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

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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 knockout 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 1H NMR spectroscopy. DS-1-P was not 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) 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 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 knockout 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 1H 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) 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 Kdo₂-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 Kdo₂-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 IVα) (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 glucoinosamine-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,
The sixth step in the biosynthesis of Kdo 2-lipid A is catalyzed by the enzyme LpxK, which transfers two Kdo residues to form Kdo 2-lipid IVA. HtrB is necessary to catalyze the addition of the second Kdo residue.

**Materials**

- **UDP-N-Acetylglucosamine + R-3-hydroxymyristoyl ACP**
- **ATP**
- **ADP**
- **Kdol-P**
- **Kdol-Lipid IVA**
- **Kdol-Lipid A**
- **Lauroyl-ACP**
- **Mystearyl-ACP**
- **3′-phosphorylation of DS-1-P to yield lipid IV A, KdtA gene in lipid A biosynthesis.**

**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. pTAG2 (2) was digested with SacI and NdeI, and pMAK705 was digested with XhoI and BamHI. The 1.2-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.

**Transformation of E. coli with plasmid DNA**

Plasmid DNA was isolated from chloramphenicol-resistant clones and was digested with SacI and BamHI to verify the presence of the correct insert. One such plasmid was 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 Nsi1 site of lpxK gene. pJK2 was digested with NsiI, and pUC-4K (Amersham Pharmacia Biotech) was digested with PsiI. The 5.5-kb linearized pJK2 and the 1.2-kb kanamycin cassette were ligated together. A portion of the ligation mixture was transformed into E. coli XL1-Blue, and colonies resistant to ampicillin were selected. Plasmids isolated from ampicillin-resistant colonies were digested with Ndel and BamHI to verify the presence of the correct 2.2-kb insert. The lpxK:kan construct described above was digested with XbaI and BamHI and cloned into pMAK705 exactly as for pTAG1, yielding pTAG2.

**Construction of TG1/pTAG1, a Mutant with an Insertion in the lpxK Gene**

pNGH1-amp was constructed from pNGH1 (16). pNGH1 was digested with BamHI and SalI yielding 3.9- and 1.6-kb fragments. pACYC177 was digested with BamHI and XhoI 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 tcr promoter were ligated together to form pNGH1-amp.

To construct pTAG6, pJK2 was digested with NdeI, 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 BamHI, yielding a 985-base pair fragment with one blunt end. This fragment was then ligated with pMAK705, yielding pTAG6.

**Construction of TG1/pTAG1, a Mutant with an Insertion in the lpxK Gene**

A portion of the ligation mixture was transformed into E. coli XL1-Blue competent cells, and colonies resistant to ampicillin were selected. Plasmid DNA was isolated from ampicillin-resistant clones and was digested with EcoRV and NdeI 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 chromosome of lipX**

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 A600 of 0.6. Next, 1 × 10⁹ 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 growth, the integrated plasmid will occur either the wild type lipX or the lipX::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 XbaI and BamHI. Of the 14 colonies, 11 contained the pTAG2 insert. Three,
**Integration Mutation in E. coli lpxK Gene**

**Resolution at 30°C**

**Resolution at 44°C**

**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 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 \textsuperscript{32}P, for about two doubling times. \textsuperscript{32}P (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 0.2, the cultures were inoculated with 300 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 modifications. 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 decysylation 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 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 TG1/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 TGA1/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
phase. The final lower phase was dried down 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 (40:25:2:1, v/v). The lipids were detected by charring with 20% sulfuric acid in ethanol.

**Purification of the Accumulated Lipid A Precursor from TG1/pTAG1**

Purification of one lipid component, and it was almost pure, as judged by thin layer chromatography. Approximately 1 mg of purified material was dissolved in 0.6 ml of CDCl₃/CD₃OD (4:1, v/v), and its 1H NMR spectrum was recorded on a Varian 500 Unity spectrometer using a 500.13-Hz spectral window with a 5120-point zero-filled spectrum. A line broadening of 0.05 Hz before Fourier transformation was used to process the data.

Two-dimensional 1H correlation (COSY) spectra were recorded in the absolute value mode over the same spectral region used in the one-dimensional 1H 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/pTAG1 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 A₆₀₀ of 0.01. The temperature was then shifted to 44 °C and growth was continued with shaking at 250 rpm. To maintain logarith-
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, 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.
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 32P, 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. Accumulation of an unknown lipid migrating like DS-1-P in 32P-labeled TG1/pTAG1 grown at 44 °C.** TG1/pTAG1 and TG1/pTAG6 were labeled with 32P 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-1-32P 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-1-32P.

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 1H 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', 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 IVα 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 IVα (structure in Fig. 1) is the chemical shift of H-4'. In lipid IVα, 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
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/pJ2K, 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 IV₄ 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 IV₄, 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 *lipK* gene product is responsible for the phosphorylation of DS-1-P at position 4' in living cells of *E. coli* to generate lipid IV₄.

Rescue of the *lipK*::kan Insertion Mutation in TG1/pTAG1 with the *lipK* Homolog of *F. novicida*—F. *novicida* is a facultative intracellular bacterial pathogen (17). Mduli et al. (17) identified a locus of *F. novicida* that is required for virulence of the bacteria in mice. The locus, called *valA*, is homologous to the *msbA/lpxK* locus in *E. coli* (17). *valB* is 66.8% similar and 41.4% identical to *lipK* (8) and may encode the *F. novicida* lipid A 4'-kinase. To test this hypothesis, extracts of XLI-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 XLI-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 not *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 were ampicillin-resistant but chloramphenicol-sensitive, showing that *pTAG1* had been lost. These findings indicate that *valB* can provide 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 (*lipK*) (8) encoding the lipid A 4'-kinase. TG1/pTAG1 has a kanamycin cassette inserted into the chromosomal copy of *lipK*. This *lipK::kan* allele is complemented by a hybrid plasmid containing *lipK* and a temperature-sensitive origin of replication (15).

By using TG1/pTAG1, we have confirmed the essential nature of *lipK*, originally suggested by Karow and Georgopoulos (12) prior to the identification of the function of the gene (8). Once the *lipK* 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).

In our earlier work, DS-1-P was characterized only after enzymatic synthesis in vitro by lipid A disaccharide synthase (the *lipB* gene product) (20, 27). However, other key lipid A precursors, such as 2,3-diacylglycerol (lipid X) (30, 31), UDP-2,3-diacylglycerol (UDP-DAG) (31), and tetraacyldisaccharide 1,4-bis-phosphate (lipid IV₄) (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 *lipK* variants from other bacteria. In this work, we have shown that *valB*, the *F. novicida* *lipK* homolog (17), is able to substitute for *lipK* in *E. coli*. TG1/pTAG8, a strain in which the *lipK::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* *lipK* 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 *lipK* 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 *lipK* 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 *lipK* are co-transcribed (12, 13). Although it appears that MsbA
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...
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 IVα) (10, 11, 34) might allow growth in the absence of 4-kinase. Interestingly, the 4-phosphate is not found in the lipid 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 Kdo2-lipid IVα (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 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 genome. 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.