Identification of the Gene Encoding the Escherichia coli Lipid A 4'-Kinase

FACILE PHOSPHORYLATION OF ENDOTOXIN ANALOGS WITH RECOMBINANT LpxK*

(Received for publication, April 28, 1997, and in revised form, June 20, 1997)

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The genes for seven of nine enzymes needed for the biosynthesis of Kdo₂-lipid A (Re endotoxin) in Escherichia coli have been reported. We have now identified a novel gene encoding the lipid A 4'-kinase (the sixth step of the pathway). The 4'-kinase transfers the γ-phosphate of ATP to the 4'-position of a tetraacyldisaccharide 1-phosphate intermediate (termed DS-1-P) to form tetraacyldisaccharide 1,4'-bis-phosphate (lipid IVₐ). The 4'-phosphate is required for the action of distal enzymes, such as Kdo transferase and also renders lipid A substructures active as endotoxin antagonists or mimetics. Lysates of E. coli generated using individual λ clones from the ordered Kohara library were assayed for overproduction of 4'-kinase. Only one clone, [218]E1D1, which directed 2-2.5-fold overproduction, was identified. This construct contains 20 kilobase pairs of E. coli DNA from the vicinity of minute 21. Two genes related to the lipid A system map in this region: msbA, encoding a putative translocator, and kdsB, the structural gene for CMP-Kdo synthase. msbA forms an operon with a downstream, essential open reading frame of unknown function, designated orfE. orfE was cloned into a T7 expression system. Washed membranes from cells overexpressing orfE display ~2000-fold higher specific activity of 4'-kinase than membranes from cells with vector alone. Membranes containing recombinant, overexpressed 4'-kinase (but not membranes with wild-type kinase levels) efficiently phosphorylate three DS-1-P analogs: 3-aza-DS-1-P, base-treated DS-1-P, and base-treated 3-aza-DS-1-P. A synthetic hexaacylated DS-1-P analog, compound 505, can also be phosphorylated by membranes from the overproducer, yielding [4',32P]lipid A (endotoxin). The overexpressed lipid A 4'-kinase is very useful for making new 4'-phosphorylated lipid A analogs with potential utility as endotoxin mimetics or antagonists. We suggest that orfE is the structural gene for the 4'-kinase and that it be redesignated lpxK.

Lipopolysaccharide (LPS) is the major glycolipid of the outer membrane of Gram-negative bacteria (1–5). Lipid A, or endotoxin, is the hydrophobic anchor of LPS (1–5), and it is a potent immunostimulant. It also appears to be responsible for many of the features of septic shock that can accompany severe Gram-negative infections (1–5). Lipid A is a disaccharide of glucosamine that is phosphorylated at the 1- and 4'-positions and is acylated with R-3-hydroxy-myristate at the 2-, 3-, 2',- and 3'-positions (1–5). In Escherichia coli, two additional fatty acyl chains are also esterified to the 2'- and 3'-R-3-hydroxy-myristate groups to form acyloxyacyl units (Fig. 1) (1–5).

Lipid A biosynthesis begins with the acyl-ACP dependent acylation of UDP-N-acetylglucosamine (6–10). Nine enzymes are required for the complete synthesis of Kdo₂-lipid A (Fig. 1) (2, 3, 11). Seven of the nine structural genes coding for the enzymes of lipid A biosynthesis in E. coli have been identified (3). However, the lipid A 4'-kinase gene has remained elusive (3). The 4'-kinase catalyzes the sixth step of the pathway (Fig. 1) (12). It phosphorylates the 4'-position of a tetraacyldisaccharide 1-phosphate intermediate (termed DS-1-P) to form tetraacyldisaccharide 1,4'-bis-phosphate, also known as lipid IVₐ (Fig. 1) (12–14).

Identification of the lipid A 4'-kinase gene has been hampered because mutants lacking the 4'-kinase have not been identified (3). Presumably the 4'-kinase gene, like most other genes encoding enzymes of lipid A biosynthesis, is required for growth (3). Genetic screens for conditionally lethal mutants, such as that used for the identification of the Kdo transferase gene, kdsA (15), have not been developed. Attempts to purify the kinase to homogeneity have been thwarted by the protein’s association with membranes and its instability in the presence of detergents (12, 16).

The lipid A 4'-kinase can be used to make 4'-32P-labeled lipid A precursors, such as [4',32P]lipid IVₐ and Kdo₂-[4',32P]lipid IVₐ, for biochemical analyses of late pathway reactions (12, 16–18). The 4'-kinase activity found in wild type E. coli membranes, however, is relatively inefficient and unstable, especially in the presence of low chemical concentrations of ATP (12, 16). Only 0.1–0.5% incorporation of 32P into [4',32P]lipid IVₐ is obtained when trace quantities of [γ-32P]ATP are used together with the physiological substrate, DS-1-P (12, 16–18). This low level of 32P transfer makes it virtually impossible to use the 4'-kinase for phosphorylating DS-1-P analogs that are utilized less efficiently. Identification and overexpression of the 4'-kinase gene would allow investigation of DS-1-P analogs as substrates for the 4'-kinase, possibly facilitating the synthesis of interesting lipid IVₐ analogs with potential activity as endotoxin antagonists or mimetics (2, 3). Studies of endotoxin uptake and metabolism (19–21) would be simplified if [4'-32P]lipid A or various analogs could be made with the 4'-kinase.

To identify the gene encoding the lipid A 4'-kinase, we employed the approach used by Clementz et al. (22, 23) to find the gene for the Kdo-dependent lauroyltransferase of the lipid A pathway. Individual lysates generated from the ordered Kohara library (22–24) were assayed for overproduction of 4'-kinase activity. Using this procedure, a λ clone was identified.
that spans 20 kilobase pairs near minute 21 of the E. coli chromosome and is capable of directing slight (2–2.5-fold) overexpression of 4'-kinase activity. We believe that orfE is the structural gene for the 4'-kinase and should be redesignated lpxK. orfE does not display significant sequence homology to other known kinases. The overexpressed 4'-kinase is very useful for the efficient synthesis of labeled precursors and novel lipid A analogs.

EXPERIMENTAL PROCEDURES

Materials—[32P]ATP and [γ-32P]ATP were obtained from NEN Life Science Products. 0.25-mm glass-backed Silica Gel 60 thin layer chromatography plates from Merck, yeast extract and tryptone from Difco, restriction enzymes from New England Biolabs, T4 DNA Ligase from Life Technologies—E. coli strain, and Pfu DNA polymerase from Stratagene. Solvents for thin layer chromatography were reagent grade from Mallinkrodt, and solvents for lipid preparations were high pressure liquid chromatography grade (Aldrich).

Bacterial Strains and Growth Conditions—Table I lists the strains used in this study. Cells were cultured at 37 °C in Luria Broth (LB) consisting of 10 g of NaCl, 5 g of yeast extract, and 10 g of tryptone per liter (27). Antibiotics were added, when required, at 50 μg/ml for ampicillin, 12 μg/ml for tetracycline, and 30 μg/ml for chloramphenicol.

DNA Techniques—E. coli W3110 chromosomal DNA was isolated as described by Ausubel et al. (28). Minipreparations of plasmid DNA were made using the Promega Wizard minipurification system. Large scale preparations of plasmid DNA were made using the 5–3' Bigger Prep kit. Polymerase chain reactions were optimized using the Stratagene Oligo Extender kit. DNA fragments were isolated from agarose gels using the Qiagen Qiaex II gel extraction kit. Restriction enzymes and T4 ligase were used according to the manufacturer's directions. Transformation of E. coli with plasmid DNAs was done using salt-resistant cells (29).

Lipid Substrates—DS-1-32P was made according to Radika and Raetz (30). Milligram quantities DS-1-P were enzymatically synthesized from UDP-2,3-diacylglucosamine and 2,3-diacylglucosamine disaccharide by homogenization in 10 ml of the HEPES buffer and stored frozen in a plastic vial. The base-treated, deacylated DS-1-P eluted with the 1:1 solvent mixture. The base-treated, deacylated DS-1-P was made by treating 2 mg of DS-1-P for 30 min with 0.2M NaOH in 1 ml of chloroform/methanol (2:1, v/v). The mixture was separated on a silica column equilibrated with 50 ml of chloroform/methanol (95:5). The column was washed with 25 ml of each of the following ratios (v/v) of chloroform/methanol: 95:5, 90:10, 70:30, 1:1, 30:70, and 20:80. Thirty 5-ml fractions were collected. The resolved hydroxy fatty acids and the deacylated DS-1-P were detected by spotting 5 μl of each fraction onto a thin layer chromatography plate, developing the plate in chloroform/methanol/water/acetic acid (25:15:4.2: v/v/v/v), and staining with sulfuric acid. The hydroxy fatty acids eluted with the 70:30 (v/v) solvent mixture. The base-treated, deacylated DS-1-P eluted with the 1:1 solvent ratio. The relevant fractions were pooled, and the solvent was removed by rotary evaporation. The base-treated, deacylated 3-aza-DS-1-P was prepared in the same manner. The elution profile for this compound was the same as for the base-treated, deacylated DS-1-P. For use as substrates in 4'-kinase assays, all lipid substrates were dispersed in 50 mM HEPES, pH 7.4, by sonication irradiation for 2 min.

Kohara Lambda Library Preparation and Screen for 4'-Kinase Activity—Fresh lysates of the Kohara lambda library were made following the method of Clementz et al. with slight modifications (23). The host E. coli strain, W3110, was grown overnight at 37 °C in LB medium, supplemented with 0.2% maltose and 10 mM MgSO4. The culture was diluted 1:1 with 10 mM CaCl2, 10 mM MgCl2. The λ lysates used by Clementz et al. (23) were diluted 1:100 and 1:1000 in SM buffer (5.8 g of NaCl, 2 g of MgSO4, 50 ml of 1 M Tris, pH 7.5, per liter). Using 96-well microtiter plates, 5 μl of the individual diluted lysates and 10 μl of the diluted host cell suspension were mixed and incubated at 37 °C for 15 min. LB medium supplemented with 10 mM MgSO4 (150 μl) was added to each well, and incubation continued at 37 °C. After 4 h, the A600 of each well was measured using a Molecular Dynamics Spectramax 250 microplate reader. When the cell suspension had cleared to an A600 less than 0.1, it was considered lysed and was transferred to a fresh microtiter plate at 4 °C. Lysates was evaluated every hour until 8 h after infection. The lysates originating from the 1:1000 dilution of the original stock were chosen for further analysis by a hybridization assay that utilized immobilized lysates with the 1:1000 dilutions of the original stock were generally obtained from the 1:100 dilutions of the original stock. The final lysates were stored at −80 °C overnight. The 4'-kinase activity of each lysate was assayed in a 10-μl reaction mixture containing 5 μl of lysate, 100 μM DS-1-P (1000 cpn/mmol), 1 mg/ml cardiolipin, 50 mM Tris, pH 8.5, 5 mM MgSO4, and 5 μg/ml of T4 ligase for 90 min at 30 °C. After 60 min, the reaction was stopped by spotting 5 μl onto a Silica Gel 60 TLC plate. The plates were developed in chloroform/methanol/water/acetic acid (25:15:4.2, v/v/v/v), dried, and exposed to a Molecular Dynamics PhosphorImager screen. Conversion of DS-1-32P to [1-32P]lipid IVc was quantified using ImageQuant software (Molecular Dynamics).

Construction of pJK2 Bearing orfE under the Control of a T7 Promoter—The gene encoded by the open reading frame orfE was cloned into pET3a cloning vector (Novagen). orfE was amplified by polymerase chain reaction of E. coli W3110 genomic DNA using Pfu DNA polymerase (according to the manufacturer's specifications) and the following primers: 5'-GTTTTGGCATATGATGAAATACTTG-3' and 5'-ATCATGATCTCAAACTGACGTCG-3'. The first primer introduces a NdeI site at the start codon of orfE, and the second primer introduces a BamHI site downstream of the stop codon. The polymerase chain reaction product was digested with NdeI and BamHI and ligated into a similarly cut pET3a vector. A portion of the ligation reaction was transformed into E. coli Suicide cells (Stratagene, La Jolla, CA), and colonies resistant to ampicillin were selected. Plasmid DNA was isolated from ampicillin-resistant clones and was digested with BamHI and NdeI to identify those constructs that contained the desired 1-ki-lobase pair insert. One correct plasmid was designated pJK2.

Expression of the orfE Gene Product—pJK2 was transformed into BLR(DE3)pLysS cells and grown at 37 °C in 2 liters of LB. When the cultures reached an A600 of 0.6, isopropyl-1-thio-β-D-galactopyranoside was added (final concentration of 1 mM) to induce expression of the orfE gene product. After 3 h of induction, the cells were collected by centrifugation at 10,000 × g for 15 min at 4 °C, washed with 1 liter of 50 mM HEPES, pH 7.5, and resuspended in 30 ml of the wash buffer. Cells were broken in a cold French pressure cell at 20,000 p.s.i., and unbroken cells were removed by centrifugation at 3,500 × g for 1 min at 4 °C. The membrane and soluble fractions were isolated by centrifugation of the entire cell-free extract at 150,000 × g for 60 min. The membrane fraction was washed once with phosphate buffer and the membrane pellet was resuspended in 50 ml of 50 mM HEPES, pH 7.5. The soluble fraction and the resuspended membranes were both centrifuged a second time. The final membrane pellet was resuspended by homogenization in 10 ml of the HEPES buffer and stored frozen in aliquots at −80 °C. The membrane-free cytosol was also stored in aliquots at −80 °C. The protein concentration was determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Assays for 4'-Kinase Activity—Two methods for analyzing 4'-kinase activity of various protein fractions were employed. The first (method 1) utilizes DS-1-32P as the labeled substrate. Typically, 100 μM DS-1-P (1000 cpn/mmol), 1 mg/ml cardiolipin, 50 mM Tris, pH 8.5, 5 mM ATP, 1% Nonidet P-40, and 5 mM MgCl2 are mixed with 0.5–500 μg/ml protein fraction and incubated at 30 °C for various times. Reactions were stopped by spotting a portion of the reaction onto a Silica Gel 60 thin layer chromatography plate. Plates were developed in chloroform/methanol/water/acetic acid (25:15:4.2, v/v/v/v) and analyzed as described above. Method 2 (which is not intended for the quantitative determination of specific activities) utilizes [γ-32P]ATP (∼8 × 104 cpn/mmol) as the labeled substrate. The reaction conditions are exactly the same as for method 1, except that the ATP concentration is lowered to 0.6 μM and only nonradioactive DS-1-P (final concentration of 100 μM) is added. The reactions were stopped as described above, and plates were developed in chloroform/pyridine/formic acid/water (30:70:16:10, v/v/v/v).

RESULTS

Screening for Overproduction of Lipid A 4'-Kinase in Kohara Lambda Lysates—Kohara et al. (24) have generated a library of 3400 mapped hybrid λ bacteriophage clones that covers the E. coli genome. A subset of this library containing 476 λ clones is available that covers 99% of the genome with some overlap between the clones (23, 32). Clementz et al. showed that enzyme
Massive Overexpression of 4′-Kinase Activity on a Hybrid Plasmid Bearing orfE—The gene encoding orfE was cloned behind the T7 promoter of pET3a to form pJK2. Plasmid pJK2 was transformed into BLR(DE3)pLysS cells, an E. coli strain that carries the T7 RNA polymerase as a λ lysogen (Table I). The expression of T7 RNA polymerase is induced with isopropylthio-β-D-galactopyranosidase and leads to the expression of genes from the T7 promoter. Washed membranes from BR7, an E. coli strain deficient for diglyceride kinase (12, 16), BLR(DE3)pLysS/pJK2, and BLR(DE3)pLysS/pET3a were assayed for 4′-kinase activity using DS-1-32P as the phosphate acceptor. The result of this assay is shown in Fig. 5. The 4′-kinase activity was highly overexpressed in cells with pJK2 versus strain BR7 or cells with pET3a vector alone (Fig. 5, lanes 3 and 4 versus lanes 1, 2, and 5). When assayed at a protein dilution in which product formation is linear with respect to time, overexpression of orfE led to several thousand-fold overproduction of 4′-kinase activity. Table II shows the specific activities of the 4′-kinase in cell-free extracts, membrane-free cytosols (subjected to two ultracentrifugations), and washed membranes.

seen with other enzymes of lipid A biosynthesis (23). However, there were several lysates with significantly higher activity than their neighboring lysates (Fig. 2). To choose lysates for further analysis, the mean and standard deviation values for each set of 80 were calculated. Fifteen clones, the activity of which surpassed the mean by more than two standard deviations, were reassayed (data not shown). One lysate, [218]E1D1 (marked by an asterisk in Fig. 2) consistently displayed 2–2.5-fold more kinase activity than the other lysates.

Differences in lysis time could account for some of the variation of the activities seen in the lysates. The original lysates used to make the library were not generated from a fixed titer. To control for this variation among lysates, the plaque-forming units (pfu) for lysates derived from λ clones [216]13E3, [217]6D12, [218]E1D1, and [320]15G10 were determined (Fig. 3). Matched lysates were then made by infecting E. coli W3110 with 4 × 10^–3 pfu. After 7–8 h, lysis occurred in each case, and the lysates were again assayed for 4′-kinase activity as before. The result is shown in Fig. 4. The lysate of λ [218]E1D1 persisted in having 2–2.5-fold overproduction of 4′-kinase activity, compared with controls. This finding led us to investigate further the genes on λ [218]E1D1.

Genes Present on [218]E1D1—The λ clone [218]E1D1 contains a 20-kilobase pair fragment of the E. coli genome spanning minutes 20.8–21.3 (33). Two genes in this region, msbA and kdsB, are related to the lipopolysaccharide system (33). kdsB encodes the CMP-Kdo synthase (3, 34, 35), and msbA encodes a putative LPS transporter (Fig. 3) (25, 26) with homology to mammalian Mdr proteins. msbA was first identified by Karow and Georgopoulos (25, 26) as a multicopy suppressor of htrB (36–38), the gene encoding the Kdo-dependent lauryltransferase (Fig. 1) (22, 23). msbA forms an operon with an essential downstream open reading frame of unknown function, orfE. (25). Insertion of an ß-chloramphenicol resistance cassette into the msbA gene blocks transcription of both msbA and orfE (25). As shown in Fig. 3, only about half of the msbA coding region is on λ clone [218]E1D1. In this clone, orfE is missing its native msbA promoter, and expression of this gene would be from read-through of λ genes. Given the relatively low overproduction of the 4′-kinase activity found in lysates generated with [218]E1D1 and the indication that orfE does not have its own endogenous promoter, we constructed a plasmid to overexpress orfE using the T7 RNA polymerase system.

Cloning and Overexpression of the E. coli 4′-Kinase Gene

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FIG. 1. Biosynthetic pathway for E. coli Kdo2-lipid A. Five enzymes, LpxA, LpxC, LpxD, a UDP-diacylglycerol pyrophosphatase, and LpxB, are required for the synthesis of DS-1-P, the substrate for the 4′-kinase (3). The 4′-phosphorylation of DS-1-P yields lipid IVα. The Kdo transferase, encoded by kdsB, then transfers two Kdo sugars to lipid IVα to form Kdo2-lipid IVα (3). The late acyltransferases, HtrB and MsbB (3), add laurate and myristate, respectively, to form Kdo2-lipid A. Kdo2-lipid A is sufficient to support the growth of E. coli and is fully active as an immunostimulant and as an endotoxin during Gram-negative sepsis (3). Whole cells of mutants lacking MsbB are many orders of magnitude less immunostimulatory than wild type (68).

mastic activity could be detected in E. coli lysates produced by these hybrid λ clones (23). Activities of several enzymes involved in LPS biosynthesis were detected, and lysates generated from the λ clones containing the gene coding for the enzymes of interest displayed 5–10-fold overproduction of the activities (23). Assay of each individual λ lysate for overproduction of the lauroyltransferase (Fig. 1) led to the identification of htrB as the structural gene for that enzyme (23).

We employed the same approach to identify the gene for the lipid A 4′-kinase. The 4′-kinase activity was assayed in the lysates using method 1 (DS-1-32P and 5 mM ATP). Under these conditions, product formation was linear with respect to time and protein concentration (data not shown), there were no side products, and the results were reproducible for a given lysate.

Fresh λ lysates of W3110 were prepared and assayed for 4′-kinase activity in six sets of 80. Fig. 2 shows the assay results for one set, hybrid λ clones [201]4H7 to [280]22E3. No single lysate in the collection gave the 5–10-fold overproduction activity seen with other enzymes of lipid A biosynthesis (23). However, there were several lysates with significantly higher activity than their neighboring lysates (Fig. 2). To choose lysates for further analysis, the mean and standard deviation values for each set of 80 were calculated. Fifteen clones, the activity of which surpassed the mean by more than two standard deviations, were reassayed (data not shown). One lysate, [218]E1D1 (marked by an asterisk in Fig. 2) consistently displayed 2–2.5-fold more kinase activity than the other lysates.

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Massive Overexpression of 4′-Kinase Activity on a Hybrid Plasmid Bearing orfE—The gene encoding orfE was cloned behind the T7 promoter of pET3a to form pJK2. Plasmid pJK2 was transformed into BLR(DE3)pLysS cells, an E. coli strain that carries the T7 RNA polymerase as a λ lysogen (Table I). The expression of T7 RNA polymerase is induced with isopropylthio-β-D-galactopyranosidase and leads to the expression of genes from the T7 promoter. Washed membranes from BR7, an E. coli strain deficient for diglyceride kinase (12, 16), BLR(DE3)pLysS/pJK2, and BLR(DE3)pLysS/pET3a were assayed for 4′-kinase activity using DS-1-32P as the phosphate acceptor. The result of this assay is shown in Fig. 5. The 4′-kinase activity was highly overexpressed in cells with pJK2 versus strain BR7 or cells with pET3a vector alone (Fig. 5, lanes 3 and 4 versus lanes 1, 2, and 5). When assayed at a protein dilution in which product formation is linear with respect to time, overexpression of orfE led to several thousand-fold overproduction of 4′-kinase activity. Table II shows the specific activities of the 4′-kinase in cell-free extracts, membrane-free cytosols (subjected to two ultracentrifugations), and washed membranes.
orfE encodes a 328-amino acid protein with a predicted molecular mass of 36 kDa (25). Analysis of protein fractions from BLR(DE3)pLysS/pJK2 cells by SDS-polyacrylamide gel electrophoresis shows an overexpressed protein that is not present in protein fractions from BLR(DE3)pLysS/pET3a cells (Fig. 6). The overexpressed protein migrates with the molecular mass predicted from the sequence of orfE and is associated with the membranes (Fig. 6, lane 8). This is consistent with the hydropathy profile of orfE, which predicts 1 or 2 transmembrane helices in the N-terminal region of the protein. Like the protein, the 4'-kinase activity is also associated with the membranes, consistent with the hypothesis that orfE encodes the enzyme (Table II).

Data base searches identified only two open reading frames of unknown function from other Gram-negative bacteria with significant homology to orfE. The predicted amino acid sequence of orfE is 70.2% similar and 48.4% identical to the predicted amino acid sequence of E. coli orfE. The valB gene (40) from Francisella novicida encodes a protein that is 66.8% similar and 41.4% identical to orfE. This strongly suggests that the H. influenzae open reading frame and F. novicida valB may also be genes encoding lipid A 4'-kinase variants. An alignment of the three protein sequences is shown in Fig. 7.

orfE and its homologues do not display significant sequence similarity to any other type of kinase, including those involved in carbohydrate, lipid, nucleic acid, or protein phosphorylation.

Analysis of Substrate Specificity and Generation of Novel Analogs with the Overproduced Kinase—The 4'-kinase is a useful tool for making 32P-labeled substrates for the biochemical analysis of the enzymes catalyzing the late steps of the lipid A pathway (16–18, 23). 32P-Labeled lipid A precursors and substructures are also useful for studying the interactions of lipid A-like molecules with mammalian cells (19, 20). To demonstrate the synthetic utility of the overexpressed 4'-kinase, several DS-1-P analogs were analyzed as 4'-kinase sub-
strates (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8). DS-1-P is the physiological substrate for the 4'-kinase (3, 12). 3-aza-DS-1-P has an amide-linked hydroxy-group (Fig. 8).
phorylation of this compound yields the major molecular species that constitutes *E. coli* K-12 lipid A (endotoxin) (48), as shown in Fig. 10. Compound 505 was tested in a 4'-kinase assay system using membranes from BR7 and BLR(DE3)-pLysS/pJK2. The results are shown in Fig. 11. When BR7 membranes are used with DS-1-P, a small amount of [γ-32P]lipid IV A product (representing ~ 0.5% of the input [γ-32P]ATP) is formed (Fig. 11, lane 2). Use of BLR(DE3)pLysS/pJK2 membranes leads to the formation of a large amount of lipid IV A (Fig. 11, lane 5). If compound 505 is assayed with BR7 membranes, no detectable product (4',3'-phosphid A) is formed (Fig. 11, lane 3). However, with BLR(DE3)pLysS/pJK2 membranes, about 5% of the input 32P from [γ-32P]ATP is incorporated into [4',3'-2P]lipid A (Fig. 11, lane 6). This novel, enzymatically labeled product has the same migration as lipid A isolated by pH 4.5 hydrolysis (48) from wild-type *E. coli* cells (data not shown). In summary, when greatly overexpressed, the 4'-kinase is capable of phosphorylating glucosamine disaccharides that are either more or less acylated than the native substrate, DS-1-P.

**Other Properties of the 4'-Kinase**—Consistent with previous results (12), lipid X (3, 49) and UDP-diacylglycoseamine (3, 50) were not substrates for the 4'-kinase, even when the enzyme was highly overexpressed. The enzyme apparently has a strong preference for glucosamine disaccharides.

Membranes from *Rhizobium etli* strain CE3 contain an unusual phosphatase that removes the 4'-phosphate from the lipid A precursor, Kdo 2-lipid IV A (51). Solubilized CE3 membranes2 were used to make 4'-dephosphorylated Kdo 2-lipid IV A. The 4'-dephosphorylated Kdo 2-lipid IV A is not a substrate for the overexpressed 4'-kinase (data not shown). The Kdo disaccharide may interfere with the presentation of the 4'-OH of the glucosamine disaccharide to the kinase.

2 S. Basu and C. R. H. Raetz, unpublished observations.
**Fig. 8. Chemical structure of DS-1-P analogs tested as 4′-kinase substrates.** The chemical structures of DS-1-P and three analogs are shown here. The 3-aza-DS-1-P has an amide-linked hydroxymyrystate group at the 3-position instead of an ester-linked group (relevant NH indicated in **boldface type**). Mild base hydrolysis of these compounds results in removal of the ester-linked hydroxymyrystate groups. The resulting compounds, base-treated DS-1-P and base-treated 3-aza-DS-1-P, are also shown.

**DISCUSSION**

In the present study, we have identified the gene encoding the lipid A 4′-kinase (12) as *orfE*, a previously sequenced open reading frame found near minute 21 in *E. coli* (25). Until the present work, the function of *orfE* was unknown. *orfE* is an essential downstream gene in an operon that also includes *msbA*, a recently discovered gene encoding a possible inner membrane lipid A translocator with homology to mammalian Mdr proteins (25, 26, 33). In two organisms other than *E. coli*, the *msbA* and *orfE* genes are similarly grouped together (39, 40). The *msbA/orfE* operon represents a distinct new cluster of genes involved in the processing of LPS. Other LPS-related gene clusters in *E. coli* include the *waa* operon regions for lipid A biosynthesis, the *rfa* (waa) operons for core glycosylation and the *rfb* (wba) region for O-antigen assembly (3, 35, 52, 53).

The *orfE* gene is cotranscriptionally expressed with *msbA* (25). The *msbA* gene was discovered by Karow and Georgopoulos (25) as a multicopy suppressor of the temperature sensitive phenotype of insertion mutations in the *htrB* gene. Clementz et al. (22, 23) first identified HtrB as an acyltransferase of the lipid A pathway (Fig. 1). The role of *msbA* in LPS transport has been inferred from its genetic interaction with *htrB* (25), by the finding that *htrB* mutants accumulate LPS in their inner membranes (28), and by the sequence similarity of *msbA* to the ABC family of transporters (25). The mechanism of LPS transport from the inner leaflet of the inner membrane to the outer leaflet of the outer membrane is not well understood (3, 54).

The identification of *orfE* as the lipid A 4′-kinase will require a reexamination of the *msbA* knockout phenotype. In this knockout construct, the 4′-kinase activity is deleted as well. While the lethality of this knockout is clearly due to a requirement for both *msbA* and *orfE*, the accumulation of LPS in the inner membranes of such constructs (26) must be reevaluated in light of the fact that lipid A biosynthesis is also being severely compromised by the lack of the 4′-kinase. A construct in which only *msbA* is inactivated (or the introduction of a hybrid plasmid expressing *orfE* into the *msbA/orfE* knockout) will be necessary to properly evaluate *msbA*’s role in the cell. Last, it has not escaped our attention that OrfE has two potential membrane spanning domains and could itself be a component of the export machinery.

The 4′-kinase appears to be unique in that it shares no obvious sequence homology with other kinases. This may be so because phosphorylation of glucopyranosides at the 4-hydroxyl group is a relatively uncommon event in biology. Given the massive overproduction (Table I) of 4′-kinase observed when *orfE* is expressed behind a T7 promoter, it is likely that *orfE* is the structural gene for the kinase. We therefore suggest the new name *lpxK*.

Following the first description of the 4′-kinase in 1987 (12), washed membranes from cells containing wild type levels of kinase have been used to prepare substrates useful for studying the late reactions of the lipid A pathway (Fig. 1), such as [4′-32P]lipid IV$_A$ (16, 17, 51, 55). Since it was difficult to prepare [4′-32P]lipid IV$_A$ in yields greater than 0.5% relative to the input [γ-32P]ATP (because of the instability of the kinase at low ATP concentrations), no other substrates derived from [4′-32P]lipid IV$_A$, such as [4′-32P]Kdo$_2$-lipid IV$_A$, could be generated in high radiochemical yields. Consequently, enzymatic studies of the late reactions (Fig. 1) have been limited. Because the overexpression of the 4′-kinase is so great (∼2000-fold) in membranes from the LpxK overproducer (Table I), we can now prepare [4′-32P]lipid IV$_A$ from [γ-32P]ATP and DS-1-P with at least 50% yields (Fig. 9). This development will greatly facilitate the preparation of the distal intermediates of the lipid A pathway (Fig. 1) in 32P-labeled form.

Overexpression of the 4′-kinase has also been shown to be useful for the enzymatic synthesis of 4′-32P-labeled analogs of
lipid IV<sub>A</sub>, including [4'-32P]lipid A (Figs. 9 and 11). Using the DS-1-P analogs shown in Fig. 8 and compound 505 shown in Fig. 10, we have demonstrated that membranes containing the overexpressed kinase can be used to phosphorylate DS-1-P analogs containing two, three, four, or six fatty acyl chains. These analogs are not phosphorylated to any appreciable extent when wild-type membranes are employed as the enzyme source (Figs. 9 and 11).

In the future, these phosphorylations should proceed even more efficiently when the 4'-kinase is available in homogeneous form and its kinetic properties have been characterized. It will be important to stabilize the solubilized kinase to facilitate purification and assay. From the results in Fig. 6, we anticipate that about a 20-fold purification will be required to achieve homogeneity. A histidine tagged variant of LpxK could facilitate the development of a rapid purification. The N-terminal sequence of the purified, overproduced 4'-kinase will be analyzed to verify that it is indeed the protein encoded by orfE.

In addition to the possibility of making radioactive analogs and intermediates efficiently, our demonstration of the enzymatic generation of 4'-phosphorylated lipid A analogs, using the overproduced kinase, has potential pharmaceutical implications. It may be possible to design novel enzymatic processes for the large scale 4'-phosphorylation of lipid A-like molecules. Such compounds are of great interest because certain molecules of this kind, like E5531 (43), have activity as endotoxin antagonists and are potentially useful for the therapy of endotoxin shock (56, 57). For biological activity either as agonists or antagonists, lipid A-like molecules appear to require the presence of the 4'-phosphate (4, 19, 41, 46). While E5531 has been synthesized entirely by chemical methods (43), it may yet be possible to design enzyme-based processes for large scale production. Indeed, there is an extensive literature on the use of the recombinant LpxB (3, 30, 58–60) (the disaccharide synthase that functions just before LpxK in lipid A biosynthesis) for the preparation of diverse DS-1-P analogs (31, 61, 62). None of these compounds could ever be phosphorylated previously, because the cloned, overexpressed 4'-kinase was not available, and chemical methods were not applicable (47). The 3-aza-DS-1-P (Fig. 8) is an example of such an LpxB-generated analog (31). It would be of considerable interest to examine novel 4'-phosphorylated derivatives of existing DS-1-P analogs (31, 61, 62) for endotoxin antagonist or agonist activity.

Easy access to [4',32P]lipid A (Figs. 10 and 11) and its analogs should be useful for the identification and characterization of various lipid A binding proteins in animal cell membranes (20, 63–65). For instance, lipid A-like molecules bind to surface proteins, including CD14 (63, 64, 66) and the scavenger receptor (20, 67). Recombinant LpxK may be useful in the preparation of defined, highly radioactive lipid A analogs bearing photoaffinity probes. Such probes may reveal additional, perhaps minor membrane proteins that interact with endotoxin, such as...
and such as nists from antagonists (3, 63, 64). Radioactive lipid A analogs, the elusive signaling receptor that distinguishes endotoxin ago-
A isolated after pH 4.5 hydrolysis (48) from wild type cells of
migration of lipid A formed in this reaction is the same as labeled lipid
[corporation is not detectable with BR7 membranes.

*DS-1-P and compound 505 were the acceptor lipids used, as indicated. assays were performed exactly as in the legend to Fig. 9, except that
be explored.
mals. For studies of individual cells, the synthesis of fluores-
ances of lipid A uptake, metabolism, and distribution in ani-

Acknowledgments—We thank Dr. D. Golenbock and Dr. S. Kusumoto for providing compound 505.

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