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Expression of Genes *kdsA* and *kdsB* Involved in 3-Deoxy-D-*manno*-Octulosonic Acid Metabolism and Biosynthesis of Enterobacterial Lipopolysaccharide Is Growth Phase Regulated Primarily at the Transcriptional Level in *Escherichia coli* K-12

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We have cloned and sequenced a cluster of six open reading frames containing gene *kdsA* from *Escherichia coli* K-12. The gene encodes 3-deoxy-D-*manno*-octulosonate 8-phosphate synthetase (KDO-8-phosphate synthetase), which catalyzes formation of 3-deoxy-D-*manno*-octulosonic acid (KDO), an essential component of enterobacterial lipopolysaccharide. We have also identified two other genes, *hemA* and *prfA*, at the beginning of the cluster. Deletion analysis shows that *kdsA*, the terminal gene of this putative operon, is transcribed from its own promoter located within the cluster rather than from two promoters preceding this group of six open reading frames. Northern (RNA) blot analysis as well as *lacZ* operon fusion experiments reveal that the expression of gene *kdsA* occurs maximally in the early log phase and falls to a low level in the late log and stationary phases. Hence, this gene is subjected to growth phase-dependent regulation at the transcriptional level. Similarly, we show that expression of gene *kdsB*, which codes for the CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidyltransferase (CMP-KDO-synthetase), is also growth regulated. This enzyme catalyzes the activation of KDO via formation of CMP-KDO, which is necessary for the incorporation of KDO into lipid A. We have identified the promoter of gene *kdsB*, whose expression is growth regulated in the same way as that of *kdsA*. Despite the fact that transcription of genes *kdsA* and *kdsB* is shut off as cells enter stationary phase, KDO-8-phosphate synthetase as well as CMP-KDO-synthetase activities are still present at various levels during stationary-phase growth of an *E. coli* K-12 culture.

A major component of the outer membrane of enterobacteria is lipopolysaccharide (LPS), the so-called endotoxin, which is responsible for the pathogenic properties of gram-negative bacteria (38). This macromolecule consists of two structural segments, the hydrophobic lipid A part and the hydrophilic core oligosaccharide chain to which the O-antigenic polysaccharide chains may be attached. Lipid A and the core oligosaccharides are linked through two or three molecules of the eight-carbon sugar 3-deoxy-D-*manno*-octulosonic acid (KDO) (31, 38). KDO is an essential component of LPS. This is reflected by the fact that only conditionally lethal KDO biosynthesis mutants can be isolated (25, 36, 37). KDO is enzymatically synthesized in the cytoplasm in two steps, i.e., formation of KDO-8-phosphate by the condensation of D-arabinose-5-phosphate and phosphoenol pyruvate and subsequent dephosphorylation by a specific phosphatase to give KDO. The enzymes involved in this biosynthetic pathway are 3-deoxy-D-*manno*-octulosonate 8-phosphate synthetase (KDO-8-phosphate synthetase) (32) and KDO-8-phosphate phosphatase (33). Subsequent incorporation into lipid A requires activation of KDO by CTP in a reaction catalyzed by the cytosolic enzyme CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidyltransferase (CMP-KDO-synthetase) (17, 34, 35). KDO is finally incorporated into lipid A by a KDO-transferase(s) (7). KDO-8-phosphate synthetase is encoded by gene *kdsA*, and CMP-KDO-synthetase is encoded by gene *kdsB*. Both genes from *Escherichia coli* have been cloned and sequenced (18, 50). Surprisingly, they are located at different positions on the physical map of the *E. coli* chromosome, although their gene products take part in the same biosynthetic pathway. It is known that many of the genes encoding enzymes of LPS biosynthesis are organized in operons, e.g., the *lpx* (8) and *rfa* (30) genes, which are involved in lipid A and core biosynthesis, respectively. Gene *kdsA*, which lies at 26.7 min, is thought to be the terminal gene in an operon (49, 51). There is some evidence that the *kdsB* gene, which lies at 84.7 min, is the proximal part of another operon (17). There is still little known about the regulation of LPS biosynthesis in general (41), and the expression of both enzymes has not been investigated. It has been assumed that these enzymes are formed constitutively during bacterial growth. The aim of this work was to investigate whether expression of these enzymes and their genes is regulated in *E. coli* K-12. With this goal in mind, we sequenced the whole cluster containing gene *kdsA* and the region of the operon containing gene *kdsB* in order to identify putative promoters of these genes. We present here sequence information and results of experiments investigating the regulatory aspects of the identified promoters. Our experiments demonstrate that *kdsA* and *kdsB* undergo growth phase-dependent regulation at the transcriptional level but that regulation is independent of growth rate.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. All strains, plasmids, and phages used in this work are listed in Table 1. Cultures were grown in Luria-Bertani (LB) medium containing 16 g of peptone, 10 g of yeast extract, and 5 g of NaCl with vigorous aeration at 37°C if not stated otherwise. For the selection of plasmids, ampicillin was used at a concentration of 150 μg ml^{-1}. Growth was monitored by measuring the A_{600} of the culture medium. LB agar plates containing 20 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Relevant characteristic(s)	Source (reference)
E. coli K-12 strains		
5K	F$^-$ *tre thi rpsL*$^+$ *hsdR hsdM*$^+$ *lac thr* λ$^-$	Sandoz Forschungsinstitut, Vienna, Austria
MC4100	F$^-$ *araD139*Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	T. Silhavy, Princeton, N.J.
XL1-Blue	*endA1 usdR17*(*hsdR hsdM*$^+$) *supE44 thi-1 recA1* λ$^-$ *gyrA96 relA1* Δ(*lac*)[F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet^r)]	Stratagene
MV1190	Δ(*lac-proAB*) *thi supE44* Δ(*srl-recA*)*303*::Tn*10*(Tet^r) [F′ *traD36 proAB lacI*^{q}ZΔM15]	J. Messing
MC1061	*hsdR2 hsdM*$^+$ *hsdS*$^+$ *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)X74 *galE15 galK16 rpsL*(Str^r) *mcrA mcrB1*	N. Minton
CA8224.1	*lacZ*(*L8 UV5*) *relA1 spoT1 thi-1*	CGSC[a]
KVCAS1	MC4100(λpCAS1)	This work
UFKA401	MC4100(λpDSA401)	This work
UFKMB201	MC4100(λpDSB201)	This work
Phages		
4D10	Recombinant λEMBL4 with an 18-kb chromosomal DNA insert	Kohara et al. (22)
M13KO7	p15A *ori*, Kan^r	Vieira and Messing (46)
λRS45	*bla′ lacZ*$_{sc}$*YA imm*21c*Iind*$^+$	Simons et al. (42)
Plasmids		
pBluescript SK+/−	Amp^r *lacIPOZ′*	Stratagene
pBluescript KSII+/−	Amp^r *lacIPOZ′*	Stratagene
pRS414	*bla lacZ′YA*, protein fusion vector	Simons et al. (42)
pRS415	*bla lacZYA*, operon fusion vector	Simons et al. (42)
pPR100	6.0-kb *Hin*dIII fragment from phage 4D10 in pBluescript SK-	This work
pPR300	5.6-kb *Bam*HI-*Hin*dIII fragment from pPR100 in pBluescript KSII+	This work
pPR301	Whole gene cluster containing gene *kdsA* cloned on a 7.2-kb *Xho*I-*Xba*I fragment in pBluescript KSII+	This work
pMW100	2.5-kb *Sau*3AI fragment containing *kdsA* in pBR322	Woisetschläger and Högenauer (50)
pMW101	1.5-kb *Pvu*II fragment from pMW100 in pSP64	Woisetschläger and Högenauer (50)
pCAS1	0.6-kb *Sau*3AI fragment from CA8224.1 containing the *lacL8* and *lacUV5* promoter mutations in pRS414/*Sma*I	This work
pDSA401	4.89-kb *Bgl*I-*Sna*BI fragment in pRS414/*Sma*I (*kdsA′-lacZ′* protein fusion)	This work
pDSA402	4.0-kb *Bgl*II deletion subclone from pDSA401	This work
pDSA403	2.56-kb *Hin*dII deletion subclone from pDSA401	This work
pDSA404	1.85-kb *Ava*I deletion subclone from pDSA401	This work
pDSA405	0.75-kb *Dra*I deletion subclone from pDSA401	This work
pDSA406	0.63-kb *Bsm*AI deletion subclone from pDSA401	This work
pDSA407	0.52-kb *Nde*I deletion subclone from pDSA401	This work
pDSA408	0.31-kb *Pvu*I deletion subclone from pDSA401	This work
pDSA411	4.89-kb *Bgl*I-*Sna*BI fragment from pPR301 in pRS415/*Sma*I (*kdsA′-lacZ* operon fusion)	This work
pAN200	1.7-kb *Xho*I-*Hin*dIII fragment containing *kdsB* in pBluescript SK+	This work
pDSB201	0.9-kb *Xho*I-*Fsp*I fragment from pAN200 in pRS414/*Sma*I (*kdsB′-lacZ′* protein fusion)	This work
pDSB211	0.9-kb *Xho*I-*Fsp*I fragment from pAN200 in pRS415/*Sma*I (*kdsB′-lacZ* operon fusion)	This work

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

ml^{-1} were used to distinguish between LacZ$^+$ and LacZ$^-$ phenotypes of bacterial strains.

Construction of plasmids. All plasmids harboring gene *kdsA* were derived from pPR301, a pBluescript derivative containing the whole gene cluster on a 7.2-kb *Xho*I-*Xba*I fragment. Plasmid pPR301 was constructed as follows. A 6.0-kb *Hin*dIII fragment was cloned from the recombinant Kohara phage 4D10 (22) on the basis of hybridization with a probe isolated from plasmid pMW100 containing the *kdsA* gene (49). This fragment was ligated into pBluescript SK− to create plasmid pPR100. Subsequently, a 5.6-kb *Bam*HI-*Hin*dIII fragment from pPR100 was cloned into pBluescript KSII+, creating the recombinant plasmid pPR300. Finally, plasmid pPR301 was obtained by cutting out a 0.4-kb *Nsi*I-*Cla*I fragment from pPR300 and ligating the 2.0-kb *Nsi*I-*Cla*I fragment from plasmid pMW100 (Table 1), which contains the 3′-terminal part of the cluster.

