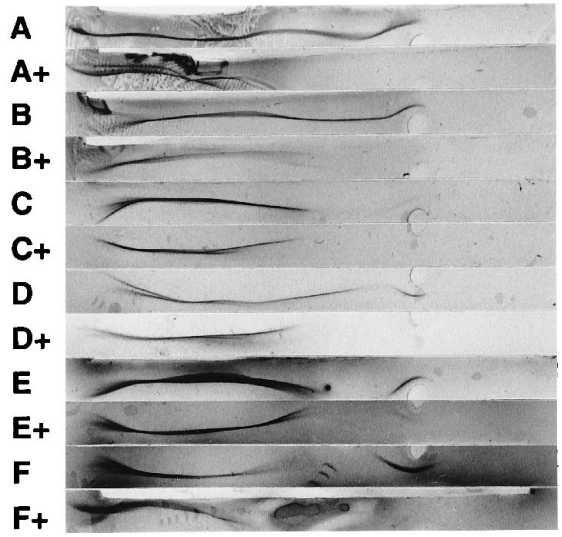

FIG. 5. Detection of lipidated polysialic acid in region 1 or 3 mutants. After agarose electrophoresis of cell extracts (0.6 A_{600} cell equivalents), wells were cut in the gel and filled with H.46 antipolysialic acid antiserum. Precipitin arcs detected after incubation at 4°C were stained with Coomassie blue and photographed. Note that the analysis is not quantitative. Each mutant was analyzed three times, with comparable results. Variation in relative intensity of precipitin arcs reflects variability of the staining techniques and, possibly, the different relative extent of lipid modification in each mutant. Lanes: A, EV36 (wild type); B, EV93 (*kpsC*::Tn*10*); C, EV94 (*kpsS*::Tn*10*); D, EV95 (*kpsT*::Tn*10*); E, EV243 (*kpsE*::*lacZ*); F, EV286 (*kpsF*::*aphT*). Lipidated polysialic acid was detected by treating samples with phospholipases prior to immunoelectrophoresis, as indicated by the plus sign. Sample origins are on the right, with electrophoresis toward the positive electrode on the left.

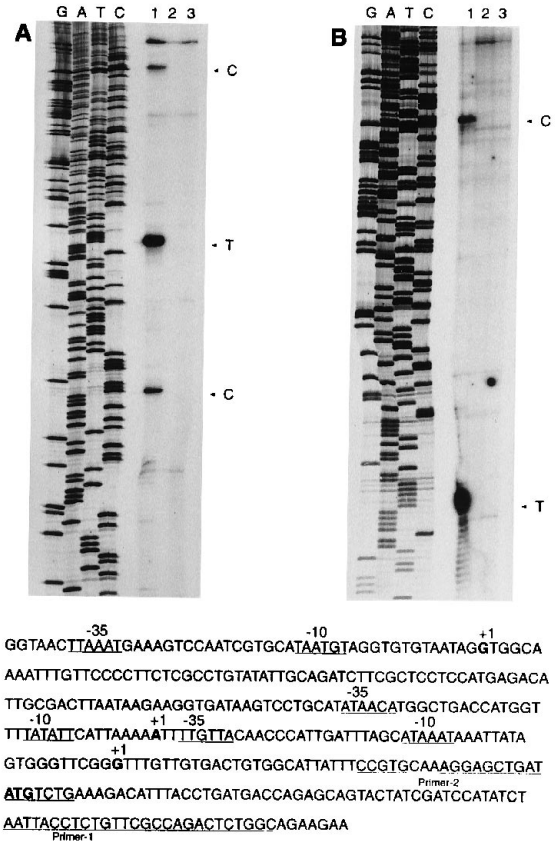

FIG. 6. Mapping and thermoregulation of the putative *kpsF* promoter. (A and B) Extensions of primers 1 and 2 (shown here by the complementary sequence), respectively, using total RNA prepared from EV36 harboring pSX362 grown at 37°C (lanes 1) or 20°C (lanes 2) or from HB101 harboring pUC18 grown at 37°C (lanes 3). Each primer was also used in standard DNA sequencing reactions with pSX362 as the template, indicated at the top by the dideoxy G, A, T, or C nucleotide used to stop each reaction. Arrows to the right of each gel identify the complement of the putative transcriptional start sites. Shown below are the 272 nucleotides 5′ to the *kpsF* translational start site (boldface letters); primers 1 and 2 and consensus −35 and −10 regions are underlined. Boldface letters with +1 superscripts indicate the apparent *kpsF* transcriptional start sites.

