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Activity of CMP-2-Keto-3-Deoxyoctulosonic Acid Synthetase in *Escherichia coli* Strains Expressing the Capsular K5 Polysaccharide: Implication for K5 Polysaccharide Biosynthesis

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The activity of the cytoplasmic CMP-2-keto-3-deoxyoctulosonic acid synthetase (CMP-KDO synthetase), which is low in *Escherichia coli* rough strains such as *E. coli* K-12 and in uncapsulated strains such as *E. coli* O111, was significantly elevated in encapsulated *E. coli* O10:K5 and O18:K5. This enzyme activity was even higher in an *E. coli* clone expressing the K5 capsule. This and the following findings suggest a correlation between elevated CMP-KDO synthetase activity and the biosynthesis of the capsular K5 polysaccharide. (i) Expression of the K5 polysaccharide and elevated CMP-KDO synthetase activity were observed with bacteria grown at 37°C but not with cells grown at 20°C or below. (ii) The recovery kinetics of capsule expression of intact bacteria, in vitro K5 polysaccharide-synthesizing activity of bacteria, and CMP-KDO synthetase activity of bacteria after temperature upshift from 18 to 37°C were the same. (iii) Chemicals which inhibit capsule (polysaccharide) expression also inhibited the elevation of CMP-KDO synthetase activity. The chromosomal location of the gene responsible for the elevation of this enzyme activity was narrowed down to the distal segment of the transport region of the K5 expression genes.

The capsular polysaccharides of *Escherichia coli* are divided into two groups, as characterized by several criteria, one of them being the chromosomal location of their genes (4, 5). Polysaccharides of group II are determined by a gene complex, *kpsA* (7–9), near the *serA* locus. With few exceptions, the polysaccharides of group II are expressed at 37°C but not at capsule-restrictive temperatures below 20°C. The K5 capsular polysaccharide is a member of the temperature-regulated group II polysaccharides. Its repeating unit, 4)-βGlcUA-(1,4)-αGlcNAc-(1, (19), is identical to that of the first polymeric intermediate in the biosynthesis of heparin (11). Consequently, the K5 polysaccharide is practically nonimmunogenic. Thus, *E. coli* strains expressing the K5 capsule are very virulent.

The organization of the genetic determinants of the K5 antigen has been analyzed (1, 16, 17). We have found that the K5 gene complex consists of three regions: region 2, directing the biosynthesis (polymerization) of the polysaccharide, and flanking regions 1 and 3, responsible for the translocation of the finished polysaccharide to the bacterial surface. Whereas region 2 is specific for the type of the capsular (K) polysaccharide, the other two regions harbor genes which determine reactions common to the expression of all group II capsular polysaccharides.

During our study on the biosynthesis of the capsular K5 polysaccharide we analyzed the product of in vitro incubations of membranes with uridine diphosphoglucuronic acid (UDPGlcA) and uridine diphospho-*N*-acetylglucosamine (UDPGlcNAc). As described elsewhere (A. Finke et al., manuscript in preparation), 2-keto-3-deoxyoctulosonic acid (KDO) was found to be the reducing sugar of the K5 polysaccharide synthesized in vitro by extension at the nonreducing end. KDO was also found to be the reducing sugar of the polysaccharide isolated from the bacteria grown in liquid culture. Since it stands to reason that the sugar on the reducing end of a polysaccharide growing at the nonreducing end is the first one to be incorporated during polysaccharide biosynthesis, we postulated as a working hypothesis that KDO participates in the initial reaction of the biosynthesis of the capsular K5 polysaccharide. The activated form of KDO is CMP-KDO, which is formed in the bacterial cytoplasm by CMP-KDO synthetase from KDO and CTP (2, 3, 13). Therefore, we analyzed the activity of this enzyme in the cytosol fraction of homogenates from *E. coli* K5 strains (13) and compared it with that in *E. coli* O111, the reference strain for the isolation of CMP-KDO synthetase (3). In this communication we report that the level of CMP-KDO synthetase activity is significantly elevated in *E. coli* K5 wild-type strains and still more in bacteria carrying the cloned K5 genes. The enzyme level is increased at 37°C but not at 18°C, indicating a temperature regulation similar to that in the biosynthesis and cellular expression of the capsular K5 polysaccharide.