All plasmids harboring gene *kdsB* were derived from pAN200, a pBluescript derivative containing a 1.7-kb *Xho*I-*Hin*dIII fragment. Gene *kdsB* was first cloned by Goldman and Kohlbrenner (18). We recloned this gene on a larger fragment by digestion of chromosomal *E. coli* DNA with *Xho*I-*Hin*dIII and transformation into *E. coli* XL1-Blue cells. Identification of positive recombinant clones was achieved by the nonradioactive digoxigenin-DNA colony hybridization technique (Boehringer, Mannheim, Germany) with a 664-bp *Hpa*II-*Bgl*II fragment (see Fig. 10) homologous to the *kdsB* gene as the probe.

For control experiments, plasmid pCAS1 was constructed by ligating the 0.6-kb chromosomal *Sau*3AI fragment from strain CA8224.1 containing the *lacZL8* and *lacZUV5* promoter mutations into the *Bam*HI site of the *lacZ* fusion vector pRS414. The region carrying the mutated *lacZ* promoter and the first 45 codons of the *lacZ* gene was cloned from chromosomal DNA of strain CA8224.1 by PCR with two oligonucleotide primers. The 0.71-kb PCR fragment obtained was cut with *Sau*3AI on both ends to give the 0.6-kb fragment.

DNA sequencing. The nucleotide sequence was determined by the dideoxy chain termination method of Sanger et al. (40) with the Sequenase 2.0 kit used according to the conditions recommended by the manufacturer (United States Biochemical Corp.). Single-stranded DNA was prepared as a template for sequencing reactions from *E. coli* XL1-Blue, carrying either of the recombinant plasmids pPR301 (*kdsA*) and pAN200 (*kdsB*) in accordance with the instructions for the phagemid vector pBluescript (Stratagene). The DNA fragments were separated on 6% polyacrylamide sequencing gels containing 8 M urea. The whole cluster containing *kdsA* was sequenced in detail by the following procedure. The insert of plasmid pPR100 was shortened from appropriate restriction sites on both ends with exonuclease III in order to construct an ordered set of deletions from both strands (21). For introducing deletions from the left end, the plasmid was cleaved with enzymes *Sac*I and *Xba*I, and for deletions from the right end, enzymes *Apa*I and *Cla*I were used. After treatment with S1 nuclease and the Klenow fragment of DNA polymerase I, the DNA fragments obtained were religated and the resulting plasmids were transformed into *E. coli* XL1-Blue. The transformants were screened by agarose gel electrophoresis for suitable deletions. Deletions from the left end were used to prepare single-stranded DNA, and deletions from the right end were used to prepare double-stranded DNA which was then purified by CsCl equilibrium centrifugation. Both strands

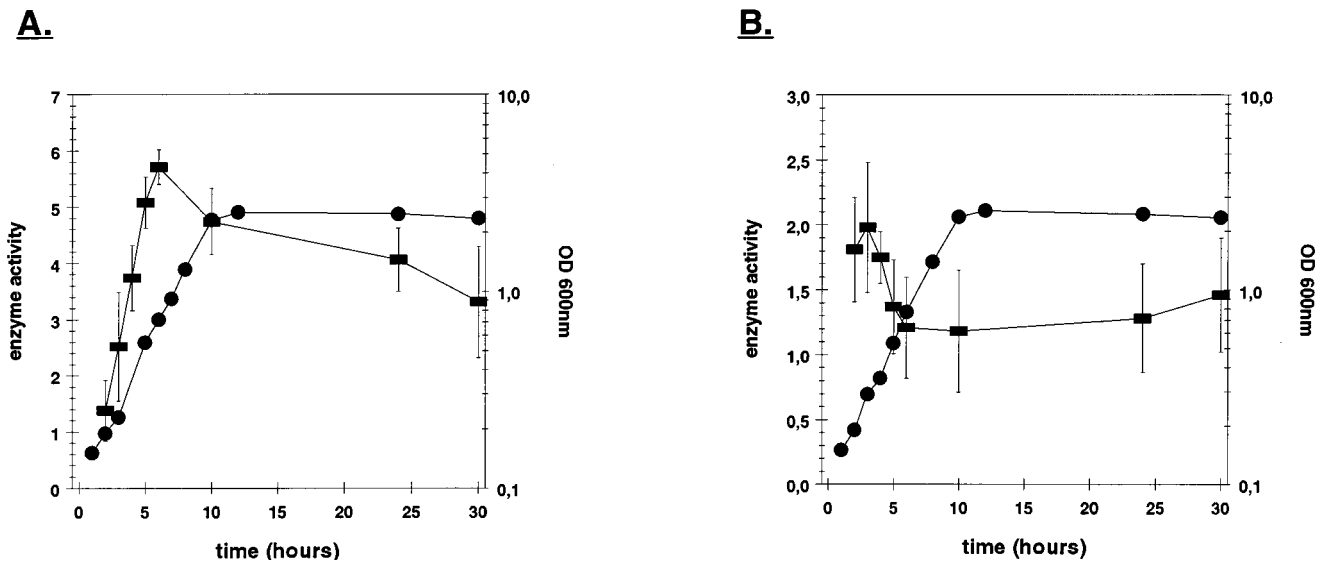

FIG. 1. Enzymatic activities of KDO-8-phosphate synthetase (A) and CMP-KDO-synthetase (B) during growth of an *E. coli* K-12 (5K) culture as a function of incubation time. Overnight cultures in LB medium were diluted ca. 100-fold to an initial OD_{600} of 0.05 and incubated at 37°C in the same medium. The first sample was taken after 2 h of incubation, before cells were entering exponential phase. Symbols: ▬, enzymatic activity measured by an in vitro assay at different incubation times; ●, growth curve of the culture in semilogarithmic scale (OD_{600}). The specific enzymatic activities are given as nanomoles of KDO-8-phosphate formed per minute per milligram of total protein for KDO-8-phosphate synthetase and as nanomoles of CMP-KDO formed per minute per milligram of total protein for CMP-KDO-synthetase. Each point is the average of three independent measurements, with standard deviations indicated by error bars.

were completely sequenced. Sequencing data were analyzed by using the sequence analysis software package of the Genetics Computer Group (16).

Recombinant DNA procedures. All DNA manipulations, including restriction enzyme cleavage, gel electrophoresis, ligation, transformation, end labeling, and filling in, were performed by using standard procedures (2, 28, 39). Restriction enzymes and all other modifying enzymes were purchased from New England Biolabs and Boehringer and were used according to the specifications of the suppliers. Restriction fragments with 5′ overhangs or 3′ overhangs were made blunt ended by the use of the Klenow fragment of *E. coli* polymerase I or T4 DNA polymerase, respectively. Dephosphorylation of linearized vector DNA prior to ligation was achieved by digestion with calf intestine alkaline phosphatase. Plasmids were constructed by ligation of agarose gel-purified (United States Biochemical Corp. Bioclean kit) restriction fragments with T4 DNA ligase and transformed either into $CaCl_2$-treated competent cells or by electroporation according to the instructions of the manufacturer (Invitrogen). Plasmid DNA was prepared by the alkaline extraction procedure of Birnboim and Doly (4), essentially as described by Ausubel et al. (2). For large-scale preparations of plasmid DNA, Qiagen columns (Qiagen Inc.) were used.

Crude cell extracts and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Overnight cultures of different *E. coli* K-12 strains were diluted ca. 100-fold to an optical density at 600 nm (OD_{600}) of 0.05 with the same medium and incubated at 37°C with continuous agitation in a rotary shaker to the desired OD_{600}. Cells were harvested by centrifugation at 7,000 rpm in a Sorvall GSA rotor for 5 min at 4°C, washed once, and resuspended in 1 to 10 ml of 10 mM Tris-HCl, pH 7.4, depending on the cell mass. The suspension was sonicated three times for 30 s each on ice, and the sonicate was centrifuged at 20,000 rpm in a Sorvall SS34 rotor for 50 min at 4°C to remove unbroken cells and cell debris. The supernatant was collected and stored frozen in small aliquots at −70°C. The quantity of total protein was measured by the method of Bradford (5), using the Bio-Rad Protein Assay reagent, with bovine serum albumin as a standard. Twenty-five micrograms of total cellular protein was separated in each lane of a sodium dodecyl sulfate (SDS)-polyacrylamide (11%) slab gel according to the method of Laemmli (24).

Enzyme assays. The specific enzymatic activity of KDO-8-phosphate synthetase was determined in crude cell extracts of different *E. coli* K-12 strains by the thiobarbituric acid assay as described by Ray (32) and represented as nanomoles of KDO formed per milligram of total protein per minute. The determination of the specific enzymatic activity of CMP-KDO-synthetase was also performed with crude cell extracts by using the thiobarbituric assay as described by Ray et al. (35) and represented as nanomoles of CMP-KDO formed per milligram of total protein per minute.

Western blot (immunoblot) analysis. The immunological procedure was essentially as described by Harlow and Lane (19). As a primary antibody we used polyclonal antisera from rabbits immunized with the purified proteins KdsA and KdsB. (The proteins were overproduced by cloning on multicopy plasmids, and the purification was done as described by Ray et al. [32, 35], with some alterations.) After electrophoresis, proteins were immediately transferred to a Hybond-C nitrocellulose membrane (pore size, 0.45 μm; Amersham) by electrophoretic blotting (Bio-Rad Trans-Blot cell). Protein transfer was for 2 h at 250 mA, and quantitative transfer was verified by Coomassie brilliant blue staining of the blotted gel. The membrane was blocked for 1 h in TBST buffer (0.05 M Tris, 0.15 M NaCl, 0.01% sodium azide, 0.05% Tween 20 [pH 7.4]) containing 5% (wt/vol) nonfat dry milk, washed twice for 5 min in TBST, and incubated with 50 μl of primary antiserum in 20 ml of blocking buffer overnight at 4°C. After two 5-min washes in TBST, the filter was incubated with 2 μl of alkaline phosphatase-conjugated sheep anti-rabbit immunoglobulin G (Sigma) in 20 ml of blocking buffer for 2 h at room temperature. After two additional washes in TBST, the filter was equilibrated by washing two times for 5 min each in AP buffer (0.1 M NaCl, 0.1 M Tris, 0.005 M $MgCl_2$ [pH 9.5]). The blot was developed by staining with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (BCIP-NBT), and the reaction was stopped by the addition of 1× phosphate-buffered saline containing 20 mM EDTA after the bands became clearly visible.