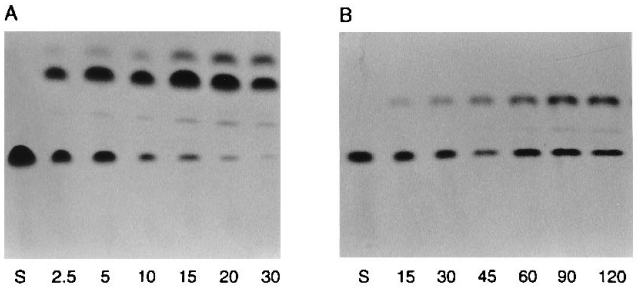

FIG. 7. Thermosensitive expression of a promoterless *cat* gene fused to *kpsF*. The *Hin*dIII fragment from pSX50 (Fig. 1) containing the 5′ end of *kpsF* was ligated in both possible orientations to *Hin*dIII-digested pKK232-8. Orientations were confirmed by restriction endonuclease digestions with *Pst*I. The *Hin*dIII fragment in pSX380 contains truncated *kpsF* fused to a promoterless *cat* gene such that chloramphenicol acetyltransferase expression is driven by sequences 5′ to *kpsF*. EV36 harboring pSX380 was grown at 37°C (A) or 20°C (B) and then assayed for enzyme activity by quantitating the conversion of ^{14}C-labeled chloramphenicol to acetylated product(s). Products migrate more rapidly than the unacetylated substrate during thin-layer electrophoresis and were quantitated by densitometry of the autoradiographic signals shown. S indicates substrate alone. Numbers at the bottom indicate timed (in minutes) aliquots removed to determine the rates of chloramphenicol acetylation. The initial rate of acetylation in cells grown at 20°C was 0.1% of the substrate consumed per min per A_{600} unit. In contrast, the linear portion of the reaction in panel A was essentially over by the first aliquot, at which time 58% of the substrate had been converted to products.

technique. More important for this communication, no unique start sites were detected with mRNA isolated from cells grown at 20°C (Fig. 6, lanes 2), indicating that *kpsF* is transcriptionally silent at the lower temperature. We tentatively concluded that thermoregulation of region 1 is exerted by transcriptional regulation of the *kpsF* promoter.

To further characterize the effect of low temperature on *kpsF* expression, the *Hin*dIII fragment from pSX50 was subcloned in both possible orientations into pKK232-8, generating pSX380 and pSX381 (Fig. 1). Chloramphenicol acetyltransferase assays were carried out with strains harboring these plasmids pregrown at 37 or 20°C. Figure 7 shows that the *kpsF* promoter activity of pSX380 was reduced at least 36-fold by growth at the nonpermissive temperature. In contrast, the activity of pSX381, containing the *kpsF* promoter region inserted in the opposite orientation relative to *cat*, was unaffected by temperature (data not shown). The pSX380 results are consistent with the preceding primer extension experiments and provide confirmatory evidence of *kpsF* thermoregulation. Since the *Nco*I fragment containing the putative *kpsF* promoter in pSX384 also expressed thermoregulated chloramphenicol resistance when cloned upstream from *cat* (Fig. 1), we conclude that the third putative promoter is not obligatory for *kpsF* expression or thermoregulation. Although promoters P_1 and P_2 are sufficient for thermoregulated expression of *kpsF*, we cannot exclude the possibility that promoter P_3 or farther upstream sequences are involved in modulating *kpsF* expression.

Thermoregulation of *kpsE*. We have previously described the isolation of a chromosomal MudJ fusion in *kpsE* (9). If our conclusions about polarity and transcriptional regulation of *kpsF* are correct, expression of *kpsE* should be reduced in the *kpsF*::*aphT* mutant and β-galactosidase activity of the *kpsE*::MudJ fusion should be thermoregulated. To determine the magnitude of polarity, total RNA was isolated from EV36 (wild type), EV286 (*kpsF*::*aphT*), and HB101 (a strain lacking the *kps* locus) and hybridized against a *kpsE*-specific DNA probe (see Materials and Methods). The results (not shown) indicated that about 16 times less *kpsE* message was produced in the mutant relative to that produced in the wild type, consistent with the reduced expression of region 1 genes as well as the small-plaque phenotype of the *kpsF* mutant, in which small amounts of region 1 gene products presumably limit surface capsule production. These results confirm that *kpsF* and *kpsE* and probably all of the remaining genes in region 1 are expressed as one major transcriptional unit controlled by the thermoregulated promoter region of *kpsF*.

