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Molecular Analysis of Region 1 of the *Escherichia coli* K5 Antigen Gene Cluster: a Region Encoding Proteins Involved in Cell Surface Expression of Capsular Polysaccharide

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The nucleotide sequence of region 1 of the K5 antigen gene cluster of *Escherichia coli* was determined. This region is postulated to encode functions which, at least in part, participate in translocation of polysaccharide across the periplasmic space and onto the cell surface. Analysis of the nucleotide sequence revealed five genes that encode proteins with predicted molecular masses of 75.7, 60.5, 44, 43, and 27 kDa. The 27-kDa protein was 70.7% homologous to the CMP-2-keto-3-deoxyoctulosonic acid synthetase enzyme encoded by the *E. coli kdsB* gene, indicating the presence of a structural gene for a similar enzyme within the region 1 operon. The 43-kDa protein was homologous to both the Ctrb and BexC proteins encoded by the *Neisseria meningitidis* and *Haemophilus influenzae* capsule gene clusters, respectively, indicating common stages in the expression of capsules in these gram-negative bacteria. However, no homology was detected between the 75.7-, 60.5-, and 44-kDa proteins and any of the proteins so far described for the *H. influenzae* and *N. meningitidis* capsule gene clusters.

In excess of 70 capsular polysaccharides (K antigens) have been described on the surface of *Escherichia coli* (29). On the basis of a number of physical, chemical, and biochemical criteria, they have been divided into groups I and II (20). Group II K antigens are expressed at 37°C but not at 18°C (20), are linked to phosphatidic acid (PA) at the reducing end of the polysaccharide (20), and are usually expressed on the surface of extraintestinal isolates of *E. coli*, and particular K antigens are associated with certain infections (21).

The cloning and detailed molecular genetic analysis of a number of group II K antigen gene clusters identified a conserved genetic organization consisting of three functional regions (31, 32). A central region, region 2, which encodes enzymes for the synthesis and polymerization of specific K antigens, is flanked by regions 1 and 3. On the basis of Southern blot analysis and complementation studies, regions 1 and 3 appeared to be constant between different group II K antigen gene clusters (31, 32, 34, 36).

The cloned K5 antigen gene cluster has been subjected to a detailed molecular genetic analysis to determine the role played by the products encoded by regions 1 and 3. Mutations in region 3 result in cytoplasmic polysaccharide unlinked to lipid (23), a phenotype consistent with the occurrence of polysaccharide synthesis on the inner face of the cytoplasmic membrane (4). Determination of the nucleotide sequence of region 3 from the cloned K5 and K1 antigen gene clusters has revealed a single transcriptional unit composed of genes *kpsM* and *kpsT* (30, 37). Analysis of the predicted amino acid sequences of the KpsM and KpsT proteins indicated that they may make up a dual-component system for the export of polysaccharide across the cytoplasmic membrane in a manner analogous to the periplasmic binding protein-dependent transport systems of gram-negative bacteria (18). Proteins homologous to KpsM and KpsT have been identified in both *Haemophilus influenzae* and *Neisseria meningitidis* and have been postulated to play an analogous role in the expression of capsules in these two bacteria (15, 22). This suggests commonality in the mechanisms of capsule expression in these gram-negative bacteria.

Previous studies on the role of region 1-encoded functions exploited deletion derivatives of the cloned K1 antigen gene cluster (6). Such mutations result in PA-linked polysaccharide in the periplasmic space (6), indicating a role for the region 1 gene products in the translocation of polysaccharide onto the cell surface. Studies on the cloned K1 and K7 antigen gene clusters identified at least five proteins encoded by this region (4, 5, 31), one of which, a protein of 60.5 kDa, is located within the periplasmic space (34).