MATERIALS AND METHODS

Chemicals. Chloramphenicol, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2-keto-3-deoxyoctonate ammonium salt (KDO), and dithiothreitol were from Sigma, Deisenhofen, Federal Republic of Germany. Tris, sodium borohydride, CTP, UDPGlcA, and UDPGlcNAc were from Serva, Heidelberg, Federal Republic of Germany. Sodium arsenate and sodium azide were from E. Merck AG, Darmstadt, Federal Republic of Germany. UDP[^{14}C]GlcA was from Amersham Buchler, Braunschweig, Federal Republic of Germany. The scintillation cocktail Rotiszint 22 was from Roth, Karlsruhe, Federal Republic of Germany.

Bacteria and cultivation. *E. coli* O18:K5:H$^-$ (2980), O10:K5:H$^-$ (20026), and O111 (2125) as well as *E. coli* K-12 strains LE392 (21241) and HB101 (20028) were used. *E. coli* O111 is an uncapsulated smooth strain which was used, together with the *E. coli* K-12 rough strains, as a basis of reference for CMP-KDO synthetase activity (13). *E. coli*

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LE392(pGB118) (21484) expressing the K5 polysaccharide as well as deletion mutants derived from the K5 clone LE392(pGB118) are described in Results. The bacteria were grown in L broth with 1% glucose, with the addition of antibiotics when necessary (see Results).

DNA manipulation. Restriction enzymes and DNA ligase (obtained from BRL Ltd.) were used in accordance with manufacturer recommendations. Electrophoretic analysis of DNA, plasmid preparation, and transformation procedures were carried out as described previously (1, 16, 17).

Preparation of cytosol fractions as a source of CMP-KDO synthetase. Bacteria were grown to the late logarithmic phase (optical density at 600 nm, 1 to 1.1; 3 to 4 h at 37°C, ca. 16 h at 18°C) and centrifuged (10,000 × *g*, 10 min, 4°C). The sediment was suspended in the same volume of 50 mM Tris (pH 8.0)–2 mM dithiothreitol–30 mM magnesium acetate and centrifuged (10,000 × *g*, 10 min, 4°C). This step was repeated once, and the final sediment was suspended in the same buffer (approximately 1/20 of the original volume; approximate density, 10^{10} bacteria per ml). The bacteria were disrupted by three passages of the dense suspension through a French pressure cell (Aminco) at 75 kg/cm^2 (11,000 lb/in^2). The homogenate was centrifuged for 10 min at 10,000 × *g*, and 4°C to remove bacterial debris and then for 60 min at 180,000 × *g* and 4°C. The clear supernatant (cytosol fraction) was kept in small portions at −20°C. The enzyme activity did not decrease over a period of several weeks.

Preparation of membranes for in vitro biosynthesis experiments. The French pressure cell homogenate described above was also used for the preparation of membranes. The sediment of the final ultracentrifugation was suspended in 50 mM Tris (pH 8.0)–2 mM dithiothreitol–30 mM magnesium acetate to a concentration of 3 to 5 mg of protein per ml. These membrane fractions were kept in portions of 0.500 ml at −80°C. They were stable for several months.

Determination of CMP-KDO synthetase. The enzyme test was performed as described by Ray and Benedict (13). In brief, samples (60 μl) of the cytosol were added to a mixture containing KDO (2 mM), CTP (10 mM), and Tris acetate (pH 9.8, 200 mM) in a total volume of 100 μl. After 15 min at 30°C, the reaction was stopped by the addition of 200 μl of ice-cold ethanol. Unreacted KDO was reduced with sodium borohydride, and the excess sodium borohydride was destroyed at 4°C with phosphoric acid (final concentration, 4 N). When the temperature was raised to 37°C the CMP-KDO formed in the enzyme reaction was hydrolyzed. The KDO thus liberated was determined with thiobarbituric acid reagent.