Measurements from one of several representative experiments showed that strain EV285 (*kpsE*::MudJ) produced 153.9 ± 3.5 U (mean ± standard deviation) at 37°C compared with 12.3 ± 0.8 U for the same strain grown at 20°C. Since the reporter gene is not translationally fused to *kpsE*, the more than 12-fold reduction most likely results from transcriptional regulation. Slow growth per se did not affect *kpsE* expression, because EV285 grown in rich medium with a doubling time of 2 h at 37°C in a Chesbro-type recycling fermentor (8) produced 125 ± 9.5 U of β-galactosidase (triplicate measurements from one of two comparable fermentations). Since cells grown in batch culture in the same medium at 20°C also have a 2-h doubling time, we conclude that the effects on *kpsE* expression are due to temperature instead of a generalized slow growth

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KpsF-E   45  YQRVLNLIMNCKGHVILSGMGKSGHVGRKMSATLASTGTPSFFIHPAEAFHGDLGMITPY 104
KpsF-H   55  FNQVIDLILACEGRLVIGGIGKSGLIGKKMVATFASTGTPSFFLHPTEAFHGDLGMLKPI 114
o328-E   48            CKGKVVVMGMGKSGHIGRKMAATFASTGTPSFFVHPGEAAHGDLGMVTPQ 107
GutQ-E   18  FVRAANIILHCEGKVVVSGIGKSGHIGKKIAATLASTGTPAFFVHPAEALHGDLGMIESR  77

KpsF-E  105  DLLILISASGETDEILKLVPSLKNFGNRIIAITNNGNSTLAKNADAVLELHMANETCPNN 164
KpsF-H  115  DIVMLISYSGETDDVNKLIPSLKNFGNKIIAVTSNKNSTLARHADYVLDITVEREVCPNN 174
o328-E  108  DVVIAISNSGESSEITALIPVLKRLHVPLICITGRPESSMARAADVHLCVKVAKEACPLG 167
GutQ-E   77  DVMLFISYSGGAKELDLIIPRLEDKSIALLAMTGKPTSPLGLAAKAVLDISVEREACPMH 136

KpsF-E  165  LAPTTSTTLTMAIGDALAIAMIRQRKFMPNDFARYHPGGSLGRRLLTRVADVMQHDVPAV 224
KpsF-H  175  LAPTTSALVTLALGDALAVSLITARNFQPADFAKFHPGGSLGRRLLCKVKDQMQTRLPTI 234
o328-E  168  LAPTSSTTATLVMGDALAVALLKARGFTAEDFALSHPGGALGRKLLLRVNDIM--EIPHV 227
GutQ-E  137  LAPTSSTVNTLMMGDALAMAVMQARGFNEEDFARSHPAGALGARLLNKVHHLMRRDIPQV 196

KpsF-E  225  QLDASFKTVIQRITSGCQGMVMVEDAEGGLAGIITDGDLRRFMEK-EDSLTSATAAQMMT 283
KpsF-H  235  LPTTNFTDCLTVMNEGRMGVALVMENEQLK-GIITDGDIRRALTANGAGTLNKTAKDFMT 293
o328-E  228  KKTASLRDALLEVTRKNLGMTVICDDNMMIEGIFTDGDLRRVFDM-GVDVRQLSIADVMT 286
GutQ-E  197  ALTASVMDAMLELSRTGLGLVAVCDAQQQVQGVFTDGDLRRWLVG-GGALTTPVNEAMTV 255