RNA isolation and Northern (RNA) hybridization analysis. Overnight cultures of different *E. coli* K-12 strains in LB medium were diluted ca. 100-fold to an OD_{600} of 0.05 with the same medium and incubated at 37°C with continuous agitation in a rotary shaker to the desired OD_{600}. Samples (5 to 30 ml, depending on the growth stage of the culture) were withdrawn from the 200-ml batch culture and rapidly chilled on ice to stop cell growth. RNA preparations were carried out as described by Emory and Belasco (14). The RNA was dissolved in 50 μl of diethyl pyrocarbonate-treated distilled water, and the concentration was determined in a Perkin-Elmer spectrophotometer at 260 nm after treatment with 30 U of RNase-free DNase I (Boehringer). For quantitative analysis, equal amounts of total RNA (10 μg) at each time point of the growth curve were suspended in RNA loading buffer containing 0.1 mg of ethidium bromide ml^{-1} and fractionated on a 1% agarose gel. The quality of the RNA preparation was controlled by UV illumination. RNA molecular weight markers (Boehringer) with the appropriate size range were used as standards. After electrophoresis, the RNA was transferred by capillary blotting with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) on a Hybond-N membrane (Amersham) and fixed via UV cross-linking (Bio-Rad GS Gene Linker). Complete transfer was verified by restaining the blotted gel with ethidium bromide. Northern blot analysis was performed according to standard procedures. Prehybridization of the filter was done in a solution of 25 mM potassium phosphate (pH 7.4), 5× SSC, 5× Denhardt's solution, and 50% deionized formamide, containing 50 μg of denatured sheared herring sperm DNA per ml, for at least 3 h at 40°C. Hybridization was done in the same solution at 42°C overnight, except that 10% (wt/vol) dextran sulfate was added. Isolated DNA restriction fragments, comprising nearly the entire coding regions of either gene *kdsA* or *kdsB*, were radiolabeled with [α-^{32}P]dCTP (NEN DuPont) by the random-priming method (15), denatured, and used to probe the RNA blots. Filters were washed once in 1× SSC–0.1% SDS for 30 min at 40°C and then subjected to a stringent wash in 0.25× SSC–0.1% SDS for 30 min at 55°C. Subsequently, a Cronex DuPont X-ray film was exposed to the filters. The lengths of the various RNA species were

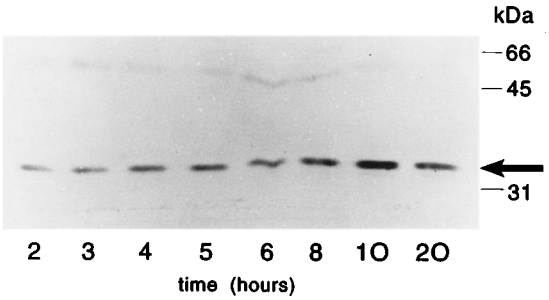

FIG. 2. KDO-8-phosphate synthetase levels in the cytoplasm during cell growth of *E. coli* 5K as analyzed by SDS-PAGE and Western blotting. The arrow indicates the position of KDO-8-phosphate synthetase (33 kDa). The positions of the protein molecular mass standards are also indicated on the right. For experimental details, see Materials and Methods.

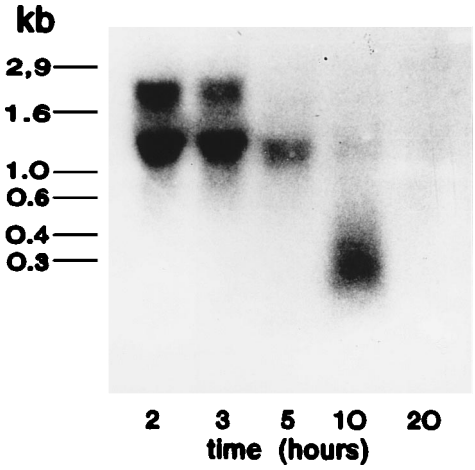

FIG. 3. Northern blot analysis of *kdsA* mRNA during cell growth. Ten micrograms of total RNA extracted from plasmid-free cells of *E. coli* 5K at various time points during cellular growth were subjected to agarose gel electrophoresis and blotted. An autoradiogram of the blot after hybridization with the 633-bp *Nru*I-*Bam*HI fragment from plasmid pMW101 as the radioactive probe is shown. The positions of the RNA molecular size standards are marked on the left.

calculated graphically by least-squares analysis of a semilogarithmic plot of molecular size as a function of mobility, on the basis of RNA molecular weight standards.

Measurement of mRNA half-life. Total cellular RNA was extracted from aliquots of cells (10 to 20 ml) withdrawn from a 200-ml *E. coli* K-12 culture at three time points along the growth curve. In each case RNA was prepared at time intervals of 1, 2, 5, and 10 min after the transcription was blocked by the addition of 0.2 mg of rifampin ml^{-1}. The first sample was taken only after 1 min to allow completion of nascent transcripts. Equal amounts (10 μg) of total RNA were separated on a 1% agarose gel and subjected to Northern blot analysis. The mRNA decay rate was determined densitometrically by scanning the autoradiogram on a Molecular Dynamics laser densitometer with Image Quant software.

Subsequently, mRNA concentrations were plotted as a function of time after transcription inhibition with rifampin. Least-squares analysis of the slope from this semilogarithmic plot was used to determine the half-life.

Primer extension analysis. Total RNA was isolated as described above, from both plasmid-free cells and cells carrying either the recombinant plasmid pPR301 (for *kdsA*) or pAN200 (for *kdsB*), after the culture reached an OD_{600} of 0.4. Total cellular RNA (10 to 20 μg) was hybridized with 0.1 pmol of oligonucleotide primer 5′ end radiolabeled with [γ-^{32}P]ATP. The primer extension assay was carried out as described previously by Sterling et al. (44). The synthetic DNA oligomers used for the primer extension were PR4 (5′-TGCTTCTGATAACG TAGCCAG-3′), which is complementary to bases 5143 to 5163 upstream of *kdsA*, and HS1B (5′-ACACTGGAATGCCATCACGCA-3′), which is complementary to bases 199 to 219 upstream of *kdsB*. The extension reaction was performed with 10 U of Moloney murine leukemia virus reverse transcriptase (RNase H Minus; Promega) in the presence of 12.5 U of human placental RNase inhibitor (Boehringer) and 0.2 mM each deoxynucleoside triphosphate at 42°C for 60 min. Equal-volume aliquots of primer-extended cDNA were analyzed on a 6% denaturing polyacrylamide gel containing 7 M urea. A DNA sequencing ladder, generated by using the same primers, was electrophoresed in parallel. The dried gel was exposed to Cronex DuPont X-ray film.

Assay of β-galactosidase. β-Galactosidase activities in samples (0.1 to 0.9 ml, depending on the OD_{600}) taken from 10-ml *E. coli* K-12 cultures at various time points of the growth curve were measured as described by Miller (29). Overnight cultures of different *E. coli* K-12 strains in LB medium were diluted ca. 50-fold to an initial OD_{600} of 0.1 in minimal medium A supplemented with 20 μg of vitamin B_1 ml^{-1}, 1 mM $MgSO_4$, 0.4% glucose, and 150 μg of ampicillin ml^{-1} in the presence of different *lacZ* fusion plasmids and grown at 37°C in a water bath with continuous shaking to the desired OD_{600}. Cells were made permeable by treatment with SDS and chloroform and were assayed for β-galactosidase activity by using 2-nitrophenyl-β-D-galactopyranoside (Serva) as a substrate. Enzymatic activities were related to cell density and expressed in Miller units.

Construction of *lacZ* protein and operon fusions. For the construction of both protein and operon fusions with the *lacZ* reporter gene, the system developed by Simons et al. (42) was used. Translational fusions of *kdsA* to the *lacZ′* gene, which lacks the first eight codons of the coding region, were constructed by using the high-copy-number protein fusion vector pRS414, and the resulting plasmids were designated pDSA. For *kdsB*, the same vector was used, and the plasmids were designated pDSB. The starting point for 5′ deletion analysis was pDSA401, which carries an in-frame fusion of the truncated *kdsA′* gene to *lacZ′* obtained by ligation of a 4.89-kb *Bgl*I-*Sna*BI fragment from the recombinant plasmid pPR301, comprising the whole cluster, into the *Sma*I site of pRS414 upstream of the *lacZ′* gene. 5′ deletions were made in pPR301 by digestion with appropriate unique restriction enzymes and religation of the filled-in fragments into the *Sma*I site of pRS414. The starting point for *kdsB* promoter analysis was pDSB201, which carries a translational in-frame fusion of the truncated *kdsB′* gene to the *lacZ′* gene on a 0.9-kb *Xho*I-*Fsp*I fragment from the recombinant plasmid pAN200 ligated into the *Sma*I site of pRS414. The various constructs were transformed by electroporation into strain *E. coli* MC4100, which was used as the host for all β-galactosidase expression assays. Positive recombinant clones were identified by their blue color on LB agar plates containing X-Gal and were further verified by restriction analysis. For constructing chromosomal single-copy fusions, the gene fusions on plasmids pDSA401 and pDSB201 were crossed onto phage λRS45 by double homologous recombination. The recombinant phages were designated λpDSA401 and λpDSB201, respectively. Phage lysates were prepared and used to lysogenize strain MC4100 by selecting for lysogens expressing β-galactosidase. For each construct up to 10 independent lysogens were isolated, purified, and tested for β-galactosidase activity. Double or triple lysogens, which produced two or three times as much β-galactosidase as single lysogens, were eliminated. The monolysogens obtained were designated UFKA401 (for *kdsA*) and UFKMB201 (for *kdsB*) and assayed for β-galactosidase expression in relation to the growth stage of the cells.

The high-copy-number operon fusion vector pRS415, carrying the complete *lacZYA* operon but lacking the *lac* promoter, was used to generate transcriptional promoter *lacZ* fusions by cloning fragments into the *Sma*I site preceding the *lacZ* gene. Plasmid pDSA411 carries a *kdsA-lacZ* transcriptional fusion obtained by ligation of the 4.89-kb *Bgl*I-*Sna*BI fragment from pPR301 into the *Sma*I site of pRS415. Plasmid pDSB211 carries a *kdsB-lacZ* transcriptional fusion obtained by ligation of the 0.9-kb *Xho*I-*Fsp*I fragment from pAN200 into the *Sma*I site of pRS415. Both constructs were also introduced into *E. coli* MC4100 by electroporation and assayed for β-galactosidase expression as described above.