Temperature upshift experiment. The bacteria were grown at 18°C to an optical density at 600 nm of 0.5, centrifuged (10,000 × *g*, 10 min, 4°C), and suspended in fresh medium to a density of 3 × 10^8/ml. Samples of this suspension (75 ml) were warmed to 37°C in a water bath. After various times (see Results) the suspensions were added to 3 volumes of ice-cold 50 mM Tris (pH 8.0)–2 mM dithiothreitol–30 mM magnesium acetate and centrifuged, and the sediments were suspended in 3 ml of the same buffer. After disintegration with the French pressure cell and ultracentrifugation, CMP-KDO synthetase activity was determined in the supernatants (cytosol fraction) as described above.

Temperature upshift experiments in the presence of inhibitors. For inhibition studies, the fresh medium in which the bacteria were suspended at 18°C prior to the temperature shift contained 150 μM chloramphenicol, 50 μM CCCP, 5 mM sodium azide, or 5 mM sodium arsenate. The suspensions were shifted to 37°C and kept at this temperature for 60 min. After that time the bacteria were collected by centrifugation and disintegrated, and the enzyme activities were determined as described above. Controls were subjected to the same treatments in the absence of inhibitors.

TABLE 1. CMP-KDO synthetase activity in *E. coli* strains grown at 18 or 37°C

Strain	Description	Activity (nmol of CMP-KDO/mg of protein in 15 min) at:	
		18°C	37°C
2125	O111	ND[a]	57.0
21241	LE392 (K-12)	55.8	35.1
20028	HB101 (K-12)	ND	33.0
20026	O10:K5	ND	391.8
2980	O18:K5	39.9	232.2
21484	LE392(pGB118)	66.3	847.2

[a] ND, Not determined.

In vitro synthesis of the K5 polysaccharide. The incubation of membranes with UDP[^{14}C]GlcA and UDPGlcNAc was performed as described in detail elsewhere (Finke et al., in preparation). In brief, a mixture containing membranes (150 to 200 μg of protein), Tris (pH 8.0) (40 mM), magnesium acetate (24 mM), and dithiothreitol (1.6 mM) was incubated with UDP[^{14}C]GlcA (5 μM, 50,000 cpm) and UDPGlcNAc (5 μM) in a total volume of 100 μl. After 30 min at 37°C, 12% acetic acid was added to terminate the reaction and the mixture was filtered through cellulose acetate filters (450-nm pore size; Sartorius). After the filters were washed with 12% acetic acid, the radioactivity retained on them was counted with a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) with Rotiszint 22 as the scintillation cocktail.

The membranes used in the in vitro assay were obtained from bacteria grown at 37 or 18°C or for various times after temperature upshift of the respective bacteria.

RESULTS

Determination of CMP-KDO synthetase in wild-type strains. Other experiments (18; Finke et al., in preparation) showed that the capsular K5 polysaccharide has KDO as the reducing sugar. Since KDO is not a constituent of the repeating unit of the K5 polysaccharide, it was assumed that this sugar participates in the initial reaction of K5 biosynthesis. Therefore, the activity of CMP-KDO synthetase was tested in the cytosol fraction of homogenates from *E. coli* strains expressing the K5 polysaccharide. As a reference, *E. coli* 2125 was chosen, since the isolation and purification of CMP-KDO synthetase have been described in this strain (3). The determination of enzyme activity was based on the fact that reduced KDO is not reactive in the thiobarbituric acid reaction (2). Thus, after incubation of the cytosol fraction containing the enzyme with CTP and KDO, unreacted KDO was reduced with sodium borohydride and excess KDO was destroyed with phosphoric acid at 4°C. The CMP-KDO formed in the enzymatic reaction was hydrolyzed, and the liberated KDO was determined with the thiobarbituric acid reaction. The CMP-KDO synthetase activity was expressed as nanomoles of CMP-KDO formed (KDO was determined after reduction and hydrolysis) per milligram of protein in 15 min.

CMP-KDO synthetase activity was very low in the cytosol

of *E. coli* 2125 (Table 1). This result is in accord with the data reported for crude CMP-KDO synthetase prior to enrichment (2). Comparably low values were found in the cytosol fractions of two *E. coli* K-12 (rough) strains, 21241 and 20028. In contrast, the cytosol fractions of two other *E. coli* strains (2980 and 20026) which express the capsular K5 polysaccharide exhibited significantly elevated CMP-KDO synthetase activity.