KpsF-E  284  REPLTLPEDTMIIEAEEKMQKDKCLNVIGDQQGK 317
KpsF-H  294  SSPKTIHQDEFLSKAEDFMKAKKIHSLV       321
o328-E  287  PGGIRVRPGILAVEALNLMQSRHITSVM        314
GutQ-E  256  GGTTLQSQSRAIDAKEILMKRKITAAPVVDENGK 289
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FIG. 8. Multiple alignment of KpsF and its homologs. Quadruple alignment of KpsF and three homologs; identical amino acids in two or more homologs are shaded. KpsF-E: *E. coli* K1, accession no. L19929. KpsF-H: *H. influenzae* Rd, accession no. L46308. o328-E: *E. coli* K-12, accession no. U18997. GutQ-E: *E. coli* K-12, accession no. U29579. All sequences noted here were deposited with GenBank.

response. When taken together with the polarity effect of *kpsF*::*aphT* on *kpsE* discussed above, the fusion results are entirely consistent with predictions made on the basis of thermoregulation of region 1 controlled by the *kpsF* promoter.

Homology analysis of KpsF. As previously noted for KpsF and GutQ, a conserved feature near the N terminus of each polypeptide shown in Fig. 8 is a structure reminiscent of the P-loop motif, GXXXXGK[TS], or Walker box A that is commonly found in ATP- or GTP-binding proteins (34). Different families of nucleotide-binding proteins have variations in this motif such that the lysine residue may be the only absolutely conserved feature (34). Since a number of regulatory proteins catalyze phosphoryl transfers, the P-loop motif consensus is consistent with a potential nucleotide binding function of KpsF and its homologs. Thus, if KpsF were involved in thermoregulating capsule expression, its effects might be difficult to detect not only because of the potential functional overlap among homologs but also because of additional components of the thermoregulatory apparatus which have not yet been identified.

In addition to the P-loop similarity, the region of KpsF from residue 108 to roughly 160 is homologous with several bacterial hexosephosphate aminotransferases (EC 2.6.1.16), including the *Rhizobium meliloti* NodM gene product which is structurally and functionally related to the glucosamine synthase encoded by *glmS* in *E. coli* (43). Since only a portion of KpsF is homologous with NodM, it is unlikely that KpsF would have glucosamine synthase activity.

DISCUSSION

Similar to the phenotype of other region 1 or region 3 mutants, we have shown that an insertion in *kpsF* blocks or at least reduces polysialic acid transport, resulting in the intracellular accumulation of lipidated polysaccharides. Since none of the mutants appears to have an absolute defect in lipidation, it is highly unlikely that this modification is sufficient in itself to signal polysaccharide translocation, because expression of the putative region 3-encoded ATP-binding cassette transporter would be intact in *KpsS* or *KpsC* mutants. Since our analysis in Fig. 5 was not quantitative, more precise analytical procedures will be necessary to determine whether the number of lipidated chains varies in the different mutants, as suggested by the reduced intensities of phospholipase-sensitive material in some of the mutants (Fig. 5). Regardless of this possibility, our observations do not lend support to the notion that KpsM and KpsT or their homologs in other bacteria function as lipid-dependent "flippases" (14). More importantly, our results indicate that none of the affected gene products in the region 1 or 3 mutants we examined appears to be obligatory for lipidation. Previous results from the K5 *kps* system have suggested that deletions of region 3 (20) or nonpolar deletions of *kpsC* or *kpsS* (7) block lipid attachment. Since the primary immunoelectrophoretic data supporting these conclusions were not presented, it is difficult to compare our results (Fig. 5), which clearly identify a slowly migrating electrophoretic variant of polysialic acid in *kpsT*, *kpsC*, or *kpsS* mutants. This conclusion is uncompromised by our use of potentially polar insertions; indeed, the demonstrated polarity on downstream expression exerted by *kpsE* (9) or *kpsF* (this study) insertions is an additionally strong indication that KpsS and KpsC are not obligatory for lipidation. In the related group B meningococcal system, LipA and LipB were thought to be capsule-lipidating enzymes (14). We previously reported that LipA and LipB are homologs of KpsC and KpsS, respectively (47), and the current results indicate that neither is obligatory for lipidation. Although the functions of LipAB may be different from those of KpsSC, we suspect that the gentle cell disruption and phospholipase treatment methods used in the present study may account for our ability to detect phospholipase-sensitive polysaccharide in mutants previously reported to be defective in this process. In addition, the previous studies with the K5 or group B systems have used extrachromosomal or heterologous expression systems in which the potential problems of gene dosage, supercoiling, and irrelevant promoters may not have been adequately controlled.