Strains that express group II K antigens have increased levels of CMP-2-keto-3-deoxyoctulosonic acid (KDO) synthetase activity at the capsule-permissive temperature (37°C) compared with either unencapsulated *E. coli* strains or those that express group I K antigens (13). In contrast, at the nonpermissive temperature (18°C), the level of CMP-KDO synthetase activity is low and essentially constant, irrespective of encapsulation (13), reflecting the presence of a CMP-KDO synthetase encoded by *kdsB* which is involved in lipopolysaccharide (LPS) biogenesis (16, 17). These findings, together with the finding of KDO at the reducing termini of a number of group II polysaccharides, has led to the hypothesis that CMP-KDO synthetase plays an important role in the expression of group II polysaccharides (14). Studies using subcloned fragments of the cloned K5 antigen gene cluster indicated that elevated CMP-KDO synthetase levels correlate with strains harboring region 1 (14). Whether the elevated levels of CMP-KDO synthetase are due to a structural gene within region 1 that encodes a capsule-specific CMP-KDO synthetase or to increased expression of the *kdsB* gene mediated by a regulator encoded within region 1 was hitherto unknown.

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic(s)	Source or reference
pGB110	$K5^+$ cosmid	31
pHEV1	Subclone of 7.7-kb *Eco*RV-*Hin*cII fragment that encodes K5 region 1 in pACYC184 (8)	This study
pCE30	Temperature-inducible expression vector	11
pCR3	Subclone of 5.4-kb *Bam*HI fragment from K5 region 1 that encodes KpsD, KpsU, and KpsC in pCE30 (11)	This study
pCR7	Subclone of 1.5-*Hin*cII fragment from K5 region 1 that encodes KpsS in pCE30 (11)	This study

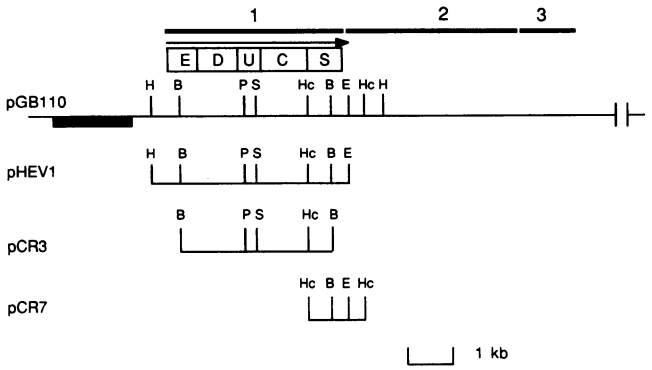

FIG. 1. Restriction map and subclones of the K5 region 1 genes. Solid lines indicate the DNA present in various plasmids described in the text. The uppermost map is part of that of pGB110, a cosmid that encodes K5 capsule production. Lines 1 to 3 indicate the functional regions within the K5 capsule gene cluster. Boxes E, D, U, C, and S are the five genes encoded in region 1, and the arrow denotes the direction of transcription. The closed box below the line refers to the plasmid vector sequence. Restriction sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; S, *Sph*I.

In this communication, we report the nucleotide sequence and organization of region 1 of the K5 capsule genes, the identification of a CMP-KDO synthetase structural gene within region 1, and the preliminary characterization of the five encoded proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. *E. coli* LE392 [F^- *hsdR51G* (m_K^- r_K^-) *supE44 supF58 lac galK2 galT22 metB1 trpR55*] was used as the host for all of the plasmids described in this report. *E. coli* DS410 was used as a source of minicells (39). *E. coli* JM101 [*thi supE* Δ(*lac proAB*) F′(*traD36 proAB*$^+$ *lacZ*ΔM15 *lacI*^{q})] was used for propagation of phages M13mp18 and M13mp19 (26, 40). The replicative forms of M13mp18 and M13mp19 were used as vectors to prepare DNA templates for sequence analysis. The plasmids used in this study are described in Table 1. All bacteria were grown in L broth supplemented with 100 μg of ampicillin per ml, 50 μg of chloramphenicol per ml, 50 μg of tetracycline per ml, or 50 μg of kanamycin per ml where appropriate.

DNA procedures. For large-scale preparations, plasmid DNA was purified by the method of Clewell and Helinski (9), while rapid small-scale purification was performed by the method of Birnboim and Doly (3). Restriction endonucleases and T4 DNA ligase were purchased from either Bethesda Research Laboratories or Pharmacia LKB Biotechnology and used in accordance with the manufacturer's instructions.