Dependence of CMP-KDO synthetase activity on growth temperature. Most group II capsular polysaccharides (4, 5), of which the K5 polysaccharide is one, are not expressed when bacteria are grown at temperatures below 20°C (6). If our assumption that the activity of CMP-KDO synthetase is related to capsule biosynthesis is correct, the possibility exists that this enzyme activity is also low in *E. coli* K5 grown at a capsule-restrictive temperature. We therefore tested the activity of CMP-KDO synthetase in the cytosol fractions of *E. coli* 2980 after growth at 18°C and found that this enzyme had only background activity, i.e., the same as in *E. coli* 2125 and 21241. The enzyme activity in the cytosol fractions of the latter remained unchanged after growth at 18°C. The values are included in Table 1.

It was found that although *E. coli* K1 and K5 (21; K. D. Kröncke et al., manuscript in preparation) do not express their K polysaccharides at 18°C, they regain the capacity of capsule expression after a shift of the growth temperature to 37°C. We wanted to know whether such a recovery of activity could also occur for CMP-KDO synthetase. Therefore, a temperature shift experiment was used for the analysis of this enzyme activity. CMP-KDO synthetase activity was measurable about 15 min after the temperature upshift, increased steadily, and was fully expressed after about 50 min (Fig. 1A). In an attempt to correlate the kinetics of the recovery of enzyme activity with the kinetics of the overall biosynthesis of the K5 polysaccharide, we studied the corresponding membrane fractions obtained at the same time periods after the temperature upshift. The recovery of K5 biosynthesis and that of CMP-KDO synthetase activity followed a similar time course (Fig. 1B). Although the recoveries could not be compared directly, it appeared as if the onset of CMP-KDO synthetase activity preceded that of the complex K5 biosynthesis.

The recovery of elevated CMP-KDO synthetase activity by the temperature upshift could be inhibited by chloramphenicol, CCCP, sodium azide, or sodium arsenate. Table 2 shows that each of these reagents inhibited the enzyme recovery almost completely. A similar inhibitory effect was observed for the in vitro polymerase activity of membranes from bacteria which had undergone a temperature upshift in the presence of the inhibitors.

CMP-KDO synthetase activity in a K5 clone and various subclones and mutants. The cloning and characterization of the K5 genes have been described previously (16, 17). *E. coli* 21484 but not 21241 fully expressed the capsular K5 polysaccharide. Electron microscopic studies (Kröncke et al., in preparation) indicated that many bacteria overexpressed the capsule, some forming community capsules which engulfed more than one bacterium. We therefore wanted to know the level of CMP-KDO synthetase activity in the cytosol fraction of *E. coli* 21484. The value found was distinctly higher than those found for the *E. coli* K5 wild-type strains (Table 1). Whereas the activity in wild-type strains was about 5 to 8 times higher than the background values (e.g., of *E. coli* 2125), that in *E. coli* 21484 was about 15 times higher. The time course for activity to recover in the temperature shift experiment was the same as that shown in Fig. 1 for the wild-type strain.

To locate the sequences in plasmid pGB118 responsible for elevated CMP-KDO synthetase activity, we constructed various subclones and deletion derivatives of this plasmid. Plasmid pGB137 was isolated by cleaving pGB118 with *Bgl*II and, following circularization of the cleavage products, DNA was introduced in LE392. Bacteria carrying the required deletion were identified by restriction enzyme analysis of plasmids in transformants. Plasmids pGB130 and pGB126 were generated by cloning either *Hin*dIII or *Bgl*II fragments into vectors pBR328 and pACYC184, respectively, while pBA2 and pBA19 were generated by cloning *Bam*HI-generated fragments of pGB118 into vector pACYC184. The structures of the recombinants were verified by restriction enzyme analysis. The plasmids are shown in Fig. 2. The CMP-KDO synthetase activity of the cytosol fractions from these strains are shown in Table 3. The results indicated that the site determining CMP-KDO synthetase is at the border between regions 1 and 2, probably still within region 1. It was evident that region 3 does not harbor the gene governing the high activity of CMP-KDO synthetase.