In addition to establishing *kpsF* as the first gene of region 1, we have demonstrated thermoregulation of what may be multiple *kpsF* promoters. Approximately 37% of known *E. coli* genes that use σ^{70} for their expression have more than one promoter, consistent with the potential for hierarchical control mechanisms (11). However, temperature is the only environmental condition known to dramatically affect polysialic acid capsule production (5, 28), and all three potential *kpsF* promoters were silent at 20°C. It may be productive in the future to investigate *kpsF* expression at temperatures intermediate between the two studied in this communication. Thermoregulation of some virulence factors and the regulation of certain exopolysaccharides involve an abundant nucleoid protein which, in *E. coli*, is encoded by *hns* (37). In unpublished experiments we did not detect an effect of *hns* (*pilG*::Tn*10*) on expression of *kpsE*::MudJ or K1 capsule production at 20 or 37°C, and Stevens et al. (39) and Keenleyside et al. (19) showed that neither an independent *hns* allele nor several other global regulators, including *rcsAB*, *himD* (Ihf β subunit), and *lrp*, affected K5 or K1 capsule production at either temperature. Stevens et al. (39) further showed that regions 2 and (probably) 3 are dependent on wild-type *rfaH* for capsule production at 37°C, suggesting that an antitermination mechanism may be involved in regulating transcription of these regions. Our results indicate that thermoregulation of region 1 is separate from that of regions 2 and 3 as well as from the other systems noted above which are modulated or controlled by previously well-characterized global regulatory circuitry. Although the reason for this apparent dichotomy is unknown, it may be that the evolution of some virulence factors has involved the modular assembly of multiple genes followed by their horizontal transfer, favoring control mechanisms independent of more integrated housekeeping or generalized survival systems. This notion is consistent with the idea that many virulence factors evolved from horizontally transferred genes under intense selection for growth of bacteria in animal hosts. Therefore, when considered in light of our present results, the regulation of previously well-characterized polysaccharide systems like alginate (16) or colanic acid (M antigen) (37) is such that it does not appear as if these other systems will be applicable models for some type II capsules. Our results thus provide the first detailed analysis of type II capsule regulation and indicate the direction of future research on this medically important biosynthetic system.

Since our results demonstrate that KpsF is not obligatory for capsule production, it is possible that this protein plays a minor role in exporting capsule or regulating other region 1 genes or gene products or that it has simply been incorporated into region 1 to provide a thermoregulated control region. Alternatively, the surprising existence of at least two KpsF homologs in the *E. coli* genome suggests the possibility of functional overlap. GutQ was predicted to be 223 residues in length (49) and thus would lack an homologous KpsF C terminus (9); however, a recent entry in the database (GenBank accession no. U29579) indicates a potential error in the *gutQ* sequence such that GutQ may actually be 308 residues. The predicted C terminus for this corrected sequence, shown in Fig. 8, is 27% identical with the homologous region of KpsF. Reinspection of the *gutQ* maxicell expression data of Yamada et al. (49) leads us to conclude that the 34-kDa band these authors observed is actually GutQ and not a dimer of the 18-kDa band as was originally proposed. In addition to GutQ and the putative polypeptide encoded by *orf328* in *E. coli* (see above), *H. influenzae* contains a hypothetical open reading frame whose product would be homologous with KpsF (13). Since the *H. influenzae* genome (GenBank accession no. U32841) apparently lacks capsule biosynthetic genes, detection of a potential KpsF homolog in this strain suggests that this protein may have a function unrelated to capsule production in this species.

The similarity of KpsF with isomerizing aminotransferases noted above suggests that KpsF could recognize glucosamine. This homology with KpsF is intriguing because glucosamine is a central structural component of several cell wall polysaccharides. Since type II capsules may compete with wall components for limiting biosynthetic intermediates (23) or translocation sites in the membrane, it is conceivable that by binding glucosamine KpsF could facilitate capsule production by modulating one or more of the competing biosynthetic systems. In this scenario, KpsF would be dispensable for polysaccharide production in vitro but critical, perhaps, for assembly of an in vivo cell wall able to resist phagocytosis or host serum resistance factors in animal hosts. While structural comparisons cannot be taken as strong evidence for function, the conserved primary structure of KpsF and its congeries implies potentially important functions for this family of novel polypeptides. Experiments are currently in progress to characterize the putative regulator(s) of *kpsF* and to determine the function(s) of KpsF.

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