As an internal control for growth phase-independent gene expression, we constructed strain KVCAS1 as follows. We cloned a 0.6-kb fragment from the chromosomal DNA of *E. coli* CA8224.1, which carries the *lacZL8* and *lacZUV5* promoter mutations, by PCR with two specific oligonucleotide primers. These mutations are known to make expression of the *lacZ* gene insensitive to growth conditions, and the specific β-galactosidase activity directly reflects the intracellular *lacZ* mRNA level (9, 47). The 0.71-kb PCR fragment obtained was cut with *Sau*3AI on both ends to give the 0.6-kb fragment which contains the *lacZL8 lacZUV5* promoter and the first 45 codons of the *lacZ* gene. Subsequently, plasmid pCAS1 was constructed by ligating this fragment into the *Bam*HI site of the *lacZ* protein fusion vector pRS414, creating an in-frame fusion to the *lacZ′* gene of the vector plasmid. This construct was transferred with the aid of phage λRS45 onto the chromosome of *E. coli* MC4100 to obtain the lysogenic strain, named KVCAS1, which was tested then for β-galactosidase activity at various times along the growth curve.

Materials. Oligonucleotides used for sequencing, primer extension analysis, and PCR were synthesized on an Applied Biosystems 381A DNA synthesizer. All radiochemicals were purchased from DuPont NEN Research Products. Synthetic KDO was a gift of the Sandoz Forschungsinstitut (Vienna, Austria). BCIP and NBT were from Boehringer and Sigma, respectively. X-Gal was obtained from Lambda Fluoreszenztechnologie (Graz, Austria).

Column material for protein purification was from Pharmacia LKB, and the purification was carried out on a fast protein liquid chromatography apparatus (Pharmacia).

Nucleotide sequence accession numbers. The nucleotide sequence of the *kdsA*-containing cluster as determined by us can be obtained from GenBank under accession number U18555. The sequence of the *kdsB*-containing region can be found in the EMBL database under accession number J02614.

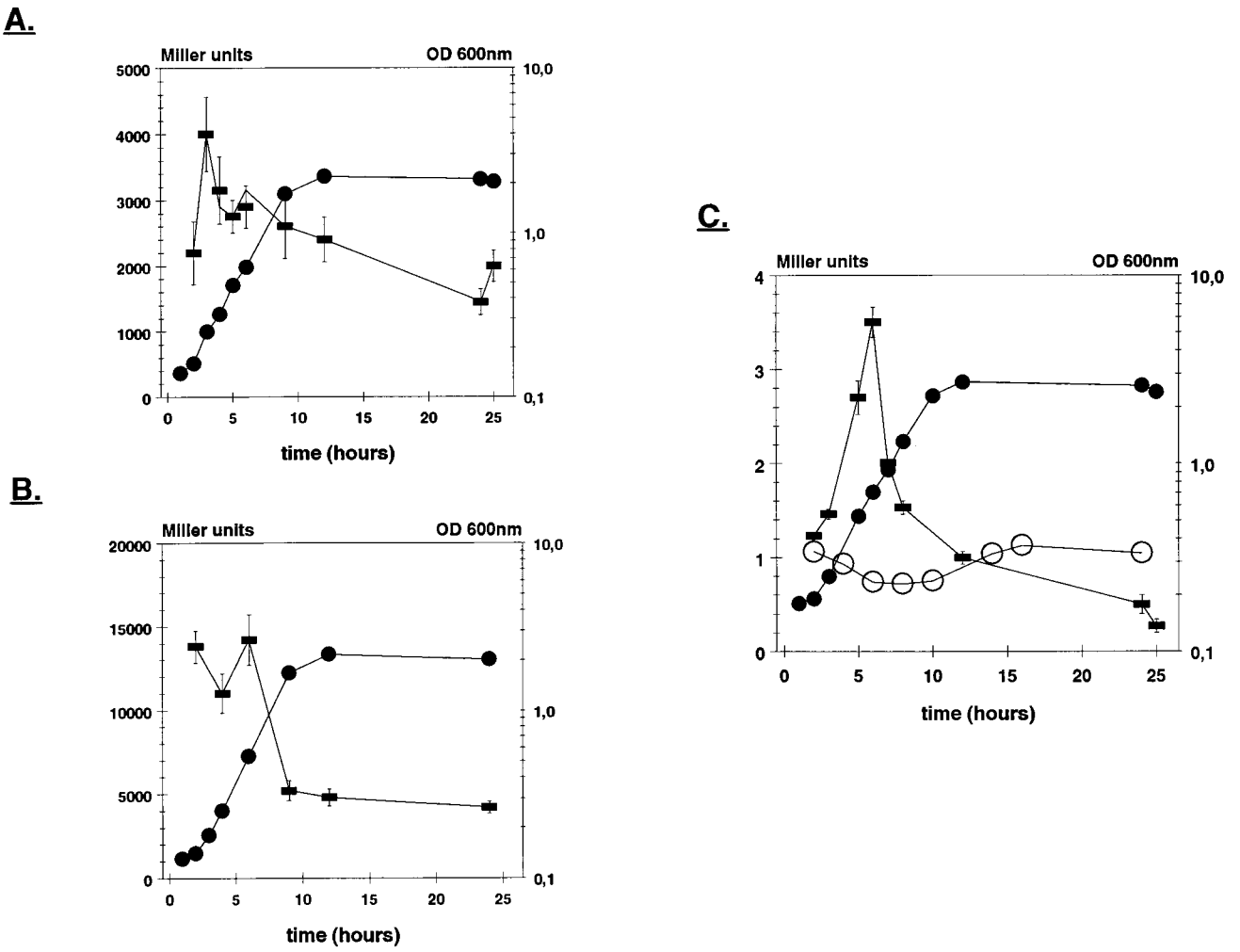

FIG. 4. *kdsA-lacZ* fusion experiment. Overnight cultures in LB medium were diluted ca. 50-fold to an initial OD_{600} of 0.1 in minimal medium A (supplemented with 20 μg of vitamin B_1 ml^{-1}, 1 mM $MgSO_4$, 0.4% glucose, and 150 μg of ampicillin ml^{-1} in the presence of various *lacZ* fusion plasmids) and incubated at 37°C. Symbols: ▬, average β-galactosidase activities (Miller units) measured at various time points during cellular growth; ●, growth curves in semilogarithmic scale. (A) MC4100 with pDSA401 (protein fusion); (B) MC4100 with pDSA411 (operon fusion); (C) UFKA401, which carries a single-copy protein fusion from pDSA401 located at the *attB* site of the chromosome of *E. coli* MC4100. Each β-galactosidase value represents the average of six independent measurements, with standard deviations indicated by error bars. The β-galactosidase activity (in 10^3 Miller units) of the control strain KVCAS1 is also shown in panel C (○).

RESULTS

Cloning and sequencing of a gene cluster containing the *kdsA* gene. We cloned and sequenced a cluster of six open reading frames in *E. coli* K-12 by hybridization with a probe isolated from plasmid pMW100, containing gene *kdsA*. In addition to *kdsA*, encoding KDO-8-phosphate synthetase, we could identify two other already known genes, *hemA* and *prfA*, by computer searches of the sequence databases. *hemA* encodes glutamyl-tRNA reductase (10, 26), and *prfA* encodes peptide release factor 1 (13, 48). These two genes are preceded by two closely linked promoters upstream of *hemA*. In the region between *hemA* and *prfA* at the beginning of the cluster and *kdsA* at the end, we found three additional open reading frames (ORF3, ORF2, and ORF1) whose functions are unknown (see Fig. 7). With the exception of ORF2, we could express their protein products in a maxicell system, but disruption of the genes in the chromosome showed that they are nonessential (not shown). Our attempts to demonstrate expression of ORF2 in a maxicell system failed, possibly because this region, lying between the *kdsA* promoter and gene, might not encode a protein but might instead have regulatory properties involved in expression of the *kdsA* gene. Alternatively, a protein may be only weakly expressed because of the high content of rare codons specifying leucine incorporation. The stop codons of *prfA* and ORF3 overlap with the initiation codons of the genes lying downstream. We have previously identified two inverted repeats at the end of the gene cluster downstream of gene *kdsA* (51) which may function as rho-independent terminators.

KDO-8-phosphate synthetase levels during growth. To measure the abundance of KDO-8-phosphate and its dependence on the growth phase, we diluted an overnight culture in LB medium ca. 100-fold to an initial OD_{600} of 0.05 in the same medium and incubated it at 37°C. Both Western blot determinations of KDO-8-phosphate synthetase levels and enzymatic activity measurements were carried out with whole-cell extracts prepared from a number of *E. coli* K-12 strains (Table 1) at various times during growth. The first sample was taken after 2 hours of cellular growth, before cells were entering exponential phase. Figure 1A shows the typical time course of

TABLE 2. Measurement of mRNA half-life

Time (h) of incubation	OD_{600}	Time (min) after addition of rifampin	Intensity[a]		Half-life[b] (min)	
			kdsA	*kdsB*	*kdsA*	*kdsB*
2	0.2	1	196.9	206.6	2.4	2.4
		2	66.0	67.8		
		5	28.6	20.3		
		10	11.3	11.5		
6	0.7	1	115.1	120.1	2.3	2.8
		2	85.9	50.0		
		5	22.9	18.8		
		10	8.3	10.7		
10	2.1	1	90.0	86.2	2.9	2.3
		2	84.9	81.7		
		5	48.9	31.1		
		10	11.3	6.4		

[a] The intensities of the bands on the autoradiogram were quantitated densitometrically by scanning on a laser densitometer. We determined the sum of the intensities of the two larger *kdsA* transcripts and the intensity of the one *kdsB* transcript at 1.0 kb. The differences in the Northern blot of Fig. 3, i.e., almost no detectable *kdsA* transcript at 10 h, can be explained by a slower growth of the culture in this experiment and the presence of rifampin prior to isolation of total RNA.

[b] Half-lives were calculated by least-squares analysis of the slope from semilogarithmic plots of densitometrically determined mRNA concentrations as a function of time after transcription inhibition with rifampin.

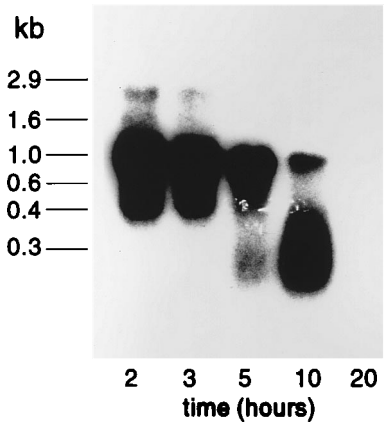

FIG. 5. Northern blot analysis of *kdsB* mRNA. Ten micrograms of total RNA extracted from plasmid-free cells of *E. coli* 5K at various time points during cellular growth was subjected to agarose gel electrophoresis and blotted. An autoradiogram of the blot after hybridization with the 664-bp *Hpa*II-*Bgl*II fragment (Fig. 10), which comprises nearly the whole *kdsB* structural gene, as the radioactive probe is shown. The positions of the RNA molecular size standards are marked on the left.

the enzymatic activity over the incubation time of a shaking culture, illustrated for strain *E. coli* 5K. The enzymatic activities, which were determined by an in vitro assay in crude cell extracts, were related to equal amounts of total protein. Figure 1A shows that the enzyme activity increased with incubation time, reached a maximum during exponential phase (about a fourfold increase), and began to decrease when cells were entering the stationary phase. The level obtained at 30 h did not change upon further incubation for 18 h (not shown). All strains tested differed in their absolute values of specific enzymatic activities but showed very similar growth phase-dependent patterns. We examined several *E. coli* K-12 strains which were grown in rich medium as well as in minimal medium, but we saw no differences in the general enzymatic growth phase-dependent pattern (data not shown). A similar result was obtained by Western blotting with a KDO-8-phosphate synthetase-specific antibody for detection (Fig. 2). Here, the maximal amount of KdsA protein was seen after 8 to 10 h, which corresponds to late log phase, and accordingly decreased again in stationary phase.

***kdsA* mRNA and growth.** In order to investigate whether increasing enzymatic activity is due to an increase in mRNA concentration over time, we performed the Northern blot experiment whose results are shown in Fig. 3. Equal amounts (10 μg) of total RNA extracted at various time points from plasmid-free *E. coli* 5K cells were subjected to agarose gel electrophoresis and, after blotting, were probed with a 633-bp *Nru*I-*Bam*HI fragment from plasmid pMW101 which comprises nearly the whole structural *kdsA* gene. After short incubation times, two mRNA species were detected. On the basis of promoter analysis, which is shown below, we conclude that the larger RNA band (2.2 kb) represents the initial mRNA molecule. This transcript disappears much earlier than the smaller, 1.2-kb mRNA, which could either be due to a processing product or represent another transcriptional start site. A surprising discovery was the abrupt disappearance of mRNA after 10 h notwithstanding the considerable quantities of KdsA protein present at this and at later growth phases. This observation indicates that the protein might be characterized by a long half-life.

***lacZ* gene fusion experiments.** The *lac*-deleted *E. coli* host strain MC4100 was transformed with plasmids pDSA401 (protein fusion) and pDSA411 (operon fusion), respectively, and β-galactosidase activities were determined at various time points of the growth curve as described by Miller (29). The β-galactosidase values, given in Miller units, over time are shown in Fig. 4. Both the translational (Fig. 4A) and the transcriptional (Fig. 4B) fusions were highly active at very early stages of cell growth and rapidly decreased upon entry of the culture into late logarithmic phase. The operon fusion showed a more drastic drop in activity upon entry into stationary phase than did the protein fusion. Taking into account that the pRS fusion plasmids and other pPR322-derived plasmids increase their copy number between three- and fivefold during the stationary phase (1), a net decrease for the expression of *lacZ* driven by the transcription regulatory sequences upstream of *kdsA* down to 10% of the initial value can be deduced. From this experiment, we conclude that *kdsA* expression is strongly growth regulated at the transcriptional level. As discussed below, this conclusion is consistent with results of the Northern blot and mRNA stability experiments.

In order to exclude copy effects of the fusion plasmids contributing to the results, we integrated the construct of pDSA401 into the chromosome of *E. coli* MC4100. This procedure involves a special λ phage derivative, λRS45. This phage carries both a β-lactamase gene and a *lacZ* gene, which enables insertion of foreign DNA via homologous recombination. The recombinant phage, designated λpDSA401, was used to lysogenize *E. coli* MC4100, creating cells with a gene fusion that is chromosomally localized at the *attB* site in a single copy. With the lysogenic strain UFKA401 we again determined the β-galactosidase activities along the growth curve and found that the data were similar to those for the plasmid construct (Fig. 4C), with the single exception that there was only weak β-galactosidase expression when the cells were in stationary phase. We also substituted glucose (0.4%) for glycerol (0.5%) as the carbon source, but the results were unaffected (data not shown).

These data are in excellent agreement with the results ob-

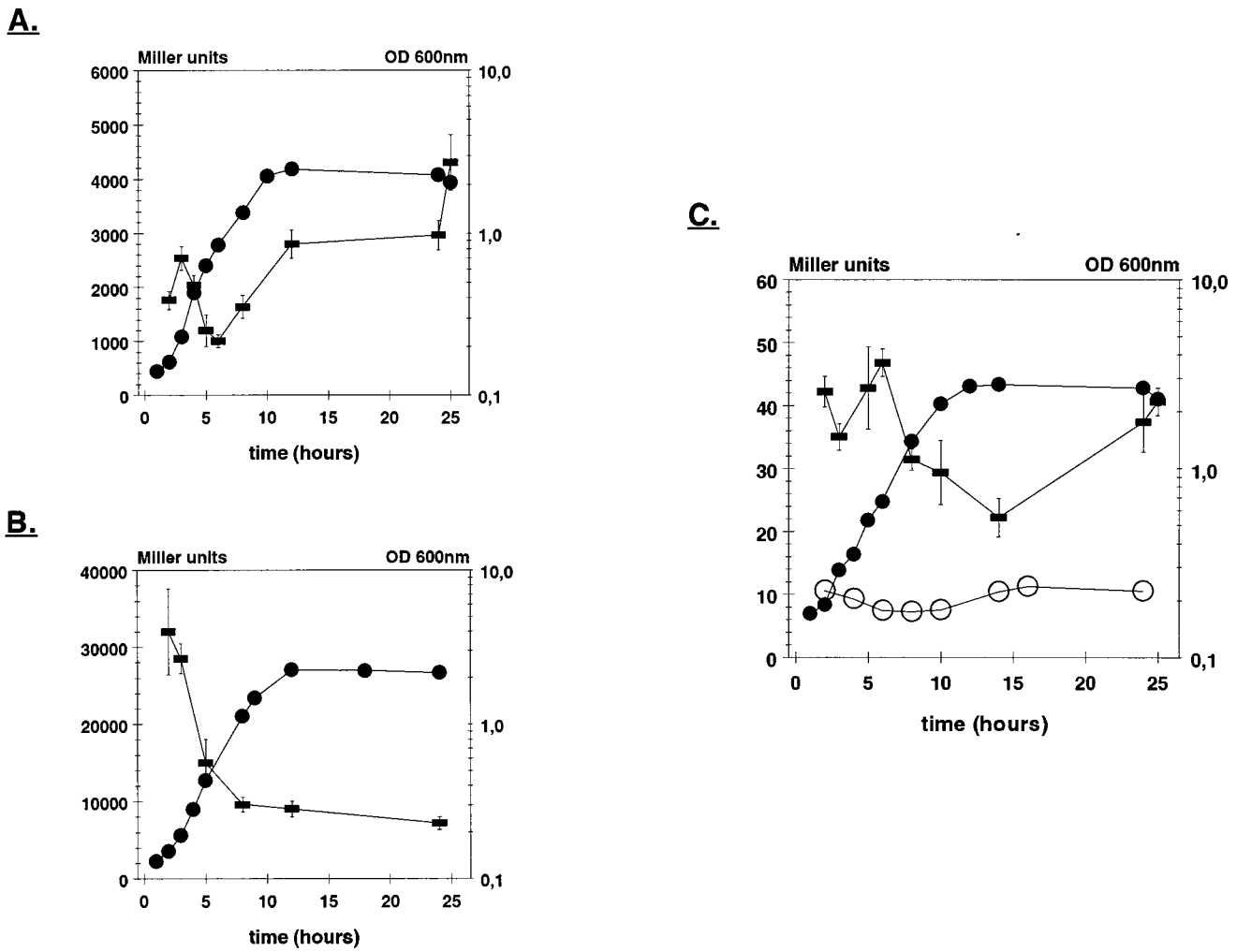

FIG. 6. *kdsB-lacZ* fusion experiment. Overnight cultures in LB medium were diluted ca. 50-fold to an initial OD_{600} of 0.1 in minimal medium A (supplemented with 20 μg of vitamin B_1 ml^{-1}, 1 mM $MgSO_4$, 0.4% glucose, and 150 μg of ampicillin ml^{-1} in the presence of various *lacZ* fusion plasmids) and incubated at 37°C. Symbols: ■, average β-galactosidase activities (Miller units) measured at various time points during cellular growth; ●, growth curves in semilogarithmic scale. (A) MC4100 with pDSB201 (protein fusion); (B) MC4100 with pDSB211 (operon fusion); (C) UFKMB201, which carries a single-copy protein fusion from pDSB201 located at the *attB* site of the chromosome of *E. coli* MC4100. Each value represents the average of six independent measurements, with standard deviations indicated by error bars. The β-galactosidase activity (in 10^2 Miller units) of the control strain KVCAS1 is also shown in panel C (○).

tained for the enzymatic activities of the KDO-8-phosphate synthetase (Fig. 1), which also showed a maximum during the late logarithmic phase of cellular growth. As a control for growth phase-independent gene expression, we tested strain KVCAS1 carrying the *lacZL8* and *lacZUV5* promoter mutations (Table 1). As expected, the β-galactosidase activity did not vary during cellular growth (Fig. 4C).

mRNA stability. In order to be able to assess the extent of mRNA degradation, mRNA stability measurements were carried out at three different time points of the growth curve, i.e., at the early and mid-log phases and at the beginning of the stationary phase. The results are shown in Table 2. At each time point rifampin was added to a culture of plasmid-free *E. coli* 5K cells, and total RNA was prepared at different time intervals as indicated in Table 2, after addition of the antibiotic. Ten micrograms of each RNA sample was separated by agarose gel electrophoresis, transferred to a nylon membrane, and probed with the same radioactive DNA fragment as used for the Northern blot analysis (Fig. 3). The intensities of the bands, which directly correspond to the mRNA concentration, were estimated by laser scanning densitometry. The results showed that mRNA half-lives were characteristic for *E. coli* and did not vary significantly (they were between 2 and 3 min) in all three phases of cellular growth.