Nucleotide sequence analysis. Single-stranded M13 DNA templates were sequenced by the dideoxy-chain termination method (33) with [α-^{35}S]thio-dATP and modified T7 DNA polymerase, Sequenase version 2.0 (USB Corp.). The DNA fragments were analyzed with buffer gradient gels (2). Nucleotide sequences were analyzed with the Wisconsin (10) and Lipman-Pearson (25) molecular biology programs on the Vax VMS Cluster.

Expression of recombinant proteins. Minicells were prepared, labeled, and analyzed as described previously (39), while in vitro transcription-translation kits were purchased from Amersham UK Ltd. and used in accordance with the manufacturer's instructions.

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been assigned GenBank accession number X74567.

RESULTS AND DISCUSSION

Nucleotide sequence analysis. Since plasmid pHEV1, a subclone of pGB110 (Table 1; Fig. 1), was the smallest subclone capable of complementing all of the deletion and insertion mutations in region 1 of both the K1 and K5 antigen gene clusters (data not shown), K5 region 1 must be encoded on this *Hin*dIII-*Eco*RV fragment (Fig. 1). Appropriate overlapping restriction endonuclease cleavage fragments from this *Hin*dIII-*Eco*RV fragment were subcloned into M13mp18 and M13mp19, and the entire nucleotide sequence was determined on both strands. This fragment was 7,721 bp long and consisted of five open reading frames (ORFs) organized in a way consistent with a single transcriptional unit (Fig. 1 and 2). Analysis of the sequence 5′ to the first ORF (ORF1) identified a putative promoter 564 bp 5′ to ORF1 (Fig. 2). This putative promoter, TCCACAT-17 bp-AATAAT, is a good match for the consensus sequence TTGACAT-16 bp-TATAAT (28). The ATG codon of ORF1 was preceded by a likely Shine-Dalgarno (SD) sequence (Fig. 2) and encoded a protein with a predicted molecular mass of 42,995 Da. There was a gap of 23 bp between ORF1 and ORF2, with a likely SD sequence 6 bp 5′ to the initiation codon (Fig. 2). ORF2 was 1,674 bp long and encoded a protein of 558 amino acids with a predicted molecular mass of 60,514 Da. There was a gap of 12 bp between ORF2 and ORF3, with a candidate SD sequence 7 bp 5′ to the initiation codon (Fig. 2). ORF3 was 738 bp long and encoded a protein of 246 amino acids with a predicted molecular mass of 27,128 Da. The initiating ATG codon for ORF4 overlapped the TGA termination codon of ORF3 (Fig. 2). ORF4 was 675 bp long and encoded a protein of 2,025 amino acids with a predicted molecular mass of 75,684 Da. There was a gap of 101 bp between ORF4 and ORF5, with a candidate SD sequence 5 bp 5′ to the initiation codon (Fig. 2). ORF5 was 1,104 bp long and encoded a protein of 368 amino acids with a predicted molecular mass of 43,987 Da. No predicted stem-loop structures were detected in the 100-bp nucleotide sequence 3′ to ORF5. However, a predicted stem-loop structure was present within ORF4, 3′ to ORF3. This had an 11-bp stem with a single mismatch and a loop of 4 bp (Fig. 2). A number of potential