DISCUSSION

The studies described here were triggered by the following results (18; Finke et al., in preparation) obtained during our studies on the biosynthesis of the capsular K5 polysaccharide, which has the structure 4)-βGlcUA-(1,4)-αGlcNAc-(1, (19). (i) Polymerization occurs at the nonreducing end of the polysaccharide. (ii) The reducing sugar of the polysaccharide is neither GlcUA nor GlcNAc but KDO, which is not a constituent of the repeating unit. These findings prompted us to assume as a working hypothesis that KDO may be the first sugar transferred in the biosynthesis of the K5 polysaccharide and may thus participate in the initial reaction. Since the activated form of KDO is CMP-KDO, formed in the cytosol from CTP and KDO by CMP-KDO synthetase (2, 3, 13), we wanted to know whether there is a correlation between the activity of CMP-KDO synthetase and the activity of the enzyme complex synthesizing the K5 polysaccharide. Since KDO is a constituent of the lipopolysaccharides in all gram-negative bacteria (15, 20) and is essential for the biosynthesis of lipid A and for membrane integrity (10, 12, 14), CMP-KDO synthetase activity is found in the cytosol of all gram-negative bacteria. The activity is, however, low and can be demonstrated to a significant extent only after enrichment, as was shown with *E. coli* O111 (2, 13). Therefore, we compared the CMP-KDO synthetase activity in the cytosol of two wild-type *E. coli* strains (2980 and 20026) with that in the cytosol of uncapsulated *E. coli* O111 (2125) and two *E. coli* K-12 rough strains (21241 and 20028). The results showed that CMP-KDO synthetase activity, which was very low in the rough and uncapsulated smooth *E. coli* strains, was significantly elevated in the two *E. coli* strains with the K5 capsule and still more in the *E. coli* clone expressing this polysaccharide.

A typical characteristic of most group II capsular polysaccharides, of which the K5 polysaccharide is one, is that they are not expressed at 20°C and below (6). If the activity of CMP-KDO synthetase and the biosynthetic system producing the K5 polysaccharide are correlated, the possibility exists that CMP-KDO synthetase activity may also be subject to temperature regulation. This was found to be the case. The activity in preparations of the two *E. coli* K5 strains grown at 18°C was as low as that in preparations from