Regulation of expression of the *kdsB* gene. Experiments similar to those described for *kdsA* were also carried out for *kdsB* to determine its expression in relation to bacterial growth. The results showed the same behavior for this gene, i.e., that it is transcriptionally regulated as a function of growth phase. The quantification of the CMP-KDO-synthetase in plasmid-free bacteria showed small changes as analyzed by measuring the enzymatic activity (Fig. 1B) and Western blotting (not shown). In contrast to the appearance of protein KdsA, which is gradually formed at early phases of growth, reaching a maximum when entering late logarithmic phase, protein KdsB is already present maximally at early growth phases. During further growth of the culture, only small changes can be detected. On entrance into stationary phase the KdsB content decreases to ca. 60%; it increases again slightly during stationary phase, reaching almost 80% of the initial value. We examined several

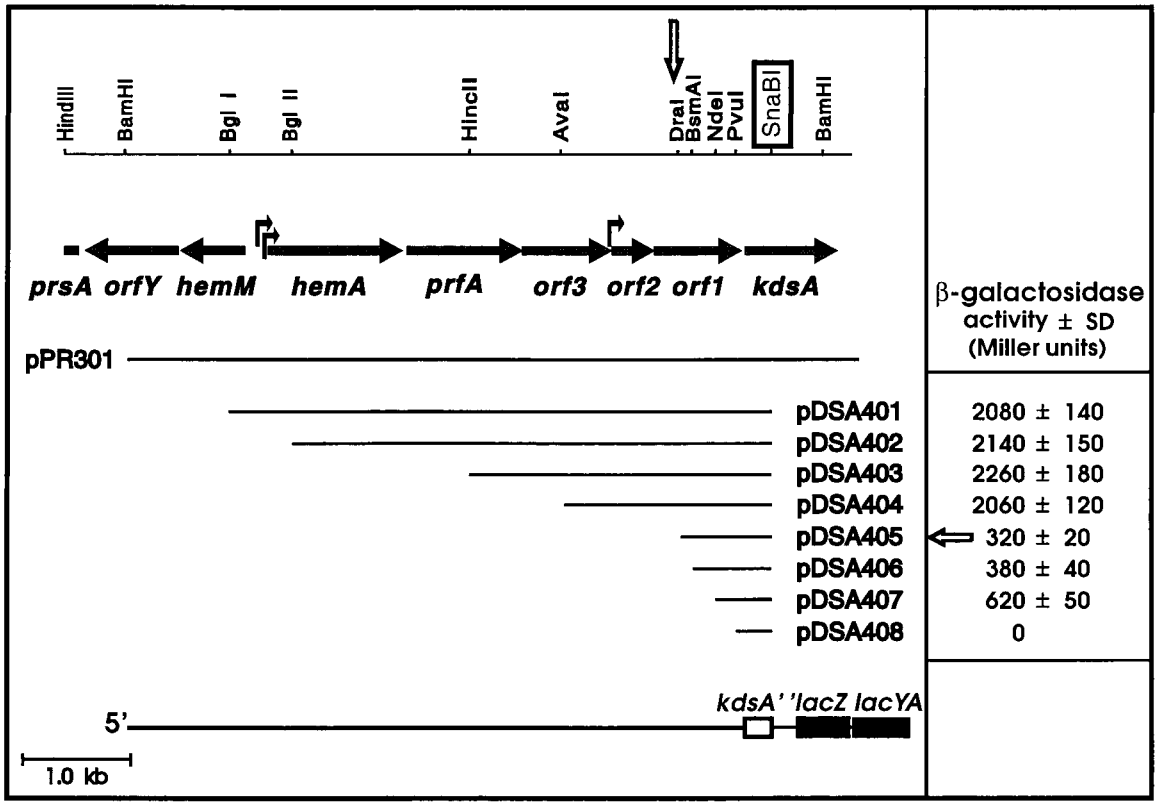

FIG. 7. Representative map of the whole cluster containing *kdsA* together with the various deletion mutants constructed in this work. The genes in the cluster are shown as solid arrows. Three genes of the neighboring operon (arrows pointing in the opposite direction) are also shown. The sizes and locations of the deletion constructs are indicated by thin horizontal lines. The *lacZ* construct of vector pRS414, used for the promoter identification, was fused at the *Sna*BI site within the *kdsA* structural gene. This fusion is shown at the bottom. The line at the top displays the positions of various restriction sites. Open arrows denote the deletion (pDS405) which resulted in a drastic reduction of β-galactosidase activity. The table on the right shows the β-galactosidase activities of the various deletion mutants measured after 2 h of cellular growth at an OD_{600} of 0.2, together with their standard deviations. Each value represents the average of six independent measurements. We obtained the same results when the β-galactosidase activities were measured after 3 h (OD_{600} = 0.3) and after 5 h (OD_{600} = 0.5) of cellular growth (data not shown). The positions of the two *hemA* promoters and of the putative *kdsA* promoter are indicated by bent arrows.

E. coli K-12 strains which were grown in rich medium as well as in minimal medium, but there were no changes in the general growth phase-dependent pattern (data not shown). The result of the Northern blot analysis of *kdsB* mRNA was quite similar to that of *kdsA*. The mRNA content decreased sharply as the cells reached stationary phase. In this analysis, we could clearly detect one mRNA species of 1.0 kb (Fig. 5). At earlier stages of growth, there was also a weak band at ca. 2.4 kb, which could represent a longer polycistronic transcriptional unit. For *lacZ* gene fusion experiments, plasmids pDSB201 (protein fusion) and pDSB211 (operon fusion) were transformed into *E. coli* MC4100 and tested for β-galactosidase activity. As with *kdsA*, we detected a drastic decrease in promoter activity during late exponential growth by the operon fusion experiment (Fig. 6B). Taking into account again that the pRS fusion plasmids and other pPR322-derived plasmids increase their copy number between three- and fivefold during the stationary phase (1), we determined a net decrease in promoter activity down to 10% of the initial value. We also integrated the translational fusion construct pDSB201 into the *attB* site of the chromosome of *E. coli* MC4100 with the aid of phage λRS45. Lysogenization of MC4100 with the recombinant phage, termed λpDSB201, gave the lysogenic strain UFKMB201. Subsequent measurement of the β-galactosidase activity showed data similar to those for the plasmid-borne protein fusion (Figs. 6A and C). The activities of both fusion constructs decreased as cells entered stationary phase but increased again in late stationary phase. Again, there was an excellent agreement with the data obtained from the measurement of the time course of the enzymatic activity of the CMP-KDO-synthetase (Fig. 1B). The values of the single-copy construct were approximately 80 times lower than those of the plasmid construct, as would be expected on the basis of the gene dosage. The mRNA stability did not change significantly during various growth phases (Table 2). Thus, the mRNA content is regulated primarily via transcriptional activity. In this regard, the expression of *kdsB* is almost identical to that of *kdsA*.

Expression of the chromosomal *kdsA*- and *kdsB-lacZ* fusions in different media. We further analyzed how our chromosomally located *lacZ* gene fusions are expressed as a function of the growth rate. For these experiments, we grew the lysogenic strains UFKA401 and UFKMB201 in a variety of different media, according to the work of Li and Cronan (27), to vary the growth rate. We were not able to detect any correlation between the expression of the genes and the growth rate of the cells (data not shown).

Localization of the *kdsA* promoter region. The *kdsA* promoter region was localized by analysis of deletion mutants. Various deletions downstream from the 5′ end of the insert of the *lacZ* fusion plasmid pDSA401 were generated by digestion with appropriate unique restriction enzymes (see Materials and Methods) and analyzed for β-galactosidase activity. The

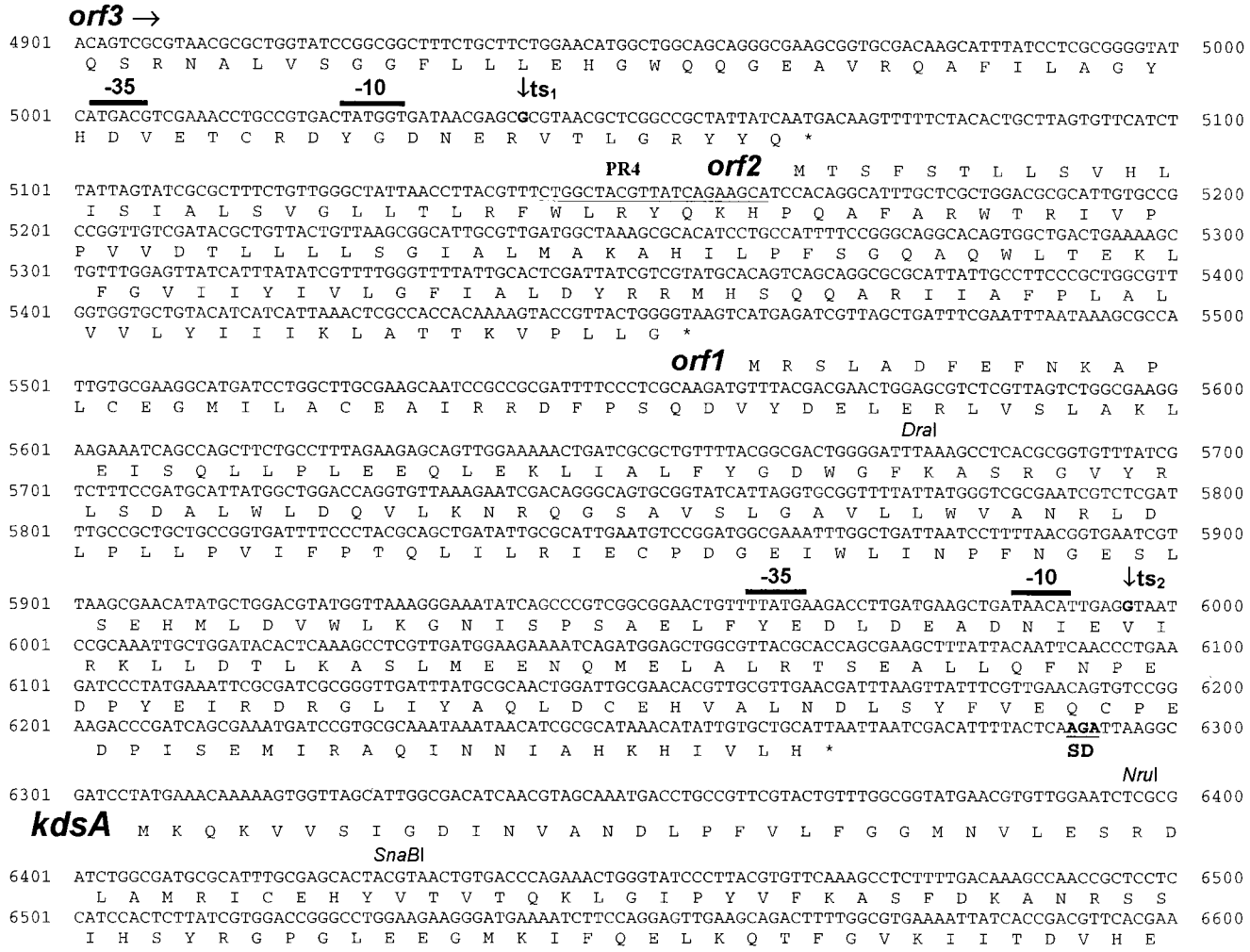

FIG. 8. Partial nucleotide sequence of the 3′-terminal part of the *kdsA*-containing cluster. The genes are marked at their beginnings. Deduced amino acid sequences are shown under the nucleotide sequences in the one-letter code. SD, ribosome binding site of gene *kdsA*. The two promoters are marked by their putative −10 and −35 regions as well as their transcriptional start sites (ts_1 and ts_2, downward-pointing arrows). PR4, oligonucleotide used for primer extension.

deletion constructs were designated pDSA402 to pDSA408 and are depicted in Fig. 7 together with their β-galactosidase activities determined after growing cells to an OD_{600} of 0.2. The promoter activity of pDSA405 as well as those of all shorter constructs decreased sharply to only ca. 15% of the activities of pDSA401 to pDSA404. Hence, the promoter which transcribes *kdsA* must be located in the region between the *Ava*I and *Dra*I sites, which corresponds to the region deleted in pDSA405. Similar results were observed when activity was tested after 3 h (OD_{600} = 0.3) and 5 h (OD_{600} = 0.5) of cellular growth (data not shown).

Since the mutants pDSA401 through pDSA404 showed nearly the same activities, we conclude that the two promoters preceding *hemA*, the first gene of the cluster, are not contributing to the expression of the *kdsA* gene. From the results of the length measurement of the *kdsA* mRNA in the Northern blot (Fig. 3), we were able to limit the promoter to a small region on the nucleotide sequence around nucleotide 5000 (Fig. 8). A more precise determination was possible by primer extension with *kdsA* mRNA as the template in a total RNA preparation. Two types of RNA were used for this experiment: chromosomally encoded RNA and RNA extracted from cells carrying plasmid pPR301. Three oligonucleotide primers, which were complementary to sequences in the region of interest, were used to search for the 5′ end of the mRNA. A positive signal was obtained with primer PR4, a 21-base oligonucleotide which generated a 123-nucleotide extension product, indicating a transcriptional start site at nucleotide 5041 (Fig. 9A). This extension product was observed with both the chromosomally coded and the plasmid-encoded RNA. Upstream of this position putative −10 (-TATGGT-) and −35 (-ATGACG-) regions which match satisfactorily with the *E. coli* σ^{70} promoter consensus sequences could be found (Fig. 8).

The weak expression of the constructs from pDSA405 to pDSA407 lacking this promoter is due to an additional promoter localized within ORF1 with a transcriptional start site at nucleotide position 5996, which we have identified previously (51). Clone pDSA408, which lacks this promoter, showed no detectable β-galactosidase activity. We have been unable to show any activity of this second promoter when the gene was in its chromosomal location. The promoter is detectable only when it is located on a plasmid being cloned on a short DNA fragment.

Localization of the *kdsB* promoter region. By means of primer extension analysis as described for *kdsA*, we were able to localize the promoter of the *kdsB* gene. With the 21-nucle-

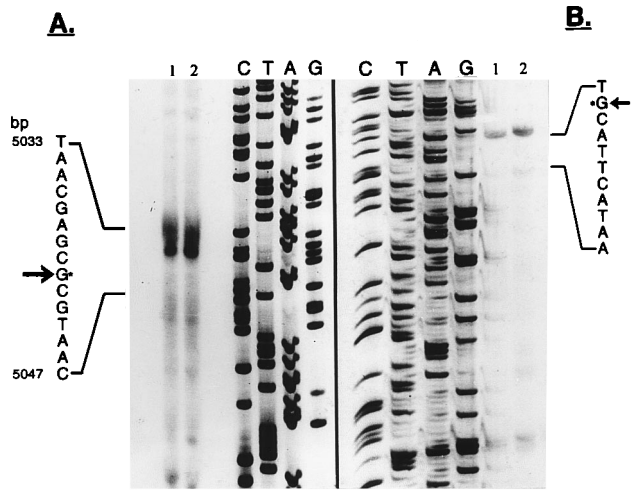

FIG. 9. Primer extension experiments to localize the promoters of *kdsA* (A) and *kdsB* (B). Autoradiograms of the electrophoretically separated primer extension products are shown. Lanes 1, chromosomally encoded template RNA isolated at an OD_{600} of 0.4 from plasmid-free *E. coli* 5K cells; lanes 2, template RNA isolated at an OD_{600} of 0.4 either from cells carrying the recombinant plasmid pPR301 (A) or from cells carrying plasmid pAN200 (B). The samples were run alongside a sequencing ladder of the corresponding region, using the same primer. The sequence as deduced from the electrophoretic run is shown at the margin in each case. The nucleotide identified as transcriptional start site is marked by an arrow and an asterisk.

otide oligonucleotide primer HS1B, we could extend a 162-nucleotide cDNA fragment by using *kdsB* mRNA as the template in a total RNA preparation. This RNA was again isolated either from plasmid-free *E. coli* 5K cells or from cells carrying plasmid pAN200. The extension product revealed the existence of a transcriptional start site at nucleotide position 58 (indicated in Fig. 9B by an arrow on the right). This start site is preceded by putative −10 and −35 regions with good homology to the *E. coli* σ^{70} promoter consensus sequences (−10, -TATGAT-; −35, -TAGACC-) (Fig. 10A). This promoter was previously predicted on the basis of a computer sequence analysis by Goldman et al. (17).

DISCUSSION

We have cloned and sequenced a cluster of six open reading frames containing gene *kdsA* in *E. coli* K-12. By sequence database searches we could identify two other genes, *hemA* and *prfA* (45, 48), at the beginning of the cluster. Gene *kdsA* encodes KDO-8-phosphate synthetase, an enzyme involved in biosynthesis of KDO of enterobacterial LPS (50, 51). It has not been clear whether all of these open reading frames are transcribed as one unit and therefore can be called an operon. No clear structural element which might act as a terminator can be found throughout the whole cluster. There are only two inverted repeats which may act as transcription terminators present after gene *kdsA* at the end of this putative operon (50). However, it has been demonstrated only that *hemA* and *prfA* are cotranscribed and therefore should lie on the same operon (11, 13). With the *lacZ* deletion analysis presented in this work we could clearly demonstrate that the two promoters in front of *hemA* do not contribute to the transcription of *kdsA* in a significant way in *E. coli* K-12 (Fig. 7). Rather, this gene is transcribed from another promoter which we found to be localized within the terminal part of the coding region of ORF3. The downstream genes ORF2, ORF1, and *kdsA* are cotranscribed into one polycistronic mRNA of 2.2 kb, and therefore, only these three genes form a transcriptional unit (Fig. 3). Our result is in agreement with the one obtained for *Salmonella typhimurium* by Elliot (12), who reported that the *hemA-prfA* operon does not extend as far as *kdsA*. As we have reported previously (51), there is also another promoter in the middle part of ORF1; however, the activity of this promoter could clearly be detected only when *kdsA* was present on the plasmid and not when the gene was chromosomally located. It is not clear if this promoter functions in vivo and, if so, under what circumstances it might be activated. We therefore regard it as a cryptic promoter. In the present work we could detect only two main mRNA species of 2.2 and 1.2 kb in the Northern blot, with the latter representing either a processing product of the longer mRNA molecule or another transcriptional start site.

We do not yet know anything about the functions of the three other open reading frames (ORF1, ORF2, and ORF3). The principal promoter for the expression of *kdsA* lies at the terminal region of ORF3. Comparison of the putative −10 and −35 regions with the *E. coli* promoter consensus sequences shows that this promoter belongs to the σ^{70} type (Fig. 8). Likewise, the promoter of the *kdsB* gene, encoding CMP-KDO-synthetase, appears to be specific for σ^{70}, judging from its similarity to the consensus hexamer (Fig. 10).

In this article we show for the first time that both genes, *kdsA* and *kdsB*, undergo transcriptional regulation as a function of growth phase but not growth rate (as discussed below). At the onset of bacterial growth, an extreme burst in transcriptional activity can be observed, which sharply declines upon entrance into stationary phase. This behavior is clearly evident from the operon fusions (Fig. 4B and 6B) as well as from the Northern blot analysis (Fig. 3 and 5). The promoter activities as measured in the operon fusions decreased to approximately 10% of the initial values as cells entered stationary phase. On the Northern blots the mRNA content gradually decreased from the beginning of bacterial growth to undetectable levels of full-length mRNA as cells entered stationary phase. Similarly, primer extension reactions gave detectable signals only with RNA isolated at early growth phases (data not shown). Since no changes in mRNA stability could be detected during the growth phase (Table 2), these data indicate that the regulation is due to changes in the rate of transcription.

The increase in enzymatic activity of KDO-8-phosphate synthetase lags behind the increase in its mRNA. The level of *kdsA* mRNA already is at its maximum at the beginning of bacterial growth, whereas the enzymatic activity of KDO-8-phosphate synthetase increases continuously from the beginning of bacterial growth and reaches a maximum value (approximately fourfold the initial value) in late log phase (Fig. 1A). On entrance into stationary phase there is a slight decrease, but even in late stationary phase three times the initial enzymatic activity still remains. That the protein is highly abundant even at late growth phases has been confirmed by immunodetection through Western blotting (Fig. 2). A different enzyme activity profile is seen for CMP-KDO-synthetase (Fig. 1B). In contrast to the increase in the enzymatic activity of KDO-8-phosphate synthetase, CMP-KDO-synthetase activity is at maximal levels at early growth phases. During further growth of the culture, only small changes can be detected. On entrance into stationary phase, enzyme activity decreases to 60%; it increases again slightly during stationary phase, reaching almost 80% of the initial maximum activity. The activity profile corresponds to the protein levels determined by Western blotting (data not shown). The specific enzymatic activity of KDO-8-phosphate synthetase was threefold higher than that of CMP-KDO-synthetase. Judging from the low content of rare codons within the reading frame, the *kdsA* gene is ex-

A.

```
                          -35                  -10        +1↓
-57  TTGCGCACCGGCTGGGAAATAGACCCCGTCGCATAAATGATTATGATAAGATTTATGCATTCATAACCCGATCCATCTGGAGGAACTATGGATCATCGTC   43

 44  TGCTTGAAATCATTGCCTGCCCGGTTTGCAACGGAAACTTTGGTATAACCAGGAAAAACAAGAGCTTATTTGCAAACTGGACAACCTCGCTTTCCCGCTG  143
          HS1B                                  SD
144  CGTGATGGCATTCCAGTGTTGCTGGAAACGGAAGCCCGCGTGCTGACTGCTGATGAGAGTAAATCATGAGTTTTGTGGTCATTATTCCCGCGCGCTACGC  243
                                                kdsB   M  S  F  V  V  I  I  P  A  R  Y  A
                HpaII
244  GTCCACGCGTCTGCCCGGTAAACCATTGGTTGATATTAACGGCAAACCCATGATTGTTCATGTTCTTGAACGCGCGCGTGAATCAGGTGCCGAGCGCATC  343
      S  T  R  L  P  G  K  P  L  V  D  I  N  G  K  P  M  I  V  H  V  L  E  R  A  R  E  S  G  A  E  R  I
344  ATCGTGGCAACCGATCATGAGGATGTTGCCCGCGCCGTTGAAGCCGCTGGCGGTGAAGTATGTATGACGCGCGCCGATCATCAGTCAGGAACAGAACGTC  443
      I  V  A  T  D  H  E  D  V  A  R  A  V  E  A  A  G  G  E  V  C  M  T  R  A  D  H  Q  S  G  T  E  R  L
                FspI
444  TGGCGGAAGTTGTCGAAAAATGCGCATTCAGCGACGACACGGTGATCGTTAATGTGCAGGGTGATGAACCGATGATCCCTGCGACAATCATTCGTCAGGT  543
        A  E  V  V  E  K  C  A  F  S  D  D  T  V  I  V  N  V  Q  G  D  E  P  M  I  P  A  T  I  I  R  Q  V
544  TGCTGATAACCTCGCTCAGCGTCAGGTGGGTATGGCGACTCTGGCGGTGCCAATCCACAATGCGGAAGAAGCGTTTAACCCGAATGCGGTGAAAGTGGTT  643
      A  D  N  L  A  Q  R  Q  V  G  M  A  T  L  A  V  P  I  H  N  A  E  E  A  F  N  P  N  A  V  K  V  V
644  CTCGACGCTGAAGGGTATGCACTGTACTTCTCTCGCGCCACCATTCCTTGGGATCGTGATCGTTTTGCAGAAGGCCTTGAAACCGTTGGCGATAACTTCC  743
      L  D  A  E  G  Y  A  L  Y  F  S  R  A  T  I  P  W  D  R  D  R  F  A  E  G  L  E  T  V  G  D  N  F  L
744  TGCGTCATCTTGGTATTTATGGCTACCGTGCAGGCTTTATCCGTCGTTACGTCAACTGGCAGCCAAGTCCGTTAGAACACATCGAAATGTTAGAGCAGCT  843
        R  H  L  G  I  Y  G  Y  R  A  G  F  I  R  R  Y  V  N  W  Q  P  S  P  L  E  H  I  E  M  L  E  Q  L
                                                                                BglII
844  TCGTGTTCTGTGGTACGGCGAAAAAATCCATGTTGCTGTTGCTCAGGAAGTTCCTGGCACAGGTGTGGATACCCCTGAAGATCTTGAGCGCGTTCGCGCT  943
      R  V  L  W  Y  G  E  K  I  H  V  A  V  A  Q  E  V  P  G  T  G  V  D  T  P  E  D  L  E  R  V  R  A
944  GAAATGCGCTAATTTCACTTCACGACACTTCAGCCAATTTTGGGAGGAGTGTCGTACCGTTACGATTTTCCTCAATTTTTCTTTTCAACAATTGATCTCA 1043
      E  M  R  *
```

B.

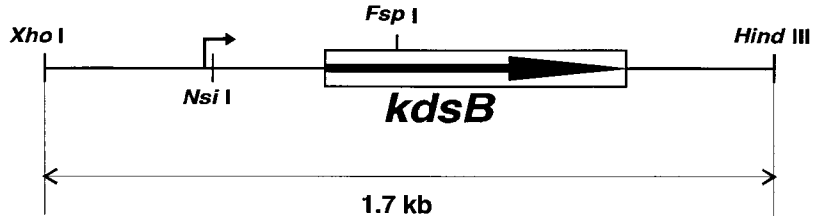

FIG. 10. Partial nucleotide sequence of the *kdsB* region (A) and representative map of the whole insert cloned on plasmid pAN200 (B). (A) The details are as described in the legend to Fig. 8. The transcriptional start site (+1) is marked with a downward-pointing arrow. HS1B, oligonucleotide used for primer extension reaction. (B) The position of the promoter near the *Nsi*I site is indicated by a bent arrow.

pected to express high levels of protein (43). The low specific enzymatic activity of the CMP-KDO-synthetase as well as the results from Western blotting indicate a low abundance of this protein in the cell, in agreement with reports in the literature that CMP-KDO-synthetase is less than 0.01% of the total cellular protein (35). Considering the short half-life, CMP-KDO might constitute a bottleneck in the overall LPS biosynthesis pathway. If the strains with the chromosomally located *kdsA*- and *kdsB-lacZ* gene fusions (UFKA401 and UFKMB201) are grown in media with different carbon sources, resulting in different growth rates, no correlation with gene expression occurs. Hence, the genes undergo no growth rate regulation despite the fact that they undergo regulation as a function of growth phase. We also substituted glucose for glycerol as the carbon source, but the results were unchanged. Thus, possible transcriptional regulation by the cyclic AMP receptor protein can be excluded.

The stationary phase represents a special situation in the life cycle of gram-negative bacteria. It is well known that cells change the morphological and biochemical characteristics of their cell walls during this period of increased environmental stress (23). Recently, it has been shown that expression of several genes is induced as cells enter stationary phase. The novel sigma factor σ^s, the product of the *rpoS* (*katF*) gene, has been identified to be involved in the induction of these genes (20). With genes *kdsA* and *kdsB*, we demonstrated that there is also a repression of gene expression at the transcriptional level as cells grow into stationary phase, despite the fact that the proteins are still abundant. Whether σ^s is involved in this down-regulation, e.g., by induction of a repressor or inefficient recognition of the *kdsA* and *kdsB* promoters, remains to be seen.

Since both *kdsA* and *kdsB* mRNA levels declined rapidly as cells entered stationary phase whereas the protein levels remained nearly constant, we conclude that both enzymes are rather stable and not readily degraded by proteases. Whether this is an intrinsic property of the proteins or whether another protein stabilizes them by complex formation remains to be answered. It is noteworthy that the specific enzymatic activity of KDO-8-phosphate synthetase decreases with increasing pro-

tein concentration in an in vitro assay, as we have reported previously (50). Complex formation with another protein(s) could account for this decrease in activity. The assembly of native KDO-8-phosphate synthetase into homotrimers has been established (32) and may also be responsible for stabilizing the protein. Interestingly, the *kdsA-lacZ* fusion hybrid protein expressed from the chromosome (Fig. 4C) initially shows the same time course of continuously increasing activity as endogenous KDO-8-phosphate synthetase (Fig. 1A), but in stationary phase its activity drops to approximately 10% of the maximum value. An explanation for the loss of protein activity may simply be degradation of the truncated protein. For the *kdsB-lacZ* fusion protein (Fig. 6C), we obtained exactly the same time course of β-galactosidase activity as was measured for the enzymatic activity of endogenous CMP-KDO-synthetase (Fig. 1B). The shapes of the curves in Fig. 4A and B correlate well, showing that *kdsA* protein and operon fusions are regulated in a similar manner. The reason for the differences in the curves in Fig. 6A and B is unclear; possibly additional regulatory effects at the level of translation play a role.

A striking result is the unexpected low level of β-galactosidase expression from the chromosomally located *kdsA-lacZ* fusion (Fig. 4C). The protein fusion of plasmid pDSA401 yielded a maximum of about 4×10^3 Miller units (Fig. 4A). Assuming a copy number of about 100 per cell, one would expect the chromosomally located construct in UFKA401 to produce ca. 40 Miller units. The observed value, however, is lower by a factor of 10. The expression of the chromosomally located *kdsB-lacZ* fusion in UFKMB201 (Fig. 6C) was as expected; i.e., it showed a ca. 80-fold reduction compared with that of the plasmid-borne gene fusion pDSB201 (Fig. 6A). We explain this low level of expression of the *kdsA* gene by the possible presence of a negative regulator affecting the *kdsA* promoter in the single-copy state but not in multiple copies, where it would be titrated out. Such a repression is not seen in the case of *kdsB*. The absence of repression of *kdsB* could also account for the increase of the β-galactosidase activity in late stationary phase as seen in Fig. 6A and C.

We report that genes *kdsA* and *kdsB* undergo growth phase-dependent regulation at the transcriptional level in *E. coli* K-12. The expression of the genes is maximal at the onset of bacterial growth. The need for LPS should probably be greatest during phases of active cell growth. Therefore, it seems logical that cells turn on expression of enzymes involved in the biosynthetic pathway of LPS as they enter exponential phase in order to maintain proper cell wall formation.

There are a large number of genes in *E. coli* that are differentially expressed as a function of growth phase or rate. The majority of growth-regulated genes are highly expressed during exponential phase. However, there are several genes which are induced in stationary phase, e.g., the *bolA* gene (1). Another group of genes is subject to what is termed the stringent response, such as genes for synthesis of rRNA and tRNA (6) or *fis*, which is induced during nutrient upshift (3). Expression of *fis* mRNA is similar to that of *kdsA* and *kdsB* mRNAs in that it is highest in early growth phases, but it is different in that both *fis* mRNA and its protein are undetectable in stationary phase, whereas proteins KdsA and KdsB remain highly abundant. Recently, a regulation pattern similar to that of *kdsA* and *kdsB* has also been reported for *acc* genes encoding acetyl coenzyme A carboxylase, which is growth rate regulated (27). Despite the fact that *acc* mRNA rapidly declined as cells entered stationary phase, the level of biotin carboxyl carrier protein (BCCP) protein remained nearly constant. Those authors could show that this is due to a long half-life of the protein.

The mechanism of the regulated expression of genes *kdsA* and *kdsB* is not known so far. Future investigations will include the search for possible *cis*- and *trans*-acting factors involved in the regulatory phenomenon.

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