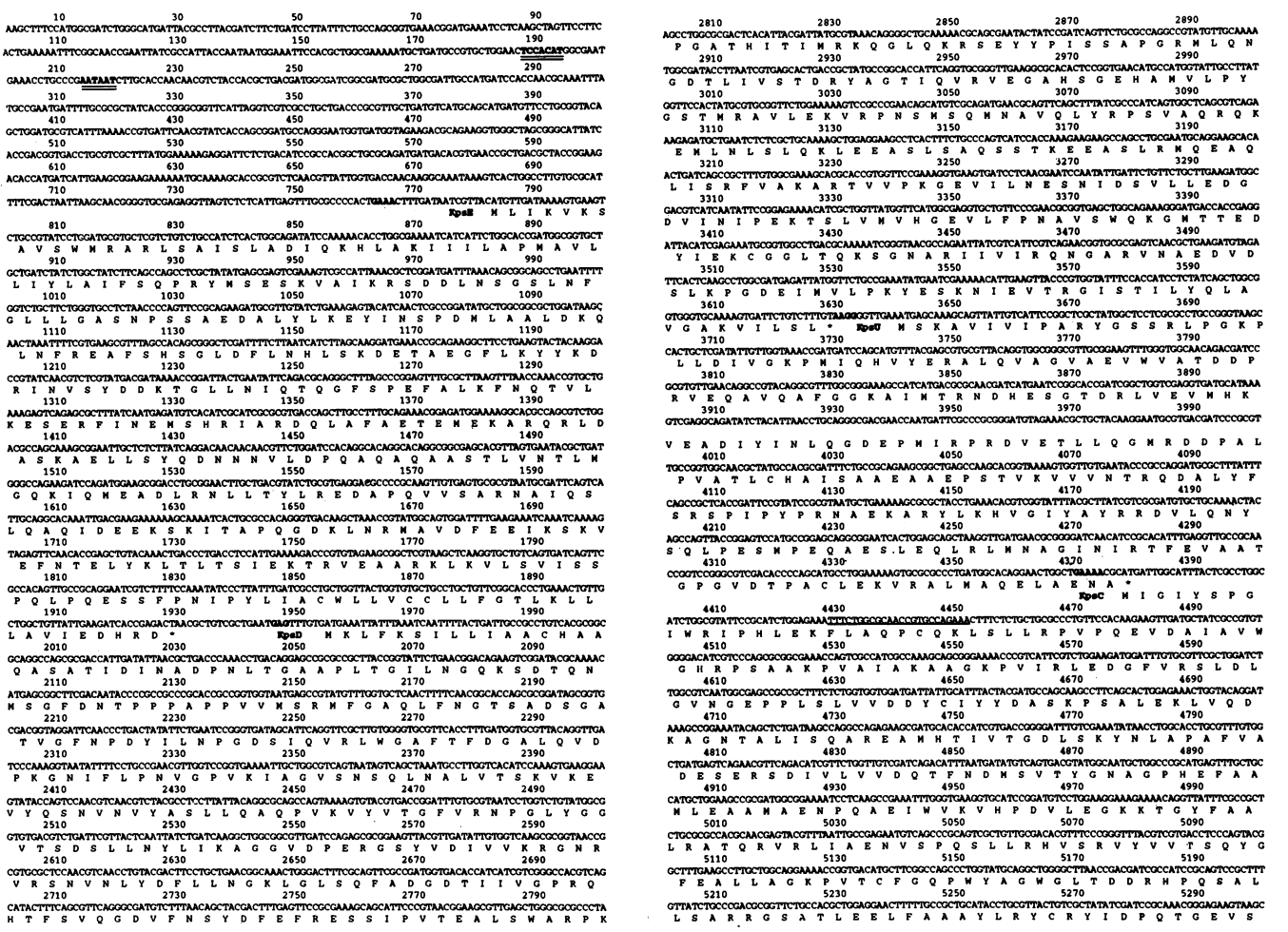

FIG. 2. Nucleotide sequence of the *Hin*dIII-*Eco*RV fragment that encodes region 1 of the *E. coli* K5 antigen gene cluster. The nucleotide sequence is numbered from 1 at the *Hin*dIII site to 7722 at the *Eco*RV site, and the predicted amino acid sequence of the five proteins is shown in the single-letter amino acid code below the nucleotide sequence. The potential −10 and −35 sequences are denoted by double underlining, and the Shine-Dalgarno ribosome-binding site for each gene is in boldface. The possible stem-loop structure within the *kpsC* gene is denoted by single underlining.

ORFs on the opposite strand which could encode small proteins with molecular masses ranging from 11.7 to 8 kDa (data not shown) were identified. No suitable SD sequences were identified 5′ to any of these small ORFs.

Expression of region 1-encoded proteins. Minicell analysis of plasmid pHEV1 (Fig. 1) revealed the presence of three non-vector-encoded proteins of approximately 27, 43, and 60 kDa (Fig. 3). This correlates well with the predicted molecular weights of the proteins encoded by the first three ORFs. To visualize the expression of the proteins encoded by ORF4 and ORF5, two additional plasmid constructs were made. First, the 5.4-kb *Bam*HI fragment containing ORF2, ORF3, and ORF4 (Fig. 1) was subcloned into temperature-inducible expression vector pCE30 (11) to generate plasmid pCR3 (Table 1). Minicell analysis of pCR3 at 42°C, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), revealed the presence of two non-vector-encoded proteins, a highly expressed protein of approximately 60 kDa and a less well-expressed protein of approximately 75 kDa (Fig. 4). No nonvector protein corresponding to the 27-kDa protein could be easily visualized, although this protein may have been masked by a number of vector-encoded proteins with similar molecular weights (Fig. 4). Second, to visualize the protein encoded by ORF5, the 1.5-kb *Hin*cII fragment from region 1 (Fig. 1) was subcloned into pCE30 to generate plasmid pCR7 (Table 1). Analysis of this plasmid in an in vitro transcription-translation system at 42°C revealed a single nonvector protein of 44 kDa (Fig. 5). Why the ORF4- and ORF5-encoded proteins could be identified only with an expression vector is unclear. Analysis of the codon usage of ORF4 and ORF5 showed it to be typical for *E. coli* (data not shown). One possibility is that the predicted stem-loop structure 3′ to the third ORF acts in part as a transcriptional terminator, thereby reducing transcription of the last two downstream ORFs. Such a system has been described for the *papB* operon in *E. coli*, where a stem-loop structure 3′ to the *papA* gene reduces transcription of the remaining downstream genes, thereby allowing differential gene expression within the *papB* operon (1). It is not clear whether this is also the case for expression of region 1-encoded proteins.

Analysis of the predicted amino acid sequences. In keeping with the nomenclature of Silver et al. (35), we propose that the 43-kDa protein be referred to as KpsE, the 60.5-kDa

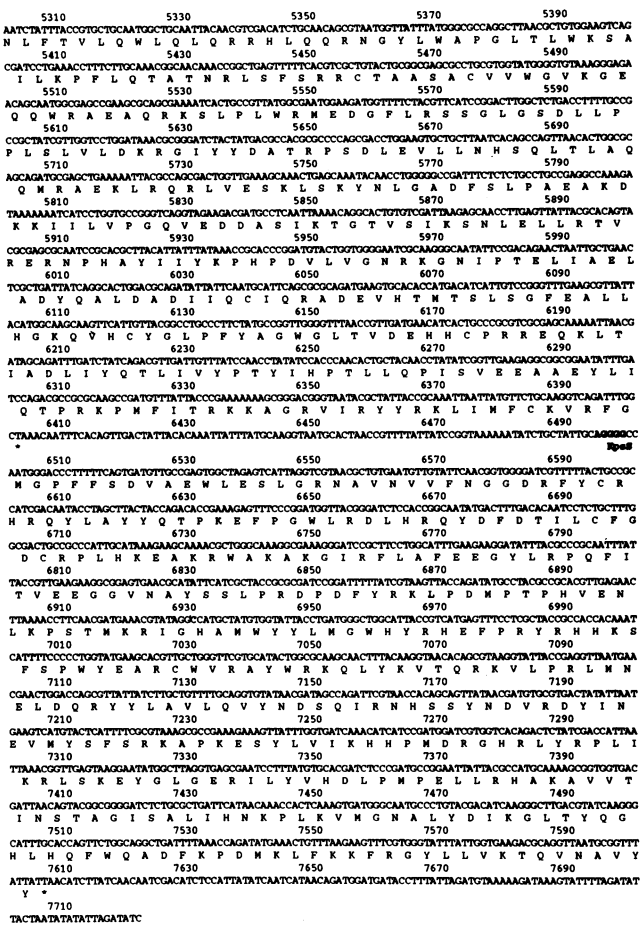

FIG. 2—*Continued.*

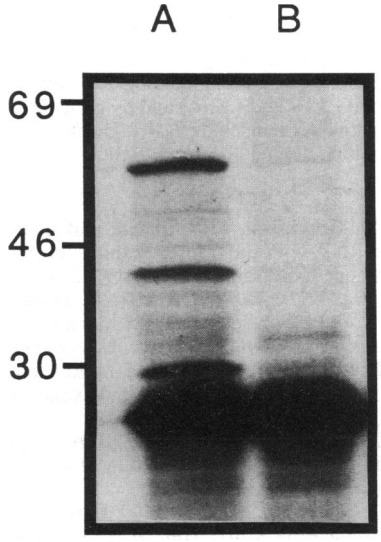

FIG. 3. SDS-PAGE analysis of proteins encoded by plasmids pHEV1 and pACYC184. Minicells were isolated from strain DS410 carrying pHEV1 (A) or pACYC184 (B) and labelled with [^{35}S]methionine, and the labelled polypeptides were separated by SDS-PAGE, which was followed by autoradiography. The numbers on the left refer to molecular mass markers in kilodaltons.

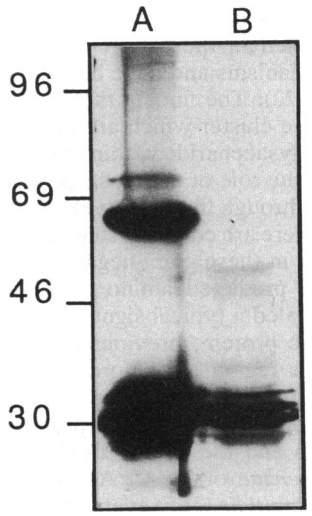

FIG. 4. SDS-PAGE analysis of proteins encoded by plasmids pCR3 and pCE30. Minicells were isolated from strain DS410 carrying pCR3 (A) or pCE30 (B) and labelled with [^{35}S]methionine, and the labelled polypeptides were separated by SDS-PAGE, which was followed by autoradiography. The numbers on the left refer to molecular mass markers in kilodaltons.

protein be referred to as KpsD, the 75.7-kDa protein be referred to as KpsC, and the 44-kDa protein be referred to as KpsS. We propose that the 27-kDa protein, which has not been identified previously, be referred to as KpsU. Analysis of the predicted amino acid sequence of KpsE showed the absence of a typical N-terminal signal sequence (Fig. 2). The hydropathy plot (24) revealed KpsE to be a relatively hydrophilic protein with N- and C-terminal hydrophobic domains (data not shown). Since both of these domains consist of approximately 20 amino acids in an α-helical configuration, they are of sufficient size to span the inner membrane and potentially act as membrane anchors for this protein. Comparisons, done with the sequence alignment program of Lipman and Pearson (25), between the predicted amino acid sequences of KpsE and BexC of *H. influenzae* and CtrB of *N. meningitidis* revealed that KpsE was 22.1% identical and 73.2% homologous to BexC over 359 amino acids and 26.8% identical and 73.2% homologous to CtrB over 355 amino acids. In addition, the hydropathy plots of the BexC and CtrB proteins are similar to that of KpsE,

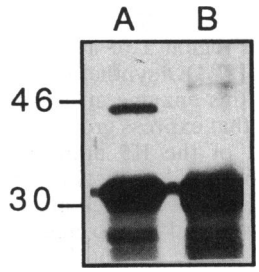

FIG. 5. SDS-PAGE analysis of proteins encoded by plasmids pCR7 and pCE30. The proteins encoded by plasmids pCR7 (A) and pCE30 (B) were detected with an in vitro transcription-translation system at 42°C, separated by SDS-PAGE, and subjected to autoradiography. The numbers on the left refer to molecular mass markers in kilodaltons.

having hydrophobic N and C termini (15, 22). These proteins have been implicated in the export of polysaccharides in these two microorganisms and have been located within the cell envelope (15, 22). The finding that deletion mutants of the K1 antigen gene cluster which are predicted to lack the *kpsE* gene have polysaccharide within the periplasmic space (6) would support the role of the KpsE protein in the export of polysaccharide through the cell envelope. Together, these data suggest that there are common steps in the translocation of polysaccharides in these gram-negative bacteria.

Analysis of the predicted amino acid sequence of the KpsD protein revealed a typical signal sequence (Fig. 2). A 60-kDa periplasmic protein, previously termed KpsD, has been identified as a region 1 gene product important in the export of group II polysaccharides (36). No significant homology was detected between the predicted amino acid sequence of KpsD and the BexA, BexB, BexC, or BexD protein of *H. influenzae* or the CtrA, CtrB, CtrC, or CtrD protein of *N. meningitidis*. However, between residues 234 to 302, KpsD was 25% identical and 67% homologous to PgpB of *E. coli*. The PgpB protein of *E. coli* is phosphatidylglycerophosphate B phosphatase, which hydrolyzes phosphatidylglycerophosphate, PA, and lysophosphatidic acid (19). Although the homology between these two proteins is low, it is localized to the hydrophilic domain of PgpB, which is thought to interact with PA (19). Since group II K antigens are linked to PA at the reducing terminus (12, 20, 23), it is possible that the KpsD protein interacts with polysaccharide in the periplasm via PA. In addition, between residues 101 and 200, KpsD was 29.3% identical and 67% homologous to the ExoF protein of *Rhizobium meliloti*. This is a periplasmic protein that is involved in the export of succinoglycan, a cell surface polysaccharide of *R. meliloti* (27). However, in the absence of a clearer picture of the functional role of ExoF in succinoglycan export, the significance of this homology is unclear.

Both the hydropathy plot (data not shown) and Gravy score analysis (24) (value, −0.22) of the predicted amino acid sequence of the KpsU protein are consistent with a cytosolic location. Protein sequence data base searches revealed 44.3% identity between KpsU and the CMP-KDO synthetase protein encoded by the *E. coli kdsB* gene, and taking conservative amino acid changes into account, the two proteins are 70.7% homologous. Comparison of the respective nucleotide sequences demonstrated 63.5% identity over 540 bp. The interpretation that KpsU is a functional CMP-KDO synthetase enzyme is supported by the finding that the predicted N-terminal amino acid sequence of the KpsU protein is in agreement with the N-terminal sequence of the purified capsule-specific CMP-KDO synthetase (32a). The identification within region 1 of a structural gene that encodes a second CMP-KDO synthetase enzyme explains the increased levels of this enzyme at capsule-permissive temperatures in strains that express group II K antigens or carry subcloned region 1 of the K5 antigen gene cluster. The relatively high DNA homology between the *kdsB* and *kpsU* genes suggests that the two genes are products of a gene duplication. The predicted amino acid sequence of KpsU was also 21.1% identical and 66.1% homologous to the CMP-*N*-acetylneuraminic acid synthetase encoded by K1 antigen region 2 (41). This is perhaps not surprising, since both enzymes catalyze the generation of CMP sugar nucleotides. The presence of *kpsU*, which encodes a second CMP-KDO synthetase enzyme, in addition to that encoded by *kdsB*, which functions in LPS biogenesis, may allow independent temperature regulation of group II capsule expression.

The hydropathy plot (data not shown) and Gravy score (24) (value, −0.21) of KpsC revealed a hydrophilic protein. This, combined with the lack of an obvious signal sequence, is consistent with a cytosolic location for this protein. Protein data base searches identified 38.1% identity and 75.9% homology between KpsC and the LpsZ protein of *R. meliloti* over 312 amino acids. LpsZ is a cytoplasmic protein which plays a poorly defined role in modifying rhizobial LPS (7). In the absence of a clearer picture of the role of LpsZ, the significance of this homology is unclear.

The predicted amino acid sequence of the KpsS protein does not contain an N-terminal signal sequence (Fig. 2). Analysis of the hydropathy plot revealed a hydrophilic protein lacking pronounced hydrophobic domains (data not shown). Data base searches showed no significant homology to any other proteins. Mutations within the *kpsS* gene of the K1 antigen gene cluster result in a decrease in endogenous sialyltransferase activity (38), and it has been postulated that the KpsS protein interacts with both the sialyltransferase enzyme and other K1-encoded proteins to form a multicomponent enzyme complex associated with the inner membrane (38). It is not clear whether this is also the case for K5 antigen expression. However, since *kpsS* appears to be conserved between different group II K antigen gene clusters (32), KpsS must be involved in a common stage in the expression of these polysaccharides. Therefore, determination of the nucleotide sequence and preliminary characterization of the predicted amino acid sequences of the encoded proteins have begun to shed light on the genetic organization of this region and on the possible functions of the encoded proteins. However, determination in more detail of the precise roles of the individual proteins encoded by region 1 requires the generation of a battery of well-characterized nonpolar mutations within this operon. Detailed biochemical analysis of the phenotypes of such mutants will then allow the role of these proteins in capsule expression to be elucidated.

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