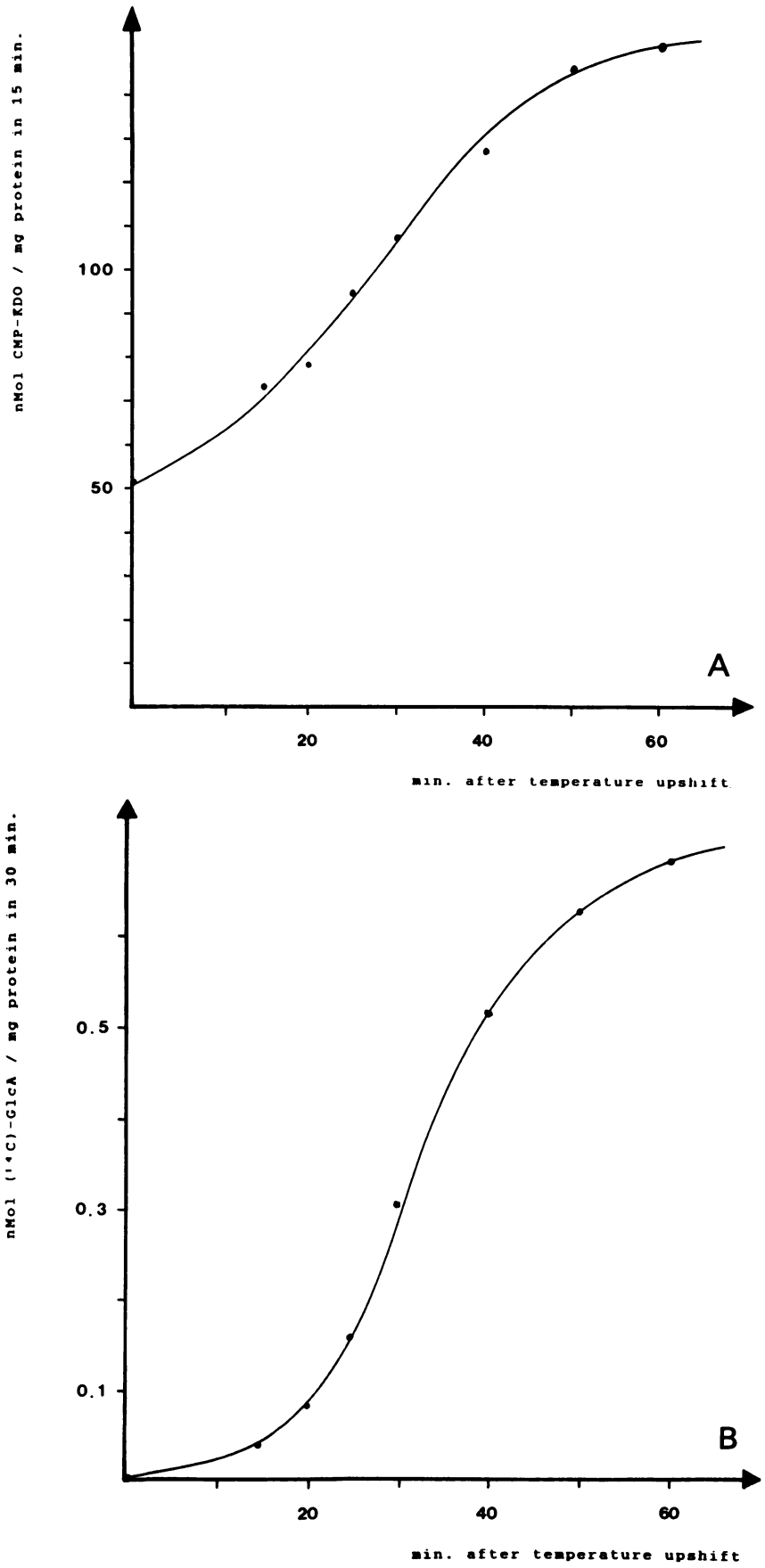

FIG. 1. Recovery kinetics of CMP-KDO synthetase (A) and glycosyltransferase (B) activities in a temperature upshift experiment with a K5 wild-type strain (2980).

TABLE 2. CMP-KDO synthetase and glycosyltransferase activities in a temperature upshift experiment with a K5 wild-type strain (2980) cultivated in the presence of different inhibitors

Inhibitor (mM)	Activity of:	
	CMP-KDO synthetase (nmol/mg of protein in 15 min)	Glycosyltransferase (pmol/mg of protein in 30 min)
None (control 1)[a]	64.2	11
None (control 2)[a]	163.6	565
Chloramphenicol (0.150)	39.6	1
CCCP (0.050)	56.0	5
Azide (5)	66.8	26
Arsenate (5)	66.8	80

[a] Control 1, No temperature upshift; control 2, temperature upshift.

the non-K5 strains studied. The time course of recovery of the CMP-KDO synthetase activity was comparable to that of the surface expression of the K5 capsule (6, 19; Kröncke et al., in preparation). The biosynthetic activity of membranes from corresponding bacterial suspensions also showed practically the same recovery kinetics.

The expression of the capsule after a temperature shift to 37°C can be blocked with inhibitors of protein biosynthesis and with energy decouplers (21; Kröncke et al., in preparation). Accordingly, we tested the activity of CMP-KDO synthetase in the cytosol of bacteria in the presence of chloramphenicol, CCCP, sodium azide, or sodium arsenate. Complete inhibition of recovery of the enzyme activity was achieved with all inhibitors. It is noteworthy that the K5 polysaccharide-synthesizing enzyme complex in membranes from *E. coli* K5 grown at 18°C and shifted to 37°C in the presence of the inhibitors was also completely blocked. Thus, CMP-KDO synthetase may be a pivotal enzyme in the biosynthesis of the K5 capsule.

TABLE 3. CMP-KDO synthetase activity of LE392(pGB118) (K5 clone) and some mutants derived from this clone (see Fig. 2)

Strain	Plasmid	Activity (nmol of CMP-KDO/mg of protein in 15 min)
21484	pGB118	847.2
21549	pGB137	973.5
21518	pGB130	27.9
21515	pGB126	527.1
21722	pBA2	33.0
21723	pBA19	39.6
21485	pGB118::Tn*1000*-1	30.9

The finding that CMP-KDO synthetase activity was very high in the K5 clone 21484 but very low in the parent strain 21241 indicated that the gene responsible for elevated CMP-KDO synthetase must be within the K5 gene complex. The increase in enzyme activity may have been due to the presence in the K5 gene complex of a gene which may have one of two functions: (i) coding for a CMP-KDO synthetase in addition to and different from the one engaged in lipopolysaccharide biosynthesis (10, 12, 14) and (ii) determining a regulator of the lipopolysaccharide-related enzyme and increasing its activity. At present we cannot distinguish between these two possibilities.

Mapping of the K5 gene complex had shown that it has the same organization as occurs in other *E. coli* strains expressing group II capsular polysaccharides (1, 16, 17). A region of about 8 kilobase pairs (region 2) which directs the polymerization of the K5 polysaccharide is flanked by two regions (region 1, of about 8 to 9 kilobase pairs, and region 3, of about 2 kilobase pairs) which direct the translocation of the polysaccharide to the bacterial surface.

Analysis of deletion mutations spanning all three regions showed that the gene responsible for increased CMP-KDO

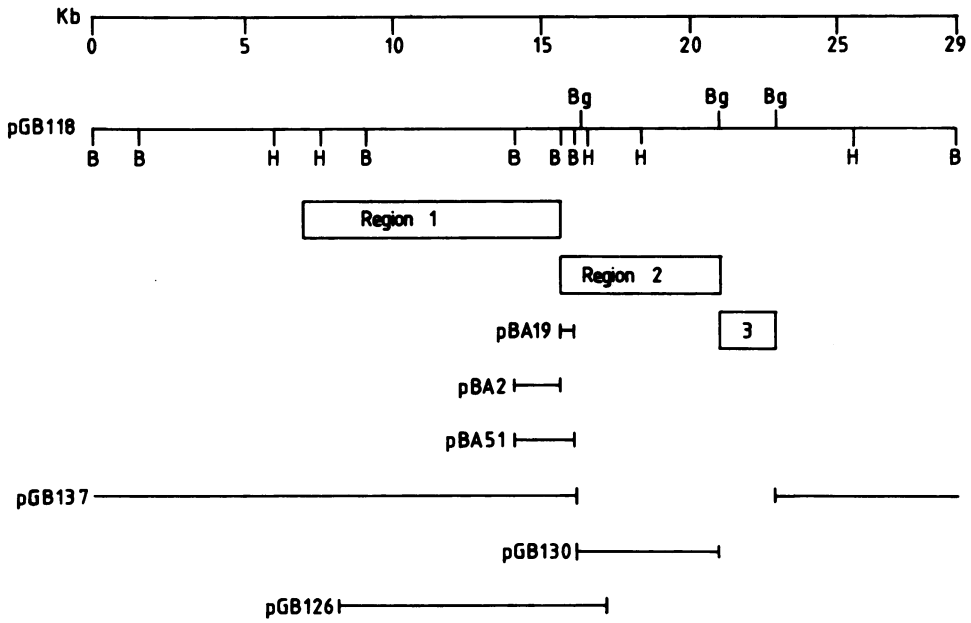

FIG. 2. Map of the K5 capsule gene cluster. The scale at the top is in kilobase pairs (Kb). Regions 1 to 3 denote regions involved in the biosynthesis of the K5 polysaccharide and are described in the text. Also shown are various subclones and deletion plasmids. The restriction sites are denoted as follows: B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II.

synthetase activity is at the site joining regions 1 and 2, possibly at the distal end of region 1. Since the presence in *E. coli* 21241 of pBA2 and pBA19, derived from this region, did not result in elevated levels of CMP-KDO synthetase activity, the determinants required probably span these two *Bam*HI fragments in pGB118.

Taken together the results indicate the participation of CMP-KDO, formed in the cytosol by CMP-KDO synthetase, in the biosynthesis of the K5 polysaccharide. The most likely stage at which KDO is incorporated into the K5 polysaccharide is during the initial reaction. We therefore assume that the biosynthesis of the K5 polysaccharide and possibly also of other group II capsular polysaccharides is initiated by the transfer of KDO from CMP-KDO to an acceptor, which is presumably a membrane constituent, the nature of which is still unknown. This hypothesis is amenable to experimental control; studies to this effect are currently being carried out in our laboratories.

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