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Accumulation of a Lipid A Precursor Lacking the 4′-Phosphate following Inactivation of the *Escherichia coli lpxK* Gene*

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Teresa A. Garrett‡, Nanette L. S. Que, and Christian R. H. Raetz§

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The *lpxK* gene has been proposed to encode the lipid A 4′-kinase in *Escherichia coli* (Garrett, T. A., Kadrmas, J. L., and Raetz, C. R. H. (1997) *J. Biol. Chem.* 272, 21855–21864). In cell extracts, the kinase phosphorylates the 4′-position of a tetraacyldisaccharide 1-phosphate precursor (DS-1-P) of lipid A, but the enzyme has not yet been purified because of instability. *lpxK* is co-transcribed with an essential upstream gene, *msbA*, with strong homology to mammalian Mdr proteins and ABC transporters. *msbA* may be involved in the transport of newly made lipid A from the inner surface of the inner membrane to the outer membrane. Insertion of an Ω-chloramphenicol cassette into *msbA* also halts transcription of *lpxK*. We have now constructed a strain in which only the *lpxK* gene is inactivated by inserting a kanamycin cassette into the chromosomal copy of *lpxK*. This mutation is complemented at 30 °C by a hybrid plasmid with a temperature-sensitive origin of replication carrying *lpxK*$^+$. When this strain (designated TG1/pTAG1) is grown at 44 °C, the plasmid bearing the *lpxK*$^+$ is lost, and the phenotype of an *lpxK* knock-out mutation is unmasked. The growth of TG1/pTAG1 was inhibited after several hours at 44 °C, consistent with *lpxK* being an essential gene. Furthermore, 4′-kinase activity in extracts made from these cells was barely detectable. In accordance with the proposed biosynthetic pathway for lipid A, DS-1-P (the 4′-kinase substrate) accumulated in TG1/pTAG1 cells grown at 44 °C. The DS-1-P from TG1/pTAG1 was isolated, and its structure was verified by ^{1}H NMR spectroscopy. DS-1-P had not been isolated previously from bacterial cells. Its accumulation in TG1/pTAG1 provides additional support for the pathway of lipid A biosynthesis in *E. coli*. Homologs of *lpxK* are present in the genomes of other Gram-negative bacteria.

Lipopolysaccharide (LPS)[1] is an essential glycolipid of Gram-negative bacteria (1–5). It is a complex molecule that forms the outer leaflet of the outer membrane and is important in forming an effective permeability barrier (3, 6, 7). The lipid A portion of LPS is required for bacterial viability and is a potent immunostimulant (1–5). Indeed, Gram-negative sepsis is thought to be mediated by over-stimulation of the immune system by bacterially derived lipid A (1–5). In *Escherichia coli* K12, lipid A is a disaccharide of glucosamine that is phosphorylated at the 1- and 4′-positions and acylated at the 2-, 3-, 2′-, and 3′-positions with (*R*)-3-hydroxymyristate (Fig. 1) (1–5). Two additional fatty acyl chains are esterified to the 2′- and 3′-hydroxymyristoyl chains to form acyloxyacyl moieties characteristic of lipid A (1–5).

The biosynthetic pathway for making lipid A in *E. coli* is well understood (1–3). Nine enzymes are required to synthesize Kdo_2-lipid A (Fig. 1) (1–3). With the recent identification of the gene encoding the lipid A 4′-kinase (8), the genes encoding 8 of the 9 enzymes required for the biosynthesis of Kdo_2-lipid A have been identified (3). The lipid A 4′-kinase catalyzes the transfer of the γ-phosphate from ATP to the 4′-position of tetraacyldisaccharide 1-phosphate (DS-1-P) to form tetraacyldisaccharide 1,4′-*bis*-phosphate (lipid IV_A) (Fig. 1) (9). Phosphorylation of the 4′-OH group is necessary for the action of distal biosynthetic enzymes, such as the Kdo transferase (10, 11), and for recognition of lipid A by mammalian cells during endotoxin stimulation (5).

The lipid A 4′-kinase gene was recently identified as *orfE* (a previously reported open reading frame of unknown function) (12), and it is now referred to as *lpxK* (8). *lpxK* forms an operon with an essential upstream gene, called *msbA*, which has homology to ABC transporters and mammalian Mdr proteins (12, 13). *msbA* has been implicated in the transport of lipid A from its site of biosynthesis on the inner surface of the inner membrane to the outer membrane (12, 13, 43). Georgopoulos and co-workers (12, 13) constructed a strain with an Ω-cam cassette inserted in the *msbA* gene. Because *lpxK* is co-transcribed with *msbA* (12, 13), this insertion stops expression of both *msbA* and *lpxK*. Complementation analysis showed that both *msbA* and *lpxK* were required for growth (12). It has also been found that glucosamine-labeled LPS precursors accumulate in the inner membrane of *msbA*/*lpxK* knock-outs (13). This phenomenon was attributed to the loss of the putative transport protein, MsbA. However, given the fact that *lpxK* plays an integral role in the biosynthesis of lipid A (8), the apparent accumulation of LPS in the inner membrane might be due to the build up of lipid A precursor(s), which could accumulate when the 4′-kinase is inactivated. These precursors may not be efficiently transported to the outer membrane by the putative lipid A transport machinery and may even inhibit transport of lipid A. Direct evidence for the function of MsbA as a lipid A transporter in strains bearing extra copies of *lpxK* is presented in the accompanying manuscript (43).

In the present work, we have constructed a strain with an insertion mutation in only the *lpxK* gene. In strain TG1/pTAG1 the chromosomal copy of *lpxK* is inactivated by insertion of a kanamycin resistance cassette into the center of the *lpxK* gene. This insertion mutation is complemented by a plasmid carrying *lpxK*$^+$ and a temperature-sensitive origin of replication. Thus,

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§ To whom correspondence should be addressed. Tel.: 919-684-5326; E-mail: raetz@biochem.duke.edu.

[1] The abbreviations used are: LPS, lipopolysaccharide; DS-1-P, tetraacyldisaccharide 1-phosphate; HPTLC, high performance thin layer chromatography; kb, kilobase pair(s); PBS, phosphate-buffered saline; COSY, two-dimensional ^{1}H correlation spectroscopy; Kdo, 3-deoxy-D-*manno*-octulosonic acid; UDP-DAG, UDP-2,3-diacylglucosamine.

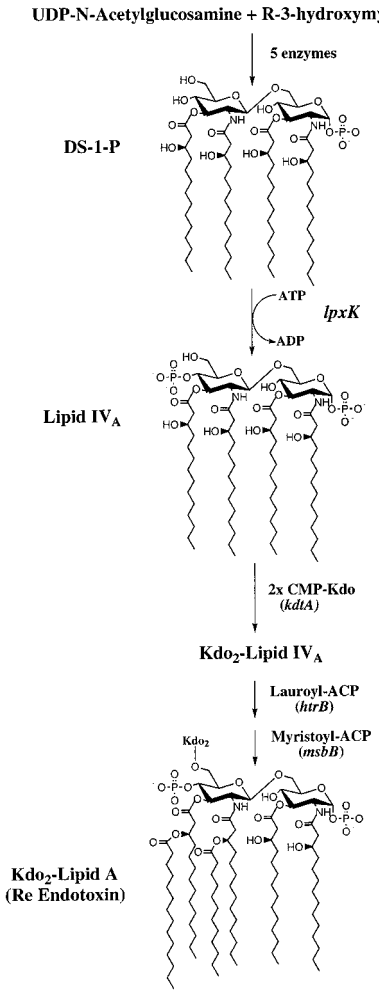

FIG. 1. **Role of the *lpxK* gene in lipid A biosynthesis.** LpxK catalyzes the sixth step in the biosynthesis of Kdo_2-lipid A (3, 9), a truncated form of LPS sufficient to support growth of *E. coli*. Five enzymes are required for the formation of DS-1-P, the 4′-kinase substrate (3). After 4′-phosphorylation of DS-1-P to yield lipid IV_A, KdtA catalyzes transfer of two Kdo residues to form Kdo_2-lipid IV_A (3). HtrB and MsbB then catalyze the addition of laurate and myristate, respectively, to generate Kdo_2-lipid A (3).

the *lpxK* knock-out genotype can be induced by growth at 44 °C. At this temperature the hybrid plasmid carrying $lpxK^+$ is no longer maintained in the cells, and both the *lpxK* gene and its product are gradually lost. Under these conditions, the phenotype of cells with no lipid A 4′-kinase can be examined. We now show that the 4′-kinase (LpxK) is indeed required for growth, and upon its depletion, the expected precursor, DS-1-P, accumulates in cells.

EXPERIMENTAL PROCEDURES

Materials—$^{32}P_i$ was obtained from NEN Life Science Products; 0.25-mm glass-backed Silica Gel 60 thin layer chromatography plates and high performance thin layer chromatography (HPTLC) plates were from E. Merck; yeast extract and tryptone were from Difco; restriction enzymes and Klenow DNA polymerase (large fragment) were from New England Biolabs; T4 DNA ligase was from Life Technologies, Inc.; shrimp alkaline phosphatase was from U. S. Biochemical Corp.; DEAE-cellulose (DE52) was from Whatman; and octadecylsilane (C18) silica was from Baker. All solvents were reagent grade from Malinckrodt. $CDCl_3$ and CD_3OD were purchased from Aldrich.

Bacterial Strains, Growth Conditions, and DNA Techniques—Table I lists the *E. coli* K-12 strains used in this study. Cells were cultured at 30, 37, or 44 °C in Luria Broth (LB) consisting of 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter (14). Antibiotics were added as necessary at 50 μg/ml for ampicillin, 12 μg/ml for tetracycline, 30 μg/ml for chloramphenicol, and 30 μg/ml for kanamycin. Mini preparations of plasmid DNA were made using the Qiaprep Spin Miniprep Kit (Qiagen). Large scale preparations of plasmid DNA were made using the Bigger Prep kit from 5 Prime → 3 Prime, Inc., Boulder, CO. DNA fragments were isolated from agarose gels using the Qiagen Qiaex II gel extraction kit. Restriction enzymes, T4 DNA ligase, and Klenow DNA polymerase were used according to the manufacturers' instructions. Transformation of *E. coli* with plasmid DNA was done using salt-competent cells (14).

Plasmid Constructions—Table I lists all of the plasmids used in this study. pTAG1 contains the *lpxK* gene cloned into pMAK705, a vector with a temperature-sensitive origin of replication (15). pJK2 (8) and pMAK705 were digested with *Xba*I and *Bam*HI. The 1-kb *lpxK* gene from pJK2 and the 6-kb linearized pMAK705 were gel-purified from a 1% agarose gel. The *lpxK* gene was ligated into pMAK705. A portion of the ligation mixture was transformed into competent *E. coli* XL1-Blue (Stratagene), and colonies resistant to chloramphenicol were selected. Plasmid DNA was isolated from chloramphenicol-resistant clones and digested with *Xba*I and *Bam*HI to identify those constructs with the desired insert. This plasmid is called pTAG1. This plasmid was tested for its ability to promote LpxK expression.

A plasmid analogous to pTAG1 was constructed with a kanamycin cassette inserted into the *Nsi*I site of *lpxK* gene. pJK2 was digested with *Nsi*I, and pUC-4K (Amersham Pharmacia Biotech) was digested with *Pst*I. The 5.5-kb linearized pJK2 and the 1.2-kb kanamycin cassette from pUC-4K were gel-purified and ligated together. A portion of the ligation was transformed into *E. coli* XL1-Blue, and colonies resistant to ampicillin were selected. Plasmids were isolated from ampicillin-resistant colonies and digested with *Nde*I and *Bam*HI to verify the presence of the correct 2.2-kb insert. The *lpxK::kan* construct described above was digested with *Xba*I and *Bam*HI and cloned into pMAK705 exactly as for pTAG1, yielding pTAG2.

pNGH1-amp was constructed from pNGH1 (16). pNGH1 was digested with *Bam*HI and *Sal*I yielding 3.9- and 1.6-kb fragments. pACYC177 was digested with *Bam*HI and *Xho*I yielding 2.5- and 1.4-kb fragments. The 2.5-kb pACYC177 fragment which contains the β-lactamase gene and 1.6-kb pNGH1 fragment which contains the *trc* promoter were ligated together to form pNGH1-amp.

To construct pTAG6, pJK2 was digested with *Nde*I, and the 5′-overhang was filled in with Klenow DNA polymerase according to manufacturer's directions (New England Biolabs). The *lpxK* gene was excised by further digestion with *Bam*HI, yielding a 985-base pair fragment with one blunt end and one *Bam*HI sticky end. pNGH1-amp was digested with *Sma*I and *Bam*HI. The *lpxK* fragment was ligated into the digested pNGH1-amp. A portion of the ligation was transformed into XL1-Blue competent cells, and colonies resistant to ampicillin were selected. Plasmid DNA was isolated from ampicillin-resistant clones and was digested with *Bam*HI and *Sac*I to verify the presence of the correct insert. One such plasmid was called pTAG6.

pTAG8 is the *Francisella novicida* 4′-kinase gene, *valB*, cloned into pACYC177. pKEM14-5 (17) was digested with *Nde*I, and the 5′-overhang was filled in with Klenow DNA Polymerase (New England Biolabs). A fragment containing *valB* and a portion of the downstream *polA* gene was excised by further digestion with *Hin*dIII, yielding a ~1800-base pair fragment with one blunt end and one *Hin*dIII sticky end. pACYC177 was digested with *Sma*I and *Hin*dIII. The *valB* fragment was ligated into the digested pACYC177. A portion of the ligation was transformed into XL1-Blue competent cells, and colonies resistant to ampicillin were selected. Plasmid DNA was isolated from ampicillin-resistant clones and was digested with *Eco*RV and *Nde*I to verify the presence of the correct insert. One such plasmid was called pTAG8.

Construction of TG1/pTAG1, a Mutant with an Insertion in the Chromosomal Copy of lpxK—TG1/pTAG1 was constructed following the method of Hamilton *et al*. (15) (Fig. 2). Competent MC1061 cells (18) were transformed with pTAG2 and grown at 30 °C to an A_{600} of 0.6. Next, 1×10^5 cells were plated on prewarmed LB plates containing 30 μg/ml chloramphenicol and incubated at 44 °C. This selects for cells in which pTAG2 has integrated into the genome. A single colony was used to inoculate 1 ml of LB containing chloramphenicol and grown at 30 °C to stationary phase. A portion of the culture was diluted 1:1000 into fresh LB containing chloramphenicol and again grown at 30 °C to stationary phase. The above outgrowth was repeated once more. During this outgrowth, the integrated plasmid will occasionally excise carrying either the wild type *lpxK* or the *lpxK::kan* allele (Fig. 2) (15). The cells were plated on LB containing chloramphenicol at 30 °C. Cells in which the plasmid had excised were identified by their inability to grow at 44 °C in the presence of chloramphenicol. Plasmids were then isolated from 14 such temperature-sensitive strains and digested with *Xba*I and *Bam*HI. Of the 14 colonies, 11 contained the pTAG2 insert. Three,

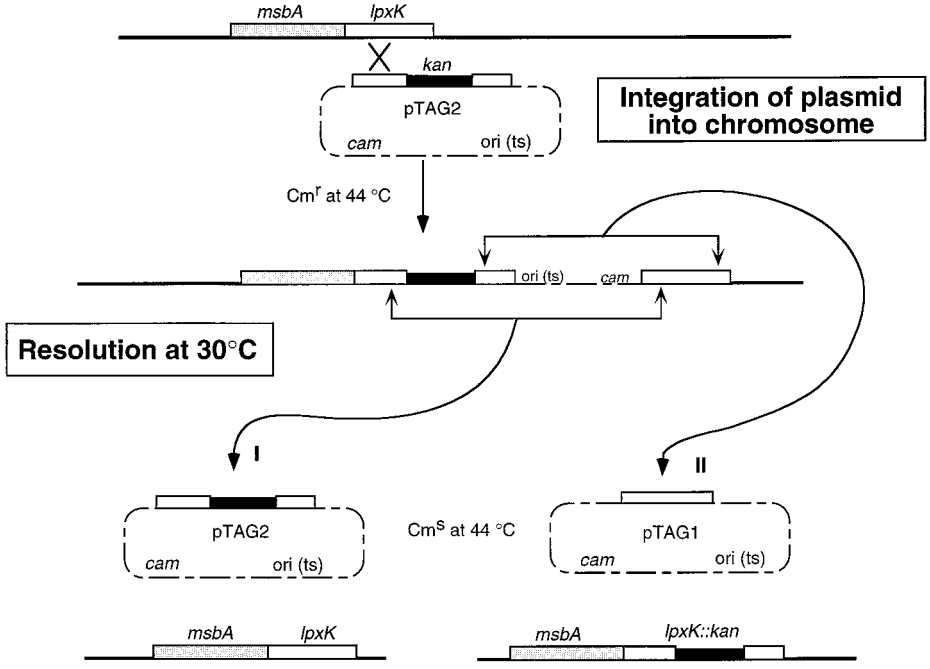

FIG. 2. **Construction of strain TG1/pTAG1, a mutant with a *kan* insertion in the chromosomal copy of *lpxK*.** The chromosomal insertion mutation in *lpxK* was made using the method of Hamilton *et al.* (15). MC1061 was transformed with pTAG2 and chromosomal integrants selected by their resistance to chloramphenicol at 44 °C. A cointegrate that could result from homologous recombination is shown. The plasmid can then excise itself from the genome after prolonged growth at 30 °C by homologous recombination. The two possible resolutions products are shown. Resolution *I* leads to restoration of the original genotype with the wild type copy of *lpxK* remaining on the chromosome and the *lpxK::kan* insertion on the plasmid. Resolution *II* leads to the *lpxK:kan* insertion on the chromosome and the wild type *lpxK* on the plasmid. One such strain was identified, made *recA*$^-$ by P1 transduction of *recA*::Tn10, and designated TG1/pTAG1 to indicate the presence of a plasmid that should be the same as pTAG1. Cell-free extracts from cells with pTAG1 contain about 10-fold more 4′-kinase activity per mg protein than cell-free extracts from cells with vector alone (data not shown).

however, had the pTAG1 insert, indicating that the *lpxK::kan* allele had replaced the wild type *lpxK* gene on the chromosome (15). One of these strains was made *recA*$^-$ by P1 transduction using BLR(DE3)pLysS (Novagen) as the donor. The presence of the recA$^-$ phenotype was verified by the strain's sensitivity to UV light (19). This strain is designated TG1/pTAG1.

Construction of TG1/pTAG6—Strain TG1/pTAG6 was made by transforming TG1/pTAG1 with pTAG6 and selecting for transformants at 30 °C on LB plates containing ampicillin. Single colonies were then streaked to LB plates containing ampicillin to 44 °C. pTAG1 is then lost, and pTAG6 (which is not temperature-sensitive for replication) provides wild type *lpxK*. TG1/pTAG8 was constructed in a similar manner.

Preparation of Cell-free Extracts and Assay for 4′-Kinase Activity—Cell-free extracts for determination of 4′-kinase activity in cells grown at 44 °C were prepared as follows. Single colonies of TG1/pTAG1 and TG1/pTAG6 were inoculated into 5 ml of LB medium containing the appropriate antibiotics and grown at 30 °C overnight. Portions of the overnight cultures were diluted into 200 ml of LB broth (containing no antibiotics) to A_{600} of 0.01, and the growth temperature was shifted to 44 °C. When the cultures reached A_{600} of 0.2–0.3, portions were diluted 10-fold into fresh pre-warmed medium, and the growth cycles were continued. The cells from the remaining cultures (about 180 ml) were collected by centrifugation at 10,000 × *g* for 15 min at 4 °C. The cell pellets were washed with 200 ml of 50 mM HEPES, pH 7.4; the centrifugation was repeated, and the final cell pellets were resuspended in 1 ml of the same buffer. Meanwhile, the growth cycles of the diluted cultures were continued as above, diluting further as necessary to maintain logarithmic growth. The volumes of the cultures were adjusted to provide 180–200 ml of cell culture at A_{600} of about 0.3 for preparation of cell-free extracts at the indicated times after the shift to 44 °C.

To prepare cell-free extracts, cells were broken in a French pressure cell at 20,000 p.s.i., and unbroken cells were removed by centrifugation at 3500 × *g*. The protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard. 4′-Kinase activity was assayed as described previously (8). Typically, 100 μM DS-1-^{32}P (1000 cpm/nmol), 1 mg/ml cardiolipin, 50 mM Tris chloride, pH 8.5, 5 mM ATP, 1% Nonidet P-40, and 5 mM $MgCl_2$ were mixed with 2 to 0.05 mg/ml protein fractions and incubated at 30 °C for various times. Reactions were stopped by spotting a portion onto a Silica Gel 60 thin layer chromatography plate. Plates were developed in chloroform/methanol/water/acetic acid (25:15:4:2, v:v), dried, and exposed to a Molecular Dynamics PhosphorImager screen. Conversion of DS-1-^{32}P to 1-^{32}P-lipid IV_A was quantified using ImageQuant software (Molecular Dynamics). DS-1-^{32}P and carrier DS-1-P were prepared as described previously (20).

Analysis of the Lipid A to Glycerophospholipid Ratio—Single colonies of TG1/pTAG1 and TG1/pTAG6 were inoculated into separate 3-ml cultures of LB medium containing the appropriate antibiotics and grown overnight at 30 °C. Each overnight culture was then diluted into two 25-ml portions of fresh LB medium (containing no antibiotics) to an A_{600} of 0.01. One was grown at 30 °C and the other at 44 °C. The cells were labeled with $^{32}P_i$ for about two doubling times. $^{32}P_i$ (5 μCi/ml) was added to the cultures grown at 30 °C when the A_{600} reached 0.15 and grown to an A_{600} of 0.5. The cultures grown at 44 °C were diluted 10-fold into fresh prewarmed medium whenever the A_{600} reached ~0.2. When the cumulative growth yield was 13.4 for TG1/pTAG1 (the point at which the growth of TG1/pTAG1 begins to slow down) and 27.5 for TG1/pTAG6, both cultures were labeled with 5 μCi/ml $^{32}P_i$. TG1/pTAG1 was labeled for 3 h, and TG1/pTAG6 was labeled for 1.5 h. Each culture was split into three tubes (~8 ml per tube). The cells were collected by centrifugation at 3000 × *g*, and the pellets were frozen at −20 °C for further analysis. Using one tube of each labeled culture, the lipid A to glycerophospholipid ratio was then determined as described previously (16, 21) with the following modification. Samples were analyzed by thin layer chromatography in a system containing chloroform/methanol/water/ammonia (40:25:4:2, v/v). HPTLC plates were used for rapid chromatography in this solvent system because the formation of an ammonia-catalyzed deacylation product was minimized.

Extraction and Detection of a Lipid A Precursor That Accumulates at 44 °C in TG1/pTAG1—A large batch of TG1/pTAG1 that had been shifted to 44 °C was prepared as follows. Overnight cultures of TG1/pTAG1 and TG1/pTAG6 grown at 30 °C were used to inoculate LB medium to an A_{600} of 0.01. Cultures were then grown at 44 °C and were diluted 10-fold as necessary to keep the optical density below 0.3 for 10.5 h. TG1/pTAG1 cultures were diluted into successively larger volumes to a final volume of 3 liters. A TG1/pTAG6 culture was maintained at 50 ml. Cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4 °C, washed once with PBS (1 liter for TG1/pTAG1 and 10 ml for TG1/pTAG6), and resuspended in PBS (30 ml for TG1/pTAG1 and 2 ml for TG/pTAG6).

Lipid A precursor accumulation was examined in the non-labeled cells of TG1/pTAG1 and TG1/pTAG6 shifted to 44 °C for 10.5 h. TG1/pTAG1 cells (200 μl of the above 30-ml suspension) were brought to a volume of 2 ml by the addition of PBS. These TG1/pTAG1 cells and the entire 2-ml suspension of TG1/pTAG6 (as prepared above) were then extracted with a neutral single phase Bligh Dyer system (chloroform/methanol/PBS, 1:2:0.8) (22, 23) (9.5 ml total volume). Cell debris was removed by centrifugation at 3000 × *g* for 10 min. The supernatant was converted to a two-phase Bligh-Dyer system by the addition of chloroform and PBS to make the final solvent proportions 2:2:1.8 (chloroform/methanol/PBS) (22, 23). The phases were resolved by centrifugation, and the lower phase was washed with fresh pre-equilibrated upper

TABLE I
Plasmids and E. coli strains used in this study

Plasmid or strain	Relevant genotype	Source
pJK2	*lpxK* in pET3a	8
pMAK705	cm^r, temperature-sensitive replicon	15
pKEM 14–5	valAB construct in pTZ18U, amp^r	17
pUC4K	kan^r	Amersham Pharmacia Biotech
pNGH1	Low copy, lac promoter, cm^r	16
pACYC177	Low copy, amp^r	New England Biolabs
pNGH1-amp	pNGH1 promoter region with pACYC origin and amp^r cassette	This work
pTAG1	pMAK705 with *Xba*I/*Bam*HI fragment from pJK2, cm^r	This work
pTAG2	pTAG1 with *Pst*I excised *kan* cassette from pUC4K in *Nsi*I site, cm^r, kan^r	This work
pTAG6	pNGH1-amp with *Xba*I/*Bam*HI fragment from pJK2, amp^r	This work
pTAG8	*valB* only, *Nde*I/*Hin*dIII fragment from pKEM14–5 in pACYC177, amp^r	This work
MC1061	Wild type, *rec* A^+	18
XLI-Blue	*recA1*, *endA1*, *lac*	Stratagene
BLR(DE3)pLysS	Δ(*srl-recA*) 306::Tn10	Novagen
TG1/pTAG1	*lpxK::kan*, *recA*::Tn10, pTAG1 (cm^r)	This work
TG1/pTAG6	*lpxK::kan*, *recA*::Tn10, pTAG6 (amp^r)	This work
TG1/pTAG8	*lpxK::kan*, *recA*::Tn10, pTAG8 (amp^r)	This work

phase. The final lower phase was dried under nitrogen and redissolved in 50 μl of chloroform/methanol (4:1). About 100 μg of total extracted lipid was loaded onto each lane of the HPTLC plate, which was developed in chloroform/methanol/water/ammonia (40:25:4:2,v/v). The lipids were detected by charring with 20% sulfuric acid in ethanol.

Purification of the Accumulated Lipid A Precursor from TG1/pTAG1 Shifted to 44 °C—TG1/pTAG1 cell pellets (7.5 ml) from cultures that had been grown at 44 °C for 10.5 h, as described above, were used to prepare the accumulated lipid A precursor. PBS was added to bring the volume to 80 ml, and the suspension was distributed into four 150-ml Corning glass centrifuge bottles equipped with Teflon-lined lids. A single phase Bligh-Dyer system (22, 23) was made by adding 25 ml of chloroform and 50 ml of methanol to each bottle. The suspension was dispersed with ultrasound in a bath sonicator for 2 min and then centrifuged at 2500 × *g* for 20 min. The supernatant was divided equally into four fresh glass bottles, and 25 ml of chloroform and 25 ml of PBS were added to each bottle to form a two-phase Bligh-Dyer system. After thorough mixing, the suspensions were centrifuged at 2500 × *g* for 15 min, and the upper phases were removed. Each of the lower phases was washed twice with 20 ml of fresh pre-equilibrated upper phase. The centrifugation was repeated to resolve the phases after each wash. The lower phases were collected, and the solvent was removed by rotary evaporation. The dried lower phase material was re-dissolved in 5 ml of chloroform/methanol/water (2:3:1, v/v) and loaded onto a 1-ml DEAE-cellulose column (Whatman DE52), pre-equilibrated in the same solvent (20). The column was washed with 3 ml of chloroform/methanol/water (2:3:1, v/v). Unexpectedly, the sample flowed through the column in the initial chloroform/methanol/water (2:3:1, v/v) wash. We attributed this behavior to residual salts in the concentrated lower phases. Therefore, the run-through fractions (5.2 ml) were pooled and converted into a two-phase Bligh-Dyer system by changing the solvent ratios to 2:2:1.8 (chloroform/methanol/PBS, v/v). The lower phase was then washed again with fresh pre-equilibrated upper phase (5 ml) and dried under N_2. The resulting material was redissolved in 1 ml of chloroform/methanol/water (2:3:1, v/v), and it was loaded again onto a 1-ml pre-equilibrated DEAE-cellulose column. This time the unknown lipid that accumulates at 44 °C in the TG1/pTAG1 was retained by the column. The column was washed with 3 ml of chloroform/methanol/water (2:3:1, v/v), then with 6 ml of chloroform/methanol, 60 mM ammonium acetate (2:3:1, v/v), and 2 ml each of chloroform/methanol/aqueous ammonium acetate (2:3:1, v/v) containing sequentially 70, 80, 90, 100, 120, and 480 mM ammonium acetate in the water component. Fractions (1 ml) were collected, and a portion of each was spotted onto an HPTLC plate. The plate was developed in chloroform/methanol/water/ammonia (40:25:4:2, v/v) and charred, as described above. The compound that accumulated in cells of TG1/pTAG1 grown at 44 °C eluted with 60 mM ammonium acetate as the aqueous component, and it was almost pure, as judged by thin layer chromatography and charring. The pooled fractions (4 ml) were then converted to a two-phase Bligh-Dyer system by changing the solvent proportions to 2:2:1.8 (chloroform/methanol/water, v/v). The phases were resolved by centrifugation at 3000 × *g* for 10 min. The lower phase was washed once with 3 ml of fresh pre-equilibrated upper phase, and the centrifugation was repeated. The final lower phase was dried down with N_2 and stored at −20 °C.

The lipid obtained from the DEAE-cellulose column was further purified by reverse phase chromatography on octadecylsilane silica gel (C18 silica), as described previously for the preparation of lipid IV_A (24).

Analysis of the Accumulated Lipid as DS-1-P by ^{1}H NMR Spectroscopy—Approximately 1 mg of purified material was dissolved in 0.6 ml of $CDCl_3/CD_3OD$ (4:1, v/v), and its ^{1}H NMR spectrum was recorded on a Varian 500 Unity spectrometer using a 5001.3-Hz spectral window with the 5-mm probe set at 20 °C. Chemical shifts were referenced to the methyl protons of internal tetramethylsilane (0.00 ppm). A line broadening of 0.05 Hz before Fourier transformation was used to process the data.

Two-dimensional ^{1}H correlation (COSY) spectra were recorded in the absolute value mode over the same spectral region used in the one-dimensional ^{1}H NMR spectrum. Two hundred fifty six time increments were collected and zero-filled to 2048 points with sine-bell weighting along both dimensions. One hundred eighty scans were collected per increment, and the relaxation delay was 1 s.

RESULTS

Temperature Sensitivity of TG1/pTAG1 on Plates—Strain TG1/pTAG1 is a mutant with a kanamycin cassette inserted into the chromosomal copy of *lpxK*, constructed by homologous recombination (Fig. 2 and Table I) (15). The mutation is covered by a plasmid, pTAG1, bearing *lpxK*$^+$ and a temperature-sensitive origin of replication. Strain TG1/pTAG6 is similar to TG1/pTAG1, except that *lpxK*$^+$ is on a plasmid with a non-temperature-sensitive origin of replication (Table I). Strains TG1/pTAG1 and TG1/pTAG6 were tested for their ability to grow at 44 °C. A single colony of each was streaked onto two LB plates containing kanamycin and tetracycline. One plate was incubated at 30 °C and the other at 44 °C. TG1/pTAG1 is able to grow and form single colonies at 30 °C but not at 44 °C, indicating that loss of the *lpxK* gene product is lethal (data not shown). Strain TG1/pTAG6 is able to grow and form single colonies at 30 and 44 °C (data not shown). This result is consistent with the finding by Karow and Georgopoulos (12) that *orfE/lpxK* is an essential gene.

Temperature-sensitive Growth of TG1/pTAG1 in Liquid Medium—To quantify the effects of *lpxK* inactivation on lipid A biosynthesis, cells were studied in shaking culture at 44 °C. Overnight cultures of MC1061/pTAG1, MC1061/pTAG6, TG1/pTAG1, and TG1/pTAG6 were first grown at 30 °C in the presence of the appropriate antibiotics (Table I). The cultures were diluted into 25 ml of LB without antibiotics to a final A_{600} of 0.01. The temperature was then shifted to 44 °C and growth was continued with shaking at 250 rpm. To maintain logarith-

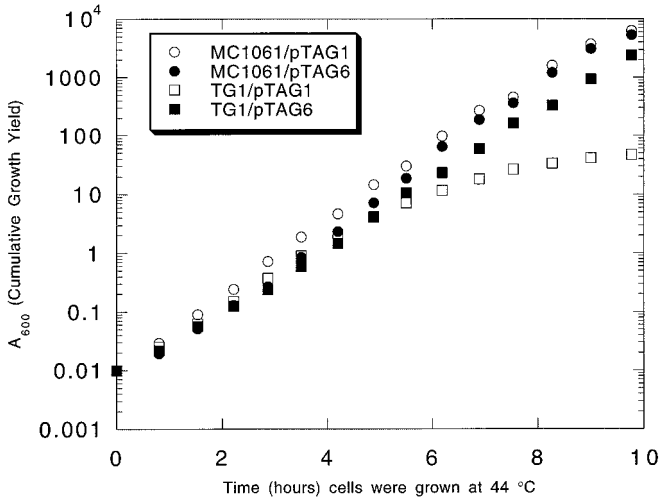

FIG. 3. **Temperature-sensitive growth of TG1/pTAG1.** Overnight cultures of MC1061/pTAG1, MC1061/pTAG6, TG1/pTAG1, and TG1/pTAG6 were grown at 30 °C in LB medium supplemented with the appropriate antibiotics (see Table I). Cultures were diluted into LB medium (with no antibiotics) to an A_{600} of 0.01, and the temperature was shifted to 44 °C. Cells in shaking culture were maintained in logarithmic growth by diluting 10-fold into fresh pre-warmed medium whenever the A_{600} reached 0.2–0.3. The A_{600} therefore represents the cumulative growth yield.

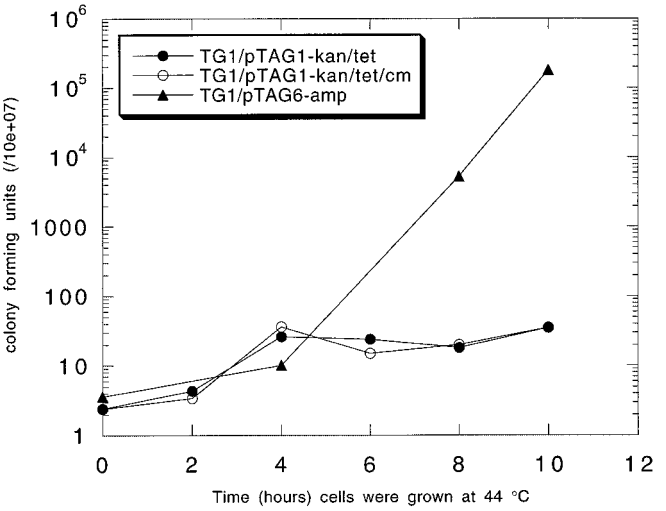

FIG. 4. **TG1/pTAG1 does not gain plating efficiency after prolonged growth at 44 °C.** A portion of the cultures grown for the experiment in Fig. 3 were diluted and plated at various times onto LB agar containing the antibiotics indicated. The plates were incubated at 30 °C overnight. The number of colony forming units was determined and is plotted as a function of time after the shift to 44 °C. TG1/pTAG6 continued to gain plating efficiency for the duration of the 10-h growth experiment in parallel to the A_{600}. TG1/pTAG1, however, only gained plating efficiency during the first 4 h after the shift to 44 °C. The presence of chloramphenicol in the plates (at 30 °C) had no effect on the plating efficiency, indicating that the lack of colony forming units was due to inviability of the cells in the absence of pTAG1. Plating of the control culture MC1061/pTAG1 (data not shown) on LB plates with or without chloramphenicol indicated that significant plasmid loss sets in after about 4 h of growth at 44 °C. MC1061/pTAG1 plated on LB without chloramphenicol gained plating efficiency in parallel to the increase in A_{600} to the same extent as TG1/pTAG6 (data not shown).

mic growth, the cultures were diluted 10-fold whenever the A_{600} reached 0.2–0.3. The results of one such experiment are shown in Fig. 3, in which A_{600} is the cumulative growth yield corrected for dilution. MC1061 containing either *lpxK* on a temperature-sensitive or a non-temperature-sensitive plasmid grows logarithmically at 44 °C for the duration of the experiment (10 h). TG1/pTAG6, a strain with the insertion mutation in the chromosomal copy of *lpxK* covered by a non-temperature-sensitive plasmid bearing $lpxK^+$, grows nearly as well as MC1061/pTAG1 or MC1061/pTAG6. However, growth of TG1/pTAG1 slows after about 4.5 h at 44 °C and stops altogether after 10 h. This result is consistent with *lpxK* being required for growth.

The growth inhibition of TG1/pTAG1 at 44 °C (Fig. 3) may be because cells lacking a 4′-kinase are not viable under all conditions or because such mutants grow very slowly at 44 °C. To address this question, plating efficiencies were determined at 30 °C at different times after the shift to 44 °C for shaking cultures of both TG1/pTAG1 and TG1/pTAG6. A portion of each culture was collected, diluted, and plated at 30 °C in the presence of the antibiotics indicated in Fig. 4. TG1/pTAG6 continues to gain colony forming units with time (Fig. 4) in parallel to the increased A_{600} when grown at 44 °C. The plating efficiency of TG1/pTAG1 also increases slightly in the first 4 h at 44 °C (Fig. 4) but then remains constant. The plating efficiency of TG1/pTAG1 (at 30 °C) is the same whether or not chloramphenicol is present (Fig. 4), indicating that only those cells that still contain the covering plasmid pTAG1 are viable. This indicates that the *lpxK* gene product is required for cell viability at 30 °C as well as 44 °C.

The plating efficiency of the control culture MC1061/pTAG1 (Fig. 3) on LB agar with or without chloramphenicol at 30 °C indicates that pTAG1 loss is detectable about 4 h after the shift to 44 °C (data not shown).

Assays of 4′-Kinase in Extracts Prepared from TG1/pTAG1 Grown at 44 °C—We next wanted to determine if loss of the *lpxK* gene leads to loss of lipid A 4′-kinase activity in cell extracts. Cultures of TG1/pTAG1 and TG1/pTAG6 were grown logarithmically at 44 °C as in Fig. 3. At regular intervals, portions of the cells were harvested. Cell-free extracts were prepared and assayed for 4′-kinase (Fig. 5). Extracts from TG1/pTAG6 contained measurable 4′-kinase after prolonged growth at 44 °C. However, the specific activity was 3-fold lower

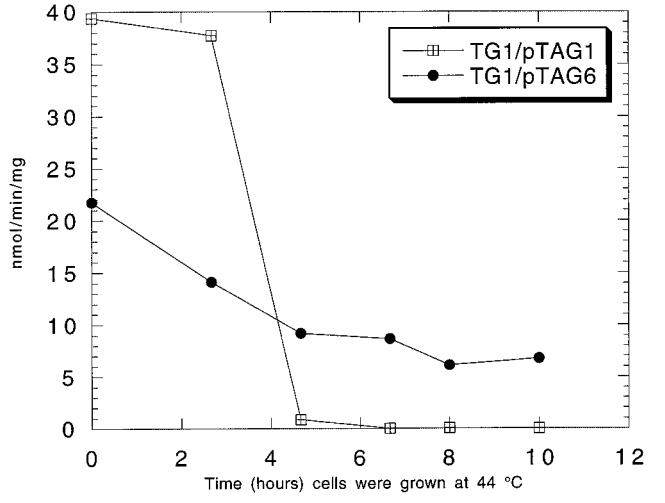

FIG. 5. **Loss of 4′-kinase specific activity in extracts of TG1/pTAG1 cells grown at 44 °C.** TG1/pTAG1 and TG1/pTAG6 were grown at 44 °C; cell-free extracts were prepared, and 4′-kinase activity was assayed at 30 °C as described under "Experimental Procedures." The specific activity of the 4′-kinase was determined for each extract and is plotted as a function of the time that the cells were grown at 44 °C. TG1/pTAG6 extracts display a high level of 4′-kinase throughout the temperature shift (compared with a specific activity of about 1 nmol/min/mg in wild type). The 2-fold reduction in kinase-specific activity seen in extracts of TG1/pTAG6 grown at 44 °C is probably due to a change in plasmid copy number. TG1/pTAG1 extracts lose most of their 4′-kinase activity after 4 h of growth at 44 °C. The 4′-kinase activity was barely detectable (0.01 nmol/mg/min) in the extracts prepared from TG1/pTAG1 cells grown at 44 °C for 10 h.

TABLE II
Lipid A (LA) to glycerophospholipid (GPL) ratio for TG1/pTAG1 and TG1/pTAG6 grown at 30 and 44 °C

Strain	Growth temperature	LA/GPL
	°C	
TG1/pTAG1	30	0.12
	44	0.10
TG1/pTAG6	30	0.11
	44	0.16

than in extracts of 30 °C grown TG1/pTAG6 (0-min time point in Fig. 5) but still 7-fold higher than in extracts of wild type cells (not shown). Extracts of TG1/pTAG1 have high 4′-kinase levels when cells are grown at 30 °C (0 min time point in Fig. 5). However, extracts of TG1/pTAG1 cells grown for 5 h at 44 °C display drastically lower 4′-kinase activity. After 10 h of growth at 44 °C, the kinase is barely detectable. The finding that loss of *lpxK* leads to loss of 4′-kinase activity supports the view that *lpxK* is the structural gene for the enzyme.

Lipid Composition of TG1/pTAG1 and TG1/pTAG6 Grown at 30 and 44 °C—The lipid A to glycerophospholipid ratio of wild type *E. coli* is 0.10–0.17, depending on the strain and growth conditions (16, 21, 25). This ratio reflects the necessary balance between the biosynthesis of LPS and glycerophospholipids in the cell. Over- or under-production of these biomolecules affects membrane biogenesis and cell viability (16, 21, 25). One would expect lipid A biosynthesis to be compromised in cells grown under conditions in which the 4′-kinase is depleted. In addition, the lack of the 4′-kinase activity might lead to the accumulation of the kinase substrate, DS-1-P (Fig. 1), a metabolite that has not been isolated previously from cells because of its low abundance.[2]

TG1/pTAG1 and TG1/pTAG6 were grown at both 30 and 44 °C and labeled for several hours with $^{32}P_i$, as described under "Experimental Procedures." The lipid A to glycerophospholipid ratio was determined (Table II) to be 0.1 in TG1/pTAG1 grown at 44 °C, as compared with 0.16 in the control TG1/pTAG6 grown at the same temperature. This analysis suggests that lipid A biosynthesis is slightly compromised under the labeling conditions employed.

Fig. 6 shows a PhosphorImager analysis of a thin layer chromatogram of the lipids extracted directly by neutral Bligh-Dyer partitioning (22, 23) from the labeled cells without prior acid hydrolysis (21). TG1/pTAG6 grown at 30 or 44 °C and strain TG1/pTAG1 grown at 30 °C all display a normal pattern of labeled glycerophospholipids, with cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine as the predominant species (26). However, the total lipids extracted from labeled cells of TG1/pTAG1 grown at 44 °C contain large amounts of an additional unknown, more slowly migrating material. This substance has the same R_F as a DS-1-^{32}P standard, prepared enzymatically (20). Thus, TG1/pTAG1 appears to accumulate large amounts of DS-1-P, the substrate of the 4′-kinase, after growth at 44 °C and loss of the *lpxK* gene. The very high levels of DS-1-P may account for the considerable residual production of lipid A (Table II) in the face of declining 4′-kinase (Fig. 4) during the time of labeling.

Isolation and ^{1}H NMR Spectroscopy of the Compound That Accumulates in TG1/pTAG1—While migration of the radiolabeled unknown with a DS-1-P standard upon thin layer chromatography (Fig. 6) is suggestive, further structural analysis was needed to confirm that the accumulated metabolite was indeed DS-1-P. Unlabeled TG1/pTAG1 and TG1/pTAG6 cells were therefore grown at 44 °C on a larger scale, and the cell

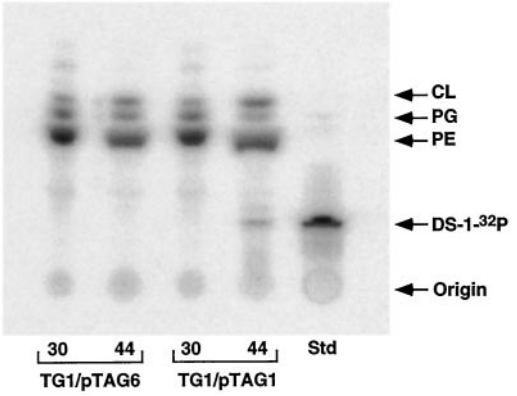

FIG. 6. **Accumulation of an unknown lipid migrating like DS-1-P in $^{32}P_i$-labeled TG1/pTAG1 grown at 44 °C.** TG1/pTAG1 and TG1/pTAG6 were labeled with $^{32}P_i$ for several hours, as described under "Experimental Procedures." Equal counts of the crude phospholipid fractions obtained by Bligh Dyer extraction of intact cells were loaded onto a high performance thin layer plate and developed in the solvent chloroform/methanol/water/ammonia (40:25:4:2, v/v). The lane marked *Std* is DS-1-^{32}P synthesized enzymatically according to Radika and Raetz (20). Phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), and cardiolipin (*CL*) are present in all of the labeled samples, and their ratios are typical of wild type cells (26). However, in the crude glycerophospholipid fraction of TG1/pTAG1 grown at 44 °C, an additional metabolite accumulates to about 9% of the total that migrates with standard DS-1-^{32}P.

pellets were extracted, as described under "Experimental Procedures." The crude lipids were first separated by chromatography on a high performance thin layer plate and charred with sulfuric acid (Fig. 7). *Lanes 1* and *4* show the positions DS-1-P standards, prepared enzymatically from lipid X and UDP-diacylglucosamine (20). *Lanes 2* and *3* show the total lipids extracted from TG1/pTAG1 and TG1/pTAG6, respectively, grown at 44 °C. A substance migrating with about the same R_F as the authentic DS-1-P standard was detected by charring the lipids of TG1/pTAG1, but not of TG1/pTAG6, confirming the findings with the ^{32}P-labeled cells (Fig. 6).

Next, milligram quantities of the substance that accumulates at 44 °C in TG1/pTAG1 were purified by DEAE-cellulose and reverse phase chromatography. This material was then analyzed by two-dimensional ^{1}H correlation (COSY) spectroscopy. Both the one- and the two-dimensional spectra are shown in Fig. 8. The overall features of the spectra and the connectivities are diagnostic of DS-1-P (20, 27), a β-1′,6-linked glucosamine disaccharide bearing four (*R*)-3-hydroxyacyl chains at positions 2, 3, 2′,b and 3′, and a phosphate residue at the 1-position in the α anomeric configuration (Fig. 8). All the protons of both glucosamine ring systems can be assigned in the two-dimensional spectrum, and their connectivities can be traced, as indicated in Fig. 8. Scalar coupling connectivities in the COSY spectrum for each glucosamine ring system were established starting with the characteristic cross-peaks between H-1 (~5.45 ppm) or H-1′ (~4.7 ppm) and their respective neighbors H-2 or H-2′.

The one-dimensional spectrum of the isolated lipid (Fig. 8) also shares many common features with that published previously for lipid IV_A dissolved in similar solvent mixtures (9, 10). However, the key resonance that distinguishes the spectrum of the lipid isolated from TG1/pTAG1 (Fig. 8) and that of lipid IV_A (structure in Fig. 1) is the chemical shift of H-4′. In lipid IV_A, H-4′ resonates at about 4.3 ppm (9, 10), but in Fig. 8 H-4′ is observed at 3.58 ppm. This chemical shift strongly suggests that the 4′-OH is unsubstituted in the material isolated from TG1/pTAG1, as in authentic DS-1-P made enzymatically (27).

Accumulated Lipid Isolated from TG1/pTAG1 Is a Substrate

[2] C. E. Bulawa and C. R. H. Raetz, unpublished observations.

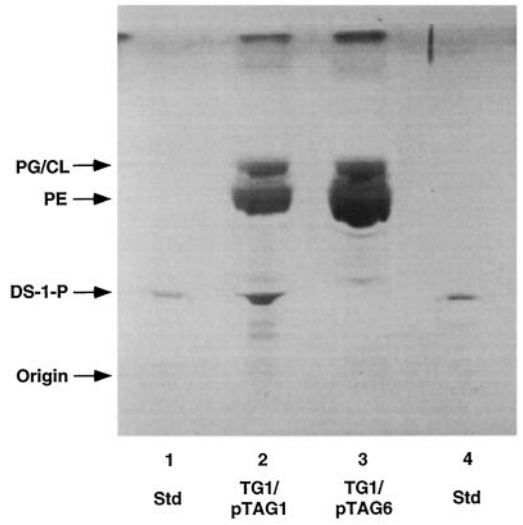

FIG. 7. **Accumulation of a lipid migrating like DS-1-P in TG1/pTAG1 at 44 °C as detected by sulfuric acid charring.** TG1/pTAG1 and TG1/pTAG6 were grown as described under "Experimental Procedures." Portions of the cell pellets were extracted with a neutral single-phase Bligh-Dyer mixture (22, 23), converted into a neutral two-phase Bligh-Dyer system using PBS as the aqueous component, and loaded onto an high performance thin layer plate, which was developed in chloroform/methanol/water/ammonia (40:25:4:2, v/v) and charred with 20% sulfuric acid in ethanol. *Lanes 1* and *4* are a DS-1-P standard (about 5 μg) synthesized enzymatically (20). *Lane 2* is a crude lipid extract from TG1/pTAG1 cells grown at 44 °C. *Lane 3* is a matched extract from TG1/pTAG6 grown at 44 °C.

for the 4′-Kinase—If DS-1-P is indeed accumulating in TG1/pTAG1 grown at 44 °C then, when purified, it should serve as a substrate for the 4′-kinase *in vitro*. We attempted to phosphorylate the material isolated from TG1/pTAG1 with membranes of strain BLR(DE3)pLysS/pJK2, a strain that overexpresses 4′-kinase activity about 3000-fold compared with wild type (8). Like synthetic DS-1-P (data not shown), the material isolated from TG1/pTAG1 serves as an excellent substrate for the 4′-kinase reaction (Fig. 9, *lane 4*). Quantitative conversion of the isolated material to a substance migrating like lipid IV_A is possible, as judged by sulfuric acid charring following thin layer chromatography (Fig. 9). The reaction is dependent upon the presence of ATP (Fig. 9, *lane 3 versus 4*). In *lane 5*, authentic lipid IV_A, isolated from a Kdo-deficient mutant of *Salmonella* (28, 29), was spotted as a standard.

The combined results of Figs. 6–9 provide unequivocal proof for the hypothesis that the *lpxK* gene product is responsible for the phosphorylation of DS-1-P at position 4′ in living cells of *E. coli* to generate lipid IV_A.

Rescue of the lpxK::kan Insertion Mutation in TG1/pTAG1 with the lpxK Homolog of F. novicida—F. novicida is a facultative intracellular bacterial pathogen (17). Mdluli *et al.* (17) identified a locus of *F. novicida* that is required for virulence of the bacteria in mice. The locus, called *valAB*, is homologous to the *msbA*/*lpxK* locus in *E. coli* (17). *valB* is 66.8% similar and 41.4% identical to *lpxK* (8) and may encode the *F. novicida* lipid A 4′-kinase. To test this hypothesis, extracts of XL1-Blue cells harboring pKEM14–5, a plasmid containing *valAB* and a portion of *polA* (17), were assayed for 4′-kinase activity. These extracts possess about 10 times more 4′-kinase activity than crude extracts of XL1-Blue cells containing vector alone (17) (data not shown). To determine whether *valB* can rescue TG1/pTAG1 grown at 44 °C, pTAG8, which contains *valB* and about one-third of *polA* (but no *valA*), was constructed. pTAG8 and pACYC177 were transformed separately into competent TG1/pTAG1, and transformants were selected on LB plates at 30 °C containing ampicillin. Single colonies were then streaked to LB plates containing ampicillin and grown at 30 and 44 °C. Cells that contained pACYC177 were able to grow and form single colonies at 30 but not 44 °C. Cells that contained pTAG8 were able to grow and form single colonies at both 30 and 44 °C. The latter cells were ampicillin-resistant but chloramphenicol-sensitive, showing that pTAG1 had been lost. These findings indicate that *valB* can provides the necessary 4′-kinase activity to promote growth of *E. coli*. A growth experiment in liquid medium was also performed, as in Fig. 3. TG1/pTAG8 was able to grow at 44 °C, exactly like TG1/pTAG6 (data not shown).

DISCUSSION

We have constructed and characterized an *E. coli* strain (TG1/pTAG1) with an insertion mutation in the structural gene (*lpxK*) (8) encoding the lipid A 4′-kinase. TG1/pTAG1 has a kanamycin cassette inserted into the chromosomal copy of *lpxK*. This *lpxK::kan* allele is complemented by a hybrid plasmid containing $lpxK^+$ and a temperature-sensitive origin of replication (15).

By using TG1/pTAG1, we have confirmed the essential nature of *lpxK*, originally suggested by Karow and Georgopoulos (12) prior to the identification of the function of the gene (8). Once the *lpxK* gene and its product are depleted, cell growth and viability are reduced (Figs. 3 and 4), and 4′-kinase activity is lost (Fig. 5). The lipid A to glycerophospholipid ratio decreases (Table II), indicating gradual inhibition of lipid A biosynthesis. Furthermore, when TG1/pTAG1 cells are grown at 44 °C, an additional substance accumulates to high levels in the lipid fraction (Figs. 6–9) that we have identified unequivocally as DS-1-P. This is the first demonstration of DS-1-P as a natural product, given its low abundance in wild type cells (3, 26).[2] In our earlier work, DS-1-P was characterized only after enzymatic synthesis *in vitro* by lipid A disaccharide synthase (the *lpxB* gene product) (20, 27). However, other key lipid A precursors, such as 2,3-diacylglucosamine (lipid X) (30, 31), UDP-2,3-diacylglucosamine (UDP-DAG) (31), and tetraacyldisaccharide 1,4-*bis*-phosphate (lipid IV_A) (28, 29, 32), have been isolated from various strains of *E. coli* and *Salmonella*.

The accumulation of DS-1-P in cells lacking the 4′-kinase strongly supports the hypothesis that DS-1-P is the physiological lipid acceptor for the 4′-kinase (9). Until this work, other schemes for the incorporation of the 4′-phosphate into lipid A could not be excluded. For instance, one alternative possibility was the 4′-phosphorylation of UDP-DAG. Given the massive accumulation of DS-1-P *in vivo* in TG1/pTAG1 at 44 °C and the fact that UDP-DAG does not serve as a substrate for the cloned 4′-kinase *in vitro* (8), this possibility is now rendered very unlikely. The NMR spectrum of the accumulated lipid isolated from TG1/pTAG1 (Fig. 8) clearly shows that DS-1-P, not UDP-DAG, accumulates *in vivo* in the absence of 4′-kinase.

TG1/pTAG1 can be used to assess the function of *lpxK* variants from other bacteria. In this work, we have shown that *valB*, the *F. novicida lpxK* homolog (17), is able to substitute for *lpxK* in *E. coli*. TG1/pTAG8, a strain in which the *lpxK::kan* insertion is covered by *valB* on a non-temperature-sensitive plasmid, is able to grow at 44 °C as well as TG1/pTAG6, an analogous strain with the *E. coli* $lpxK^+$ on a non-temperature-sensitive plasmid (data not shown). Rescue of TG1/pTAG1 at 44 °C will also be useful in evaluating the function of *lpxK* truncations (for instance those lacking the hydrophobic N-terminal domain of LpxK) in the search for an active, soluble form of the kinase. Likewise, expression of His-tagged variants of *lpxK* in the TG1/pTAG1 background could be used to determine if His-tagged LpxK is active and is tightly bound to other proteins in the absence of competing wild type LpxK.

One protein that may interact with LpxK is MsbA. *msbA* and *lpxK* are co-transcribed (12, 13). Although it appears that MsbA

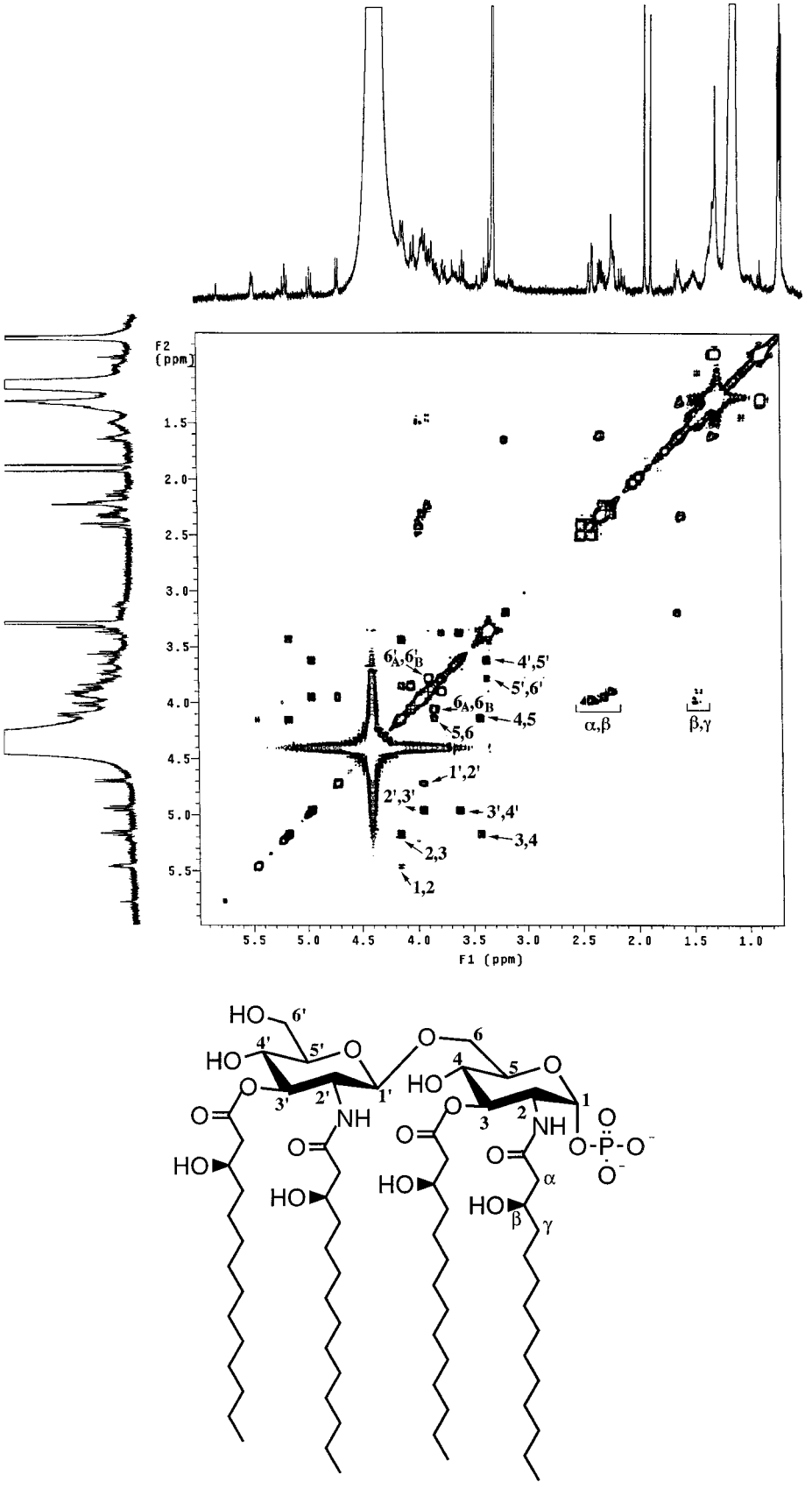

FIG. 8. **Two-dimensional ^{1}H correlation (COSY) spectrum of the lipid that accumulates in TG1/pTAG1.** The one-dimensional spectrum from 0.7 to 6 ppm of the purified material is shown along the *x* and *y* axes, and the assignments of the cross-peaks arising from the protons of two glucosamine residues and of the α, β, and γ protons of the acyl chains are indicated. The resonances at approximately 5.5, 5.2, 5.0, 4.7, and 3.6 ppm represent H-1, H-3, H-3′, H-1′, and H-4′, respectively, and are characteristic of DS-1-P standards synthesized enzymatically (27). The structure at the *bottom* indicates the locations of the key carbon atoms to which the protons indicated in the spectrum are attached.

plays an important role in lipid A transport (43), a role for LpxK in transport (in addition to its enzymatic function as the 4′-kinase) cannot yet be excluded. For instance, LpxK might form part of the putative membrane channel through which MsbA mediates lipid A flip-flop (43). Other heterodimeric membrane channels, such as the CydCD ABC transporter for periplasmic cytochrome *c* assembly, have the expression of their protein components tightly linked in operons (33), like

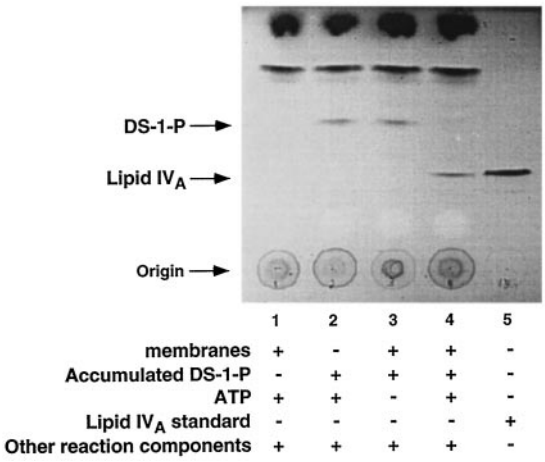

FIG. 9. **The lipid that accumulates in TG1/pTAG1 at 44 °C is a substrate for the 4′-kinase.** The purified lipid, isolated as described under "Experimental Procedures," was used in a 4′-kinase assay system together with membranes isolated from the kinase overproducer BLR(DE3)pLysS/pJK2 (8). A reaction mixture (10 μl final volume and 500 μg/ml membranes) was set up so that the final reaction conditions were exactly as described for the standard kinase assay (*i.e.* 100 μM acceptor lipid). Components were mixed as shown with any remaining volume compensated for with water. After 30 min at 30 °C, the entire sample was spotted onto an HPTLC plate, which was developed as in Fig. 7. Lipids were detected by charring with 20% sulfuric acid in ethanol.

msbA and *lpxK*. It is difficult to ask these questions at present because the one available mutation in the kinase is an insertion (Table I), which would also likely impair its putative role in a transport complex with MsbA. If MsbA and LpxK do form a complex, it might be possible to immunoprecipitate MsbA together with LpxK or to isolate *lpxK* point mutations that retain 4′-kinase activity but do not effectively interact with MsbA.

The identification of second site suppressors that might allow cells to grow without a 4′-kinase could be useful in further understanding lipid A biosynthesis, transport, and function. One possible class of second site suppressors might be mutations in the *kdtA* gene (11, 34). A mutation in KdtA that allows efficient transfer of Kdo from CMP-Kdo to DS-1-P (rather than the preferred substrate lipid IV_A) (10, 11, 34) might allow growth in the absence of 4′-kinase. Interestingly, the 4′-phosphate is not found in the lipid As of all Gram-negative bacteria (35–38). In *Rhizobium leguminosarum*, this phenomenon is attributed to the presence of a specific membrane-bound 4′-phosphatase that removes the 4′-phosphate after the 4′-kinase and Kdo transferase have generated Kdo_2-lipid IV_A (39).

The recent surge in microbial genomics is greatly facilitating the study of lipid A biosynthesis in a broad range of Gram-negative bacteria besides *E. coli*. The completed genomes of *Hemophilus influenzae* (40) and *Helicobacter pylori* (41) contain variants of all the known genes of the *E. coli* lipid A pathway, including *lpxK*. However, the genome of *Synechocystis* is very peculiar in that it contains only the genes encoding the first five enzymes of lipid A biosynthesis, leading to the formation of DS-1-P (42). *Synechocystis* lacks the genes for the 4′-kinase, the Kdo transferase, the late acyltransferases, and the enzymes required for generating CMP-Kdo (42). Although biochemical studies are very limited, LPS isolated from *Synechocystis* lacks the 4′-phosphate on its lipid A and does not contain Kdo (35), consistent with the genomics. How DS-1-P would be processed in the absence of a 4′-kinase and a Kdo transferase in *Synechocystis* is unclear. The identification of the unique genes and enzymes that make distinct lipid A variants in diverse bacteria will facilitate the modification of lipid A-like molecules in living cells and may provide insights into why lipid A is necessary for the viability of *E. coli*